Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the nonexclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

Phillip L. Price Date

Characterizing Motor Neuron-specific mRNA Processing Defects in an Intermediate Mouse Model of Spinal Muscular Atrophy

By

Phillip L. Price
Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Neuroscience

_____

Gary J. Bassell, Ph.D.
Advisor

_____

Wilfried Rossoll, Ph.D.
Advisor

_____

Arthur English, Ph.D.
Committee Member

_____

Francisco Alvarez, Ph.D.
Committee Member

_____

Yue Feng, M.D., Ph.D.
Committee Member

Accepted:

_____

Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____

Date

Characterizing Motor Neuron-specific mRNA Processing Defects in an Intermediate Mouse Model of Spinal Muscular Atrophy

By

Phillip L. Price
B.S., Sam Houston State University, 2012

Advisor: Gary J. Bassell, Ph.D.
Advisor: Wilfried Rossoll, Ph.D.

An abstract of A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in Graduate Division of Biological and Biomedical Sciences,
Neuroscience

2020

Abstract

Characterizing Motor Neuron-specific mRNA Processing Defects in an Intermediate Mouse Model of Spinal Muscular Atrophy

By: Phillip Price

Spinal Muscular Atrophy (SMA) is a neuromuscular disease characterized by a progressive loss of spinal motor neurons and consequently, a loss of locomotor abilities. SMA is directly caused by reduced levels of the ubiquitously expressed survival of motor neuron (SMN) protein, yet the molecular mechanisms by which reduced levels of SMN cause the dysfunction and degeneration of motor neurons remain elusive. By combining motor neuron–specific tagging of ribosomes with affinity purification of translating ribosomes, we have performed a comprehensive RNA-seq study to establish the profile of ribosome-bound mRNAs, or "translatome", in spinal motor neurons at pivotal time points in an intermediate mouse model of SMA. At postnatal day 9 (P9), before the onset of any disease-associated symptoms, we observed an early and persistent upregulation of transcripts involved in p53-mediated signaling pathway. We also observed a reduction of several markers of motor neuron subpopulations, including Matrix Metalloproteinase-9 (MMP9) at P9 and P19, suggesting that fast fatigable motor neurons may be more vulnerable to SMN depletion. Further analysis of splicing alterations present in our SMA translatome profiles identify novel SMN-dependent splicing perturbations that may influence SMA pathology in motor neurons. Finally, we adopted a powerful method to evaluate changes to the proteome specifically in SMA motor neuron *in vivo*. Taken together, data from these studies provide a comprehensive assessment of motor neuron-specific changes in mRNA processing at early and severe time-points in disease *in vivo*, revealing novel targets that may contribute to motor neuron degeneration in SMA.

Characterizing Motor Neuron-specific mRNA Processing Defects in an Intermediate Mouse Model of Spinal Muscular Atrophy

By

Phillip L. Price
B.S., Sam Houston State University, 2012

Advisor: Gary J. Bassell, Ph.D.
Advisor: Wilfried Rossoll, Ph.D.

A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in Graduate Division of Biological and Biomedical Sciences,
Neuroscience

2020

Acknowledgements

The work detailed in this thesis could not have been completed without the support of a number of individuals during my time in graduate training. I would like to thank both of my advisors on this project, Dr. Wilfried Rossoll and Dr. Gary Bassell, for the support and guidance they have given me during my thesis studies. Additionally for the willingness and encouragement for my participation and attendance at a number of training courses and conferences which expanded my understanding of the field.

I would like to also thank my thesis committee, Dr. Arthur English, Dr. Yue Feng, and Dr. Francisco Alvarez, for all of the helpful comments and guidance they have provided during the development of my project. Additionally I would like to thank all of the members of the Bassell lab and the Rossoll lab for excellent technical and experimental protocol support over the years. Particularly, I would like to thank Dr. Dmytro Morderer, Dr. Krysta Engel, and Chih-Wei Tsai for their technical support constructing our experimental model and assisting with the computational analysis. Lastly, I would like to thank you to friends, family, colleagues, and mentors who have supported me throughout this degree.

This work was supported by the US National Institute of Health (NIH) Grant and Diversity Supplement NS091749 to WR, and the National Research Service Award (NRSA) training award F31N NS098764-02 to PLP. This research project was supported in part by the Mayo Clinic Genomics Core.

Table of Contents

Chapter 1

General Introduction

Portions of this chapter were adapted from the following manuscripts:

Price, P.L., Morderer, D., and Rossoll, W. (2018). RNP assembly Defects in Spinal Muscular Atrophy. RNA Metabolism in Neurodegenerative Diseases

Khalil, B., Morderer, M., **Price, PL**., Liu, F., Rossoll, W. (2018). mRNP assembly, axonal transport, and local translation in neurodegenerative diseases. Brain Research

Abstract

Spinal muscular atrophy (SMA) is a motor neuron disease caused by mutations/deletions within the survival of motor neuron 1 (*SMN1*) gene that lead to a pathological reduction of SMN protein levels. SMN is part of a multiprotein complex, functioning as a molecular chaperone that facilitates the assembly of spliceosomal small nuclear ribonucleoproteins (snRNP). In addition to its role in spliceosome formation, SMN has also been found to act more generally as a molecular chaperone for the assembly of RNPs, including mRNP transport granules. The association of protein and RNA in RNP complexes plays an important role in an extensive and diverse set of cellular processes that regulate neuronal growth, differentiation, and the maturation and plasticity of synapses. This review discusses the role of SMN in RNP assembly and localization, focusing on molecular defects that affect mRNA processing and may contribute to SMA pathology.

1.1 SMA clinical background

Spinal muscular atrophy (SMA) is an autosomal recessive disorder characterized by an early-onset progressive degeneration of motor neurons in the anterior horn of the spinal cord and skeletal muscle atrophy (S. J. Kolb & Kissel, 2011). Impairments in synaptic maturation, sensory-motor circuitry, and synaptic transmission at the neuromuscular junction (NMJ), followed by a dying-back axonopathy, precede muscle denervation and loss of α-motor neurons in the spinal cord. In classical SMA, proximal muscles are more severely affected than distal muscles.

SMA is the leading genetic cause of death in infancy (T. W. Prior et al., 2010). Across ethnicities, an incidence of 1 in 8,000-20,000 has been estimated (Verhaart et al., 2017). Disease classification is based on the age of onset and clinical severity, the most common classification scheme distinguishes between Types I-IV, (Zerres, Wirth, & Rudnik-Schöneborn, 1997). The most common form of SMA (Type I; SMA1) typically leads to muscle weakness within the first six months and death due to respiratory failure by the age of two. Type II SMA patients present with signs of muscle weakness during the first 7-18 months. Affected children may crawl and sit unassisted, but often require support for standing and mobility. These patients typically have a life expectancy into early adulthood. Type III and IV SMA are milder forms of the disease, with patients having a normal life expectancy and displaying muscle weakness presenting in adolescence and adulthood. Type III SMA patients are able to stand unsupported and walk with moderate difficulty. Type IV (adult-onset) SMA patients are usually not diagnosed until early adulthood. These patients experience slowly progressing muscle weakness, primarily affecting the legs, hips, shoulders, arms.

1.1.2 SMA is caused by reduced SMN protein levels

In > 95% of cases, SMA is caused by homozygous deletions or compound heterozygous mutations in the survival of motor neuron 1 (*SMN1*) gene encoding the SMN protein (Lefebvre et al., 1995). Humans possess a nearly identical copy of this gene (*SMN2*), which carries a splice site mutation

in exon 7. This C>T transition in *SMN2* promotes the exclusion of exon 7 from the full-length protein, leading to the expression of only 10-20% of full length SMN protein, and 80-90% of a truncated SMN protein isoform (SMNΔ7) that is rapidly degraded (Campbell, Potter, Ignatius, Dubowitz, & Davies, 1997; Foust et al., 2009; McAndrew et al., 1997). Since SMA patients lack a functional *SMN1* gene, SMN protein is expressed only from *SMN2* gene copies, leading to reduced levels of full-length SMN protein. Thus, SMA is directly caused by a pathological reduction of functional SMN protein levels below a critical threshold, and disease severity is correlated with *SMN2* copy number (Campbell et al., 1997; McAndrew et al., 1997). Therapeutic approaches have mainly focused on raising SMN protein levels via gene therapy (Foust et al., 2009), or via increasing the splicing efficiency of *SMN2* exon 7. Small molecules (Naryshkin et al., 2014) as well as antisense oligonucleotides (ASOs) have been developed as splicing modifiers of *SMN2* (Hua et al., 2010), leading to the introduction of the ASO Spinraza (nusinersen) as the first drug approved by the US Food and Drug Administration (FDA) for the treatment of SMA (Hoy, 2017; Rigo et al., 2014; N. N. Singh, Howell, Androphy, & Singh, 2017). In 2019, the FDA approved the adeno-associated virus vector-based gene therapy Zolgensma as an alternative treatment for all types of SMA in patients less than 2 years of age (Al-Zaidy & Mendell, 2019). Though highly effective in treating SMA, these treatment options currently represent two of the most expensive drugs ever produced. The high cost for treatment, age limitations, uncertain long-term outcomes, and invasive nature of ASO delivery via lumbar puncture have created practical challenges that raise important ethical questions (Burgart et al., 2017). Although Spinraza treated patients demonstrate a marked improvement in survival, independence from permanent assisted ventilation, and motor functions, these individuals consistently score well below age-matched healthy controls on assessments of motor performance (Prasad, 2018). Furthermore, it remains unclear whether SMN replacement directed to the central nervous system will be sufficient to ameliorate non-motor symptoms in SMA patients. Continued effort will be required to provide more effective, affordable, and accessible treatment options.

1.2 Animals Models Recapitulate SMA Phenotypes

During early development, the assembly and stabilization of highly organized synaptic structures is essential for the maturation and function of the central nervous system. In neurodegenerative diseases, structural and functional abnormalities in synaptic connections often precede neuronal loss and cell death, and are thought to account for early clinical deficits (Day et al., 2006; L. R. Fischer et al., 2004; Shankar et al., 2008). The underlying cause for the vulnerability of motor neurons to reduced SMN protein levels remains unclear. Aside from *Drosophila*, *C. elegans*, and *Danio rerio* SMA animal models, several mouse models with different severity have been engineered to closely recapitulate pathological hallmarks observed in human patients (Edens, Ajroud-Driss, Ma, & Ma, 2015).

Unlike humans, mice only possess one gene encoding SMN, and a complete knockout results in early embryonic lethality (Schrank et al., 1997). To recapitulate the disease phenotype of human SMA, the introduction of the human *SMN2* transgene into the background of a homozygous deletion of murine *Smn1* has allowed for the creation of severe mouse models of SMA (Hsieh-Li et al., 2000; U. R. Monani et al., 2000). Further mouse models have been generated to represent less severe forms of SMA as important pre-clinical models for therapy development (Mélissa Bowerman, Lyndsay M. Murray, Ariane Beauvais, Bruno Pinheiro, & Rashmi Kothary, 2012; Hammond et al., 2010; Le et al., 2005).

Although born with a normal number of motor neurons, severe SMA mice experience a 35-40% loss of spinal cord and lower-brainstem motor neurons by day five (U. R. Monani et al., 2000). As described in **Figure 1**, pre-synaptic deficits include the aggregation of neurofilaments in the presynaptic terminal, poor terminal arborization, irregular distribution and positioning of synaptic vesicles, and reduced neurotransmission (Cifuentes-Diaz et al., 2002). In addition, multiple studies in SMA mouse models have observed significant impairments in mitochondrial function, including increased oxidative stress levels, organelle fragmentation, and axonal transport defects (N. Miller, H. Shi, A. S. Zelikovich, & Y.-C. Ma, 2016; C. C. Xu, Denton, Wang, Zhang, & Li, 2016).

Deleterious effects on the maturation and maintenance of NMJs support the characterization of SMA as an NMJ synaptopathy (Kariya et al., 2008). As the most commonly studied mouse model of SMA, SMNΔ7 mice carry a homozygous deletion of the murine *Smn1* gene and 2 transgenic constructs, one containing a single copy of the human *SMN2* gene locus, and a second encoding the human *SMN2* promoter driving expression of human *SMN2* cDNA lacking exon 7 (*SMN*Δ7) (Le et al., 2005). These mice have an average life span of 17.7 days, and display several phenotypes similar to those observed in SMA patients. Although most NMJs remain innervated until late in the disease time course of *SMNΔ7* mice, thorough explorations of the synapse electrophysiology and ultra-structure revealed a significant decrease in synaptic vesicle density and release probability (Kong et al., 2009). These deficits were found to be associated with a delayed maturation of NMJ terminals and myofibers, and together indicate that NMJ synaptic dysfunction precedes degeneration of the motor axon and finally the loss of motor neurons in severe SMA mouse models (Kong et al., 2009). Fewer subsynaptic clefts and lack of synaptic vesicles at the NMJ and abnormal preterminal accumulation of vesicles have also been observed in SMA I patients (Diers, Kaczinski, Grohmann, Hubner, & Stoltenburg-Didinger, 2005; Martinez-Hernandez et al., 2013).

Related to NMJ function, myotubes in SMA type 1 fetuses can display a significant retardation in growth and maturation (Martinez-Hernandez et al., 2009). One of the principal prenatal defects observed in mouse models and human SMA I patients was an arrest in acetylcholine receptor clustering into 'pretzel'-shaped structures during postsynaptic endplate maturation, compromising the structural and functional integrity of the NMJ (Martinez-Hernandez et al., 2013). In SMA mice, abnormal molecular composition, disruptions in normal satellite cell differentiation, and reductions in myofiber size, have been described in skeletal muscle (Hayhurst, Wagner, Cerletti, Wagers, & Rubin, 2012; Le et al., 2005; Lee, Mikesh, Smith, Rimer, & Thompson, 2011). Agrin, a protein best known for its role in organizing acetylcholine receptors at the NMJ, is misspliced and reduced in motor neurons of SMA mice (J. K. Kim, Caine, Awano, Herbst, & Monani, 2017). Rescue of the Z+ Agrin isoform prevented the development of several pathological phenotypes, and improved mean survival by 40% (J. K. Kim et al., 2017). Taken

together, this research points to abnormal function and maturation of the NMJ as key contributors to SMA pathogenesis, and as a potential target for therapy (Boido & Vercelli, 2016).

However, notable defects are also present within other cell-types that relay information, support motor neuron function and viability, and contribute to motor circuitry. In *SMNΔ7* mice, loss of proprioceptive sensory synaptic input onto spinal motor neurons has been observed in embryonic mice, suggesting that the disruption of the spinal motor circuitry at multiple levels is an early phenotype contributing to motor dysfunction (**Figure 1**) (Ling, Lin, Zingg, Feng, & Ko, 2010; Mentis et al., 2011). Defects in astrocyte activity and myelination may also influence the severity of SMA (Hunter, Aghamaleky Sarvestany, Roche, Symes, & Gillingwater, 2014; McGivern et al., 2013; Rindt et al., 2015; C. Zhou, Feng, & Ko, 2016). SMA astrocytes display significant deficits in stimulating neurite outgrowth and differentiation of motor neurons, but may also display potential toxic gain-of-function properties (J. E. Martin et al., 2017; Sison et al., 2017).

Although the *SMNΔ7* mouse model has provided invaluable insight into the intracellular mechanisms that contribute to SMA pathology, the aggressive progression of the disease culminating in early death due to cardiac deficits due to sympathetic innervation defects (Heier et al., HMG 2010), makes it a challenge to rescue this severe phenotype within the short time window for therapeutic intervention (**Figure 2**). To study the effects of SMN deficiency over an extended time course, a GGA to TTT substitution has been introduced within an exon splicing enhancer site in exon 7 of the *Smn1* gene ("2B mutation") (DiDonato et al., 2001; Hammond et al., 2010). The resulting alternative splicing event leads to the exclusion of exon 7 from the mature *Smn1* transcript and a 64% reduction of full-length SMN protein in the spinal cord (M. Bowerman, L. M. Murray, A. Beauvais, B. Pinheiro, & R. Kothary, 2012). Nevertheless, homozygous *SMN2B/2B* mice are, viable, fertile, and phenotypically normal. Crossing *SMNΔ7* heterozygous mice (SMNΔ7+/-) with *Smn2B* homozygous mice produces a mixed litter of phenotypically normal (*Smn2B/+*) and intermediate SMA (*Smn2B/-*) mice, indistinguishable until postnatal days 10-12. *Smn2B/+* mice retain approximately 78% of full-length SMN in the spinal cord

while *Smn2B/-* retain approximately 24.5% (M. Bowerman et al., 2012). In contrast to the *SMNΔ7* model, *Smn2B/-* mice have median life expectancy of 28 days, and gradually develop characteristic SMA phenotypes observed in the *SMNΔ7* model and human SMA patients. The delayed onset of symptoms and prolonged disease progression enhance our ability to identify and potentially treat pivotal disease mechanisms and pathways with greater temporal resolution (**Figure 2**). Additionally, the gradual progression of the disease may reveal other, non-neuronal features of SMA pathology previously masked by the early lethality of motor neuron degeneration.

1.3 SMA Pathology is Not Limited to Motor Neurons

The generation of conditional SMA mouse models with promoter-driven depletion or rescue of SMN expression has made it possible to study the pathological effects of selectively reduced SMN levels in specific cell-types and tissues. Using this approach, is has been demonstrated that depletion or restoration of SMN in motor neurons (*Hb9-Cre; ChAT-Cre*) significantly alters the functional synaptic output and excitability of the motor unit and retention of sensory-motor synapses (Gogliotti et al., 2012; Vicki L. McGovern et al., 2015). Nevertheless, restoration of SMN solely in motor neurons provides little to no improvement in life span, likely due to abnormal cardiac innervation by the autonomic nervous system in this severe SMA mouse model (Gogliotti et al., 2012). Multiple studies using a muscle-specific Cre driver (*Myf5-Cre; HSA-Cre*) to restore normal SMN in muscle of SMA mice showed that both replacement or depletion of SMN in muscle had little to no phenotypic effect on the mice (Gavrilina et al., 2008; Iyer et al., 2015; Shababi, Lorson, & Rudnik-Schoneborn, 2014). The greatest improvement in survival and function are the result of SMN restoration throughout the entire nervous system (*Nestin-Cre* and *ChAT-Cre; PrP-Cre*), affecting neurons and glia alike and largely rescuing SMA phenotypes and life span (Gavrilina et al., 2008; Gogliotti et al., 2012).

Although lower motor neurons and their circuitry are the primary targets of SMA pathology, mounting evidence suggests that SMN deficiency may contribute to defects in multiple tissues and across

additional peripheral organs (Nash, Burns, Chardon, Kothary, & Parks, 2016; Shababi et al., 2014). Indeed, a study examining pediatric SMA type I-III patients and SMA type I liver necropsy data provided strong evidence of increased susceptibility to developing dyslipidemia and liver steatosis in SMA patients as compared to healthy children (Deguise et al., 2016). Moreover, observations from human SMA patients were recapitulated in the intermediate Smn2B/- mouse model, with mice displaying elevated triglycerides, dyslipidemia, and developing nonalcoholic fatty liver disease prior to axon retraction and motor neuron degeneration (Deguise et al., 2016).

In summary, these studies highlight the necessity of SMN protein during development, and demonstrate the physiological consequences of insufficient levels of SMN on function and survival of various cell-types. While motor neuron degeneration and loss of central synapses and NMJs in the motor circuitry are the primary targets of SMA pathology, restoration of SMN in multiple cell-types may be necessary for a complete rescue of the SMA phenotype. The advent of effective therapies targeting the CNS may lead to the development of multi-organ impairment in surviving SMA patients, requiring systemic delivery of therapies (Wirth, Barkats, Martinat, Sendtner, & Gillingwater, 2015).

1.4 SMA is caused by reduced RNP assembly

SMN is an evolutionarily conserved and ubiquitously expressed protein with an essential role in RNA processing. Complete loss of SMN is lethal in all organisms and depends on maternal contribution across different species, highlighting its importance to cell development and survival (A. H. M. Burghes & C. E. Beattie, 2009). The temporal expression of SMN protein levels is developmentally regulated, with the highest expression levels during the embryonic period and a gradual decrease into the early postnatal period in mice and humans (Jablonka & Sendtner, 2017). SMN-containing granules are also present in the axons and growth cones of developing and regenerating motor neurons, and at the postsynaptic endplate of the neuromuscular junctions, (Dombert, Sivadasan, Simon, Jablonka, & Sendtner, 2014; Fallini, Donlin-Asp, Rouanet, Bassell, & Rossoll, 2016; Hao le, Duy, Jontes, & Beattie,

2015). Active bi-directional fast axonal transport of SMN has been demonstrated in primary forebrain and motor neurons (Fallini, Bassell, & Rossoll, 2010; H. L. Zhang et al., 2003).

As its best characterized molecular function, SMN facilitates the assembly of spliceosomal small nuclear ribonuclear proteins (snRNPs), bringing together specific sets of protein and RNA molecules that form the building blocks for spliceosome formation and pre-messenger RNA splicing (Gruss, Meduri, Schilling, & Fischer, 2017). More recent studies from multiple laboratories have demonstrated that SMN plays a broader role in the assembly of various RNP complexes with divergent roles in RNA processing, including mRNA splicing, turnover, and trafficking (D. K. Li, Tisdale, Lotti, & Pellizzoni, 2014b). In contrast to late onset neurodegenerative diseases that are often characterized by the accumulation of RNA-binding proteins into pathological aggregates (Ramaswami, Taylor, & Parker, 2013), SMA is set apart by an SMN-dependent deficiency in the formation of RNPs, and is therefore best described as an RNP hypo-assembly disease (Donlin-Asp et al., 2017; Shukla & Parker, 2016).

SMN associates with eight proteins (Gemins 2-8 and Unrip) to form the SMN core complex that is present in the cytoplasm and in discrete nuclear bodies called “gems”, for Gemini of Cajal bodies (or coiled bodies) (Cauchi, 2010; Otter et al., 2007; Livio Pellizzoni, 2007). The self-oligomerization of SMN and subsequent formation of the macromolecular SMN complex requires the evolutionarily conserved YG-box domain. Located at the carboxy-terminus, the YG-box provides a structural basis for the SMN complex to form higher-order complexes ranging from 20S to 80S (Brahms, Meheus, de Brabandere, Fischer, & Luhrmann, 2001; L. Pellizzoni, Yong, & Dreyfuss, 2002). A subunit of the SMN complex that includes SMN and Gemin2 recognizes Sm proteins, and assists in the ATP-dependent assembly of the heptameric Sm core complex (U. Fischer, Liu, & Dreyfuss, 1997; G. Meister, Buhler, Pillai, Lottspeich, & Fischer, 2001; L. Pellizzoni et al., 2002). Spliceosomal Sm proteins belong to a large family of Sm and Sm-like (LSm) proteins that share a conserved Sm motif necessary for protein-protein interaction, and are essential for snRNP biogenesis. Symmetrical dimethylation of a subset of Sm proteins by the protein arginine N-methyltransferase 5 (PRMT5) complex enhances their affinity for the conserved Tudor

domain within the SMN protein (Brahms et al., 2001; Friesen, Massenet, Paushkin, Wyce, & Dreyfuss, 2001). Gemin3 is a DEAD-box RNA-dependent RNA helicase and ATPase (Charroux et al., 1999). Gemin5 recognizes and interacts with large, ~50-60 nucleotide sequences or, snRNP codes, on specific spliceosomal U snRNAs (Battle et al., 2006; Lau, Bachorik, & Dreyfuss, 2009). Gemin6 and Gemin7 are thought to possess an Sm protein-like structure, facilitating the recruitment of Sm proteins into snRNPs (Ma, Dostie, Dreyfuss, & Van Duyne, 2005).

1.5 The SMN complex is an assembly machine for spliceosomal snRNPs

Although SMN and the associated Gemin2-8 proteins increase the efficiency and specificity of snRNP complex assembly, they do not become part of the final structure, thus acting as a molecular chaperone (D. K. Li et al., 2014b). Sm proteins have an intrinsic ability to associate with snRNAs *in vitro*, forming snRNP complexes with little regard to RNA specificity. The presence of the SMN complex restricts illicit associations of Sm proteins with erroneous RNAs, and promotes the recognition of snRNAs. The assembly of Sm proteins and binding of specific RNA requires a coordinated interaction between the SMN complex and the PRMT5 complex (Gunter Meister, Eggert, & Fischer, 2002). The PRMT5 complex consists of PRMT5, pICln, and WD45 (Mep50), and pre-assembles specific sets of Sm proteins via the pICln subunit (Friesen, Paushkin, et al., 2001; G. Meister et al., 2001). pICln is displaced from these recruited Sm proteins by the SMN complex, which promotes the transfer of Sm proteins from an intermediate RNP complex onto snRNA to form U snRNPs (Chari et al., 2008; Gruss et al., 2017; Gunter Meister et al., 2001). Therefore, the SMN complex functions as an assemblysome that regulates snRNP biogenesis, structure, and function (Paushkin, Gubitz, Massenet, & Dreyfuss, 2002). Recognition and binding of splice sites require the association of several small nuclear RNAs (snRNAs) and Sm proteins. Typically, uridine-rich snRNAs (U1, U2, U4, U5 U6, U11, U12, U4atac, and U6atac) are assembled with a set of seven Sm proteins (Sm B/B′, D1, D2, D3, E, F and G) into different classes of heptameric snRNP core complexes that are essential to the catalytic activity of the spliceosome (L. Pellizzoni et al., 2002; Wan et al., 2005). The U2-dependent major spliceosome comprised of U1, U2,

U4/6, and U5, is the predominant machinery responsible for the accurate removal of canonical 'GT-AG' introns from most eukaryotic transcripts, whereas the U12-dependent minor spliceosomal complex comprised of U11, U12, U4atac/U6atac, and U5, removes rare 'AT-AC' introns. Despite U12 introns representing only < 1% of all human introns, the U12-dependent spliceosome is essential for the viability and development of many multicellular organisms, including humans (A. A. Patel & Steitz, 2003). U12-type introns have been identified mainly in genes with a role in DNA replication and repair, transcription, RNA processing, and translation, but can also be found in genes related to vesicular transport, cytoskeletal organization and assembly, and voltage-gated ion channel activity (Turunen, Niemelä, Verma, & Frilander, 2013). As discussed below, alterations to U12-dependent spliceosomal activity may have particularly deleterious effects on the morphology and physiology of neurons. Of note, a mutation in the gene encoding U12 snRNA has been identified as the potential cause of early onset cerebellar ataxia in one pedigree (Elsaid et al., 2017), whereas mutations of core spliceosomal factors are typically associated with severe developmental disorders (Bacrot et al., 2015; R. K. Singh & Cooper, 2012).

Aside from its role in spliceosomal snRNP assembly, SMN has also been shown to be involved in the assembly of related RNP complexes with diverse roles in RNA metabolism (D. K. Li et al., 2014b). Unlike the U2 and U12 complexes, U7 snRNPs function not in splicing, but in the unique 3′-end processing of replication-dependent histone mRNAs that comprise the most abundant class of intronless and non-polyadenylated transcripts in metazoans (Marzluff, Wagner, & Duronio, 2008). Facilitated by the SMN complex, U7 snRNA associates with LSm proteins LSm10 and LSm11 instead of SmD1 and SmD2, to form the heptameric Sm core characteristic of snRNPs complexes (Tisdale et al., 2013). Interactions between U7 snRNA and the stem-loop binding protein (SLBP) mediate the recruitment and positioning of the trans-acting factors that cleave histone pre-mRNA (Marzluff et al., 2008). It remains to be seen whether SMN also plays a role in the assembly of the structurally related but functionally distinct LSm2–8 and LSm1–7 complexes, which play a role in pre-mRNA processing and mRNA decay (Vindry et al., 2017).

While it is well established that the SMN complex promotes snRNP assembly, and ultimately spliceosome formation, several questions regarding the arrangement and association of SMN with Sm proteins remain. Further examination into the structural arrangement and functions of these complexes *in vivo* and their regulation by cellular signaling pathways are necessary to fully understand the physiological relevance of these complexes in development and disease.

1.5.1 SMA deficiency causes widespread splicing defects

The extensively examined role of SMN in snRNP biogenesis and pre-mRNA splicing led researchers to hypothesize that SMA phenotypes are the result of SMN-dependent alterations in snRNP biogenesis and splicing, and that SMA can be described as a general splicing disease (Z. Zhang et al., 2008). Evidence supporting this hypothesis is substantial, yet incomplete. A potential direct link between defective snRNP assembly activity and SMA phenotypes was provided by a study showing that injection of purified U snRNPs could rescue embryonic arrest and SMA-like axon degeneration caused by a reduction of SMN or Gemin2 in zebrafish embryos (Winkler et al., 2005), although later studies in zebrafish have not found low Gemin2 levels to cause specific motor axon defects, arguing for a separate role for SMN in the SMA disease process that is snRNP independent (McWhorter, Boon, Horan, Burghes, & Beattie, 2008). Moreover, studies have shown a reduction in SMN-dependent snRNP activity in SMA patient tissue and animal models, demonstrating a correlation between snRNP activity and disease severity, but no selectivity for vulnerable cell types or tissues was found (F. Gabanella et al., 2007; Wan et al., 2005; Z. Zhang et al., 2008).

As previously described, the SMN complex facilitates snRNP assembly of the major (U2-dependent) and minor (U12-dependent) spliceosomes, as well as the U7 histone processing complex. Accordingly, several studies exploring how SMN deficiency influences the assembly and activity of each pathway have provided insight into the relationship between SMN-dependent snRNP activity and SMA phenotypes. Interestingly, changes in snRNP assembly in mouse models of SMA primarily affect the

(U12 dependent) minor spliceosome pathway (F. Gabanella et al., 2007). Caused by a deficiency in SMN, an inability of U11 snRNP to accumulate and form the U12 spliceosome machinery results in increased U12 intron retention, exon skipping, and aberrant splicing events. In a *Drosophila* model of SMA, mis-splicing of the U12 intron-containing gene *Stasimon* correlated with motor neuron pathology (Lotti et al., 2012). While overexpression of Stasimon in a *Drosophila* model of SMA rescued axonal pathfinding and outgrowth defects in motor neurons, it failed to restore normal viability and locomotion (Lotti et al., 2012). **Table 1** provides a list of selected mRNAs, which are affected by SMN-dependent splicing alterations and have also been suggested to contribute to SMA pathology. SMN depletion may also affect U7 histone mRNA processing. Due to an accumulation of U7 pre-snRNA, U7 snRNP steady-state levels are significantly reduced in SMN deficient cell lines and SMA mouse tissue, decreasing the post-transcriptional regulation of histone mRNA and resulting in the accumulation of uncleaved, 3'-end-extended histones (Tisdale et al., 2013).

Although these experiments emphasize the physiological relevance of snRNP core assembly, it remains unclear whether SMN-dependent alterations to snRNP biogenesis can account for the full spectrum of pathology observed in SMA patients and disease models. Studies in *Drosophila Smn* null mutant larvae showed no appreciable defects in the splicing of mRNAs containing minor-class introns, despite significant reductions in minor-class spliceosomal snRNAs (Praveen, Wen, & Matera, 2012). These findings suggest that SMN's role in snRNP biogenesis can be uncoupled from its effect on viability and locomotion. A comparison of snRNP-dependent and SMN-specific RNA changes in SMA models suggests that defects in snRNP supply are unlikely to be the primary drivers of SMA pathophysiology, at least in *Drosophila* (Garcia, Wen, Praveen, & Matera, 2016). Despite impairment of snRNP synthesis, endogenous snRNP and snRNA levels were found to be unaltered in SMA I patient-derived fibroblasts, a chicken cell line, and a severe *Drosophila* mutant, all of which had severely reduced SMN levels (F. Gabanella et al., 2007; Rajendra et al., 2007; Wan et al., 2005). Moreover, despite a significant difference in lifespan between the severe and SMNΔ7 models (~9 days), there was no difference in snRNP

assembly activity, suggesting that the difference in disease severity is caused by differential effects on an additional function of the SMN protein (F. Gabanella et al., 2007). It should be noted that snRNAs that are not associated with Sm cores are unstable, so snRNA levels are correlated to snRNP levels (Sauterer, Feeney, & Zieve, 1988).

Taken together, this research strongly suggests that while the direct effects of SMN deficiency on altered snRNP assembly and splicing are likely to contribute to SMA phenotypes, it fails to fully explain motor neuron susceptibility and the full spectrum of phenotypes observed in SMA pathology.

1.6 RNA localization is integral to neuronal developmental and function

The biological function of RNA localization to the cell periphery is to generate an asymmetry within the cell, which provides a mechanism for regulating gene expression with precise patterns of spatiotemporal control. This evolutionary conserved mechanism is a driving force behind many biological processes, including mating type switching in yeast, body axis establishment and cell fate determination in *Drosophila* embryos and *Xenopus* oocytes, cell motility and migration in fibroblasts, and axonal pathfinding and synaptic plasticity in polarized neurons (K. C. Martin & Ephrussi, 2009). While most studies have focused on the localization of a small number of well characterized transcripts, recent studies suggest that mRNA localization may be more the rule, than an exception. Based on RNA-seq studies in hippocampal neuropil samples, it has been estimated that ca. 2,550 mRNAs are present in axons and dendrites of hippocampal neurons, suggesting that many of the proteins that form the synapse may originate from local translation that is independent of the cell soma (Cajigas et al., 2012). Recent studies using RNA-seq on compartmentalized cultures of sensory neurons and motor neurons *in vitro* (Michael Briese et al., 2016; Minis et al., 2014), and on retinal ganglion cell axons *in vivo* (T. Shigeoka et al., 2016), have identified a highly complex axonal transcriptome that includes transcripts of multiple classes, similar to the somatodendritic compartment. Overcoming limitations of previous studies using micro-

arrays, these studies have expanded considerably the known axonal transcriptome (reviewed by (A. N. Kar, Lee, & Twiss, 2017)).

Early studies in the 1960s using metabolic labeling in isolated synaptosomes (Autilio, Appel, Pettis, & Gambetti, 1968) and axons (Koenig, 1967) led to the hypothesis that mRNA localization and its local translation may provide a mechanism for autonomous temporal and spatial control of the local proteome in nerve terminals (Holt & Schuman, 2013). Due to technical limitations, it has been widely assumed for a long time that transcripts and the machinery necessary for their translation is only present in dendrites, but not in axons. Although studies found polysomes at the base of spines (Steward & Levy, 1982), ribosomes were rarely observed in adult axons, possibly due to their localization in F-actin-rich structures at the periphery of the axoplasm termed periaxoplasmic ribosomal plaques (PARPs) that were described in several related studies (Calliari et al., 2014; Koenig, Martin, Titmus, & Sotelo-Silveira, 2000; J. Sotelo-Silveira, Crispino, Puppo, Sotelo, & Koenig, 2008; J. R. Sotelo-Silveira, Calliari, Cardenas, Koenig, & Sotelo, 2004). Similar axonal clusters of ribosomes are also present in *C. elegans* neurons, suggesting that clustering of ribosomes may be a common feature throughout evolution (Noma, Goncharov, Ellisman, & Jin, 2017). Furthermore, super-resolution microscopy has revealed eukaryotic ribosomes in mature interneuron axon terminals in mouse hippocampal slices (Younts et al., 2016). Additional studies with tagged ribosomal components will be required to confirm the presence of these structures in other model systems *in vivo*. The advent of new technologies, including compartmentalized chambers and animal models that allow for cell-type specific tagging and isolation of polysomes, have revealed the presence of an increasingly complex repertoire of several thousand of axonally localized transcripts (reviewed by (A. N. Kar et al., 2017)). There is growing evidence that axonal translation is present in developing axons and in axons responding to nerve injury (Michael Briese et al., 2016; Gumy et al., 2011; Saal, Briese, Kneitz, Glinka, & Sendtner, 2014; T. Shigeoka et al., 2016; D. E. Willis et al., 2007; Zivraj et al., 2010). An approach based on the RiboTag method (E. Sanz et al., 2009; Shigeoka, Jung, Holt, & Jung, 2018) has made it possible to compare axonal ribosome-bound mRNAs, or ‘translatomes’, from axons of retinal

ganglion cells in both developing and adult mice (T. Shigeoka et al., 2016). The identification of a complex translatome in adult axons, encoding proteins with a role in axon survival, neurotransmission, and neurodegenerative disease, provides compelling evidence for an important role of axonal translation in synapse function and maintenance *in vivo*.

Beyond mechanisms of mRNA localization, there are many additional factors and mechanisms to regulate axonal homeostasis. The RNAi pathway (Hengst, Cox, Macosko, & Jaffrey, 2006; Murashov et al., 2007) and dynamic N6-methyladenosine (m6A) modification (Yu et al., 2017) are functional in axons and regulate local translation. Local protein synthesis of Robo3.2 is regulated by axonal nonsense-mediated mRNA decay, influencing axonal pathfinding (Colak, Ji, Porse, & Jaffrey, 2013). The protein synthesis machinery present in axons includes components of the endoplasmic reticulum (ER) and Golgi apparatus required for the synthesis of most secreted and transmembrane proteins (Gonzalez et al., 2016; Luarte, Cornejo, Bertin, Gallardo, & Couve, 2017; T. Merianda & Twiss, 2013; T. T. Merianda et al., 2009), as reviewed by (Cornejo, Luarte, & Couve, 2017). Axons also harbor lysosomes required for autophagosome turnover (Farías, Guardia, De Pace, Britt, & Bonifacino, 2017). Local protein synthesis is coupled with local protein degradation as a major feature that is needed to maintain growth cone responses (Deglincerti et al., 2015; Verma et al., 2005).

Because of their extraordinary length and energetic demands, highly polarized neurons are particularly vulnerable structures and are at continuous risk of damage. In the case of lower motor neurons in the spinal cord, axons can measure up to 1 m in length in adult humans, and considerably more in larger extant and extinct organisms (Smith, 2009; Wedel, 2012). The axonal volume can exceed that of the cell soma by 1000-fold or more (Goldstein, 2001). Other reasons to explain the exquisite vulnerability of neurons compared to other cell types are that postmitotic and long-lived cells are likely more vulnerable to the accumulation of cell damage, and that their interconnectedness facilitates spread of the pathology along neurites (Ramaswami et al., 2013).

Local translation in mammalian axons serves primarily two functions: (1) it provides cells with the means to respond to environmental cues and express proteins with a role in axon elongation and pathfinding during development, and synaptic function and maintenance in adults (Andreassi et al., 2010; Aschrafi, Gioio, Dong, & Kaplan, 2017; Cosker, Pazyra-Murphy, Fenstermacher, & Segal, 2013; Hengst, Deglincerti, Kim, Jeon, & Jaffrey, 2009; A. N. Kar et al., 2014; Welshhans & Bassell, 2011; K. Y. Wu et al., 2005). (2) It enables cells to respond to injury and communicate with the cell soma, by expressing transcription factors and nucleocytoplasmic transport factors that are assembled into retrograde signaling complexes that regulate transcription in the nucleus (Baleriola et al., 2014; Ben-Yaakov et al., 2012; Cox, Hengst, Gurskaya, Lukyanov, & Jaffrey, 2008; Ji & Jaffrey, 2014; Amar N. Kar, MacGibeny, Gervasi, Gioio, & Kaplan, 2013; Riccio, Pierchala, Ciarallo, & Ginty, 1997; Yan, Wu, Chisholm, & Jin, 2009). Beyond the scope of this review, local translation has been shown to play an important role in axon regeneration, as reviewed by (Twiss, Kalinski, Sachdeva, & Houle, 2016). Impaired axonal trafficking of RNA-granules has also been implicated in the pathogenesis of chemotherapy-induced peripheral neuropathy (Pease-Raissi et al., 2017). The profound effect that changes to axonal translation may have on the pathology of various neurodegenerative diseases has only recently been recognized, as reviewed by (Baleriola & Hengst, 2015; Costa & Willis, 2017; A. N. Kar et al., 2017; Wang et al., 2016).

1.6.1 Assembly and transport of neuronal mRNPs

mRNAs do not exist as isolated molecules within eukaryotic cells. From the moment that an mRNA molecule is transcribed from DNA in the nucleus, they are decorated by a set of mRNA-binding proteins (mRBPs) that determine its fate and activity, by regulating pre-mRNA splicing, capping, and poly-adenylation, followed by mRNA nuclear export, packaging, trafficking, quality control, translation, and decay (G. Singh, Pratt, Yeo, & Moore, 2015). These processes are spatially and temporally closely linked and do not occur in a strictly separated sequential manner, e.g. transcription elongation is coupled to pre-mRNA splicing and mRNP assembly and nuclear export (Moore & Proudfoot, 2009). These interconnected steps in the life time of mRNAs explain why mRBPs typically participate in multiple steps

of mRNA processing, and are often found to regulate splicing and trafficking, as well as turnover and translation of their target transcripts.

The compartmentalization of mRNA is determined by a complex network of interacting cis- and trans-regulatory elements that modulate mRNA trafficking. Cis-acting elements within the nucleotide sequence, often referred to as “zipcodes” (Kislauskis & Singer, 1992), are most commonly located in the 3' untranslated region (UTR) of the mRNA. This allows for encoding spatial information about the gene product without changing its amino-acid sequence. The variety and specification of zipcode regions are largely products of alternative splicing and polyadenylation. An analysis of local transcriptomes within neural projections and cell soma of primary neurons and neuronal cell lines found that alternative last exons often confer isoform-specific localization (Taliaferro et al., 2016). Interestingly, a shift toward gene-distal last exon isoforms during neuronal differentiation led to a coordinated induction of mRNA isoforms that preferentially localize to neurites. Alternative 3′-UTR isoforms were also found enriched in axonal ribosome-associated transcripts in mouse neurons *in vivo* (T. Shigeoka et al., 2016).

In addition to these RNA processing events and information encoded in cis-elements, transcript stability and localization is tightly regulated by the association of zipcode regions with trans-acting mRBPs. Classical mRBPs contain RNA-binding domains (RBDs) such as RNA recognition motifs (RRMs), heterogeneous nuclear RNP K-homology domains (KHs), or zinc fingers (Znf). However, an ‘interactome capture’ approach has led to the identification of additional classes of protein that directly bind mRNA but lack these RBDs, expanding the repertoire of known mRBPs (Castello et al., 2012; Castello et al., 2016). The majority of these newly identified RNA interacting proteins show intrinsically disordered regions that lack stable secondary or tertiary structure and are enriched in short amino acid motifs, such as RGG and SR repeats (Hentze, Castello, Schwarzl, & Preiss, 2018). mRBPs recognize secondary structures formed within zipcode regions, and assemble into transport-competent RNPs. A single mRBP can bind several structurally or functionally related mRNAs and coordinate sequential steps in mRNA processing. Once assembled, RNPs interact with molecular chaperones, adaptor proteins, and

motor proteins to be actively transported along microtubules into axons and dendrites (Buchan, 2014; Xing & Bassell, 2013). Formation of RNP complexes protects mRNA from premature degradation and represses translation and ensures that transcripts arrive at their appropriate destination. Axonal RNP localization and cue-dependent release of translational repression contribute to the precise spatial and temporal gene regulation that supports the development, regeneration, and plasticity of neuronal circuits (Sasaki et al., 2010; Taylor, Wu, Tai, & Schuman, 2013; Tcherkezian, Brittis, Thomas, Roux, & Flanagan, 2010; Welshhans & Bassell, 2011; Wong et al., 2017).

A growing body of evidence suggests RNP granules form through the process of liquid-liquid phase separation (Brangwynne et al., 2009; Han et al., 2012; Kato et al., 2012; Mitrea & Kriwacki, 2016). As related to intracellular RNP assembly, this phenomenon refers to the ability of specific proteins to spontaneously separate into a demixed liquid phase, forming a membrane-less compartment within the cell. Unlike other membrane-less organelles, RNP granules are highly transient, assembling and dissembling in response to changes in the environment. The dynamic nature of RNP granule formation is largely owed to the presence of intrinsically disordered regions (IDRs) and low-complexity domains (LCDs) within the protein sequence (Molliex et al., 2015). These domains establish weak and multivalent interactions between RBPs, promoting the assembly of oligomeric structures. While this propensity toward fibrillization plays a fundamental role in the assembly of stress granules, persistent cellular stress or fibril-promoting mutations in the LCD can result in excess pathological fibrillization as seen in ALS, FTD, and AD (Lin, Protter, Rosen, & Parker, 2015; Molliex et al., 2015; A. Patel et al., 2015). While most studies have focused on larger RNP granules, such as cytoplasmic stress granules and P-bodies, neuronal transport granules share some of their mRBP components, and TDP-43-containing axonal mRNA transport granules display liquid-like properties (Gopal, Nirschl, Klinman, & Holzbaur, 2017). Heat-shock proteins have been implicated in P-body and stress granule assembly, but the only evidence for the involvement of chaperones in the assembly of mRNA transport granules comes from the

identification of SMN as a molecular chaperone for the assembly of axonally-transported mRNPs (Donlin-Asp et al., 2017).

1.6.2 Mechanisms of mRNA trafficking and local translation in axons

Once an RNP granule is formed, it must be translocated to the axon terminal. Axons employ two main classes of axonal transport based on the overall speed of movement, namely fast axonal transport (up to 400 mm/day, or 1 μm/s) and slow axonal transport (<8 mm/day or 0.1 μm/s) (Maday, Twelvetrees, Moughamian, & Holzbaur, 2014). Both classes of transport utilize the same microtubule-associated molecular motors from the kinesin and dynein protein families (Hirokawa & Takemura, 2005). Most cargos are bound to both plus-end-directed kinesin motors and minus end-directed cytoplasmic dynein motors, and their overall net directionality towards the periphery or cell body is achieved through back-and-forth movements (Rezaul et al., 2016). Unique domains in RBPs determine their affinity for kinesin family members, providing an additional determinant of transcript axonal localization (Chevalier-Larsen & Holzbaur, 2006). This process of cargo-loading is often facilitated by additional molecular chaperones and adaptor proteins. Transport of the motor-cargo complex depends on the hydrolysis of ATP to generate the force necessary for movement.

Localized RNAs are often kept in a translationally-repressed state until they reach their destination, whereupon they are released and may be translated to give a localized pool of protein product. At the axon terminal, post-translational modifications of RBPs are often necessary to dissociate the RNP complex and relieve translational repression of the localized transcript. For example, IMP1/ZBP1 (insulin-like growth factor 2 mRNA binding protein 1 / zipcode-binding protein 1) is a component of RNP transport granules and regulates the axonal localization of *β-actin* mRNA in neurons (Donnelly et al., 2011; Salerno et al., 2008; Welshhans & Bassell, 2011). The nonreceptor tyrosine kinase Src phosphorylates a key tyrosine residue on IMP1, interfering with its ability to bind RNA and destabilizing the RNP complex (Huttelmaier et al., 2005; Sasaki et al., 2010).

Examples for extrinsic signals that can drive localized protein synthesis in axonal growth cones include netrin-1 and brain-derived neurotrophic factor (BDNF), which regulate IMP1/ZBP1 phosphorylation (Eom, Antar, Singer, & Bassell, 2003; Kalous, Stake, Yisraeli, & Holt, 2014; Sasaki et al., 2010; Welshhans & Bassell, 2011; J. Yao, Sasaki, Wen, Bassell, & Zheng, 2006). Stimulation of local protein synthesis in neurons via netrin-1 (Piper et al., 2015), NGF (Gracias, Shirkey-Son, & Hengst, 2014), BDNF and glutamate (Hsu et al., 2015) is achieved by activating the mammalian target of rapamycin (mTOR) signaling pathway. As discussed below, deficiency in the assembly and axonal delivery of IMP1-containing mRNPs may contribute to pathology in spinal muscular atrophy (SMA), and restoring local translation in axons by stimulating the mTOR pathway may represent a target for future therapy (Kye et al., 2014; Ning et al., 2010).

The recent advent of methodologies with increased sensitivity and precision has allowed researchers to greatly expand our knowledge of mRNA abundance and diversity within axons. Microarray and RNAseq analyses of the axonal transcriptome in multiple model organisms have provided a more elaborate and comprehensive picture of the axonal transcriptome during embryonic development and in response to injury or pathological conditions, as reviewed by (A. N. Kar et al., 2017). The cell-type specific isolation of ribosome-bound mRNAs in mouse retinal ganglion cell axons allowed for the identification of over 2,000 mRNAs in developing and mature axons *in vivo* (T. Shigeoka et al., 2016). Analysis of axonal "translatome" profiles offers invaluable insight into axonal transport and local translation that occurs *in vivo*, and provides direct evidence for the occurrence of developmental stage-specific mRNA translation in axons of the developing and mature CNS. As discussed below, disease-associated disruptions to the axonal localization or translation of mRNA may contribute to a wide variety of pathological phenotypes observed in neurodegenerative diseases.

1.6.3 SMN and Motor Neuron Susceptibility

The pathological hallmark of SMA is the loss of alpha-motor neurons in the spinal cord. However, several phenotypic consequences of low SMN occur prior to cell death. The earliest defects in SMA patients and animal models are found at central synapses in the spinal cord and neuromuscular junctions (NMJ). In mice, NMJs begin to form at embryonic day 12 and undergo a critical stabilization and maturation process until E17 (Boido & Vercelli, 2016). Despite establishing NMJs at a normal frequency, SMA patients and animal models display a lack of synapse maturation. Arrested maturation and a gradual retraction of motor neuron axons are early, detrimental events in SMA pathogenesis that strongly suggest that SMA is a synaptopathy that affects primarily motor neurons, but also other parts of the motor circuitry.

Why motor neurons are uniquely vulnerable to SMN deficiency remains unclear. As its best characterized molecular function, SMN facilitates snRNP assembly and the formation of spliceosomal complexes (Q. Liu, Fischer, Wang, & Dreyfuss, 1997). SMN-dependent splicing defects have been described in a number of SMA mouse models, suggesting a direct link between SMN deficiency and impairments in spliceosome assembly and function (Baumer et al., 2009; Custer et al., 2016; Doktor et al., 2017; Garcia et al., 2016; Lotti et al., 2012; Praveen et al., 2012; See et al., 2014; Z. Zhang et al., 2008; Z. Zhang et al., 2013). However, since these defects in spliceosome assembly are found ubiquitously throughout various tissues, it is not clear how such deficits lead to motor neuron degeneration and other SMA phenotypes (Doktor et al., 2017; Shababi et al., 2014). It has been suggested that additional roles of SMN in axonal mRNA processing may cause a specific motor neuron susceptibility and contribute to the pathophysiology of SMA, as reviewed by (M. Briese, Esmaeili, & Sattelle, 2005; A. H. Burghes & C. E. Beattie, 2009; Donlin-Asp, Bassell, & Rossoll, 2016; Fallini, Bassell, & Rossoll, 2012; Jablonka, Wiese, & Sendtner, 2004; Rossoll & Bassell, 2009a). This hypothesis is based on the finding of mRNA mislocalization in SMN-deficient axons *in vitro*, as outlined in more detail below. A recent study has found that SMN interacts with the neuron-specific RBP HuD/ELAVL4 in zebrafish motor neurons *in vivo*, and reduction of both SMN and HuD lead to reduced axonal

localization *Gap43* mRNA encoding growth-associated protein 43, a protein with a role in neurite outgrowth, regeneration, and plasticity (Frey, Laux, Xu, Schneider, & Caroni, 2000). Overexpression of HuD rescued *Gap43* mRNA levels as well as morphological motor axon and movement defects, suggesting that reduction in axonal RNPs contributes to the SMA phenotype *in vivo*, at least in the zebrafish model (Hao et al., 2017). The specific vulnerability of motor neurons may come from unique requirements of RNP localization and local translation, perhaps related to their long axons and specialized synapses (NMJs), or from cell-type specific differences in axonally localized transcripts, such as the reported enrichment of *Acta* and *Actg* in axons of cultured primary motor neurons (Moradi et al., 2017).

SMN is a ubiquitously expressed 38kDa protein that oligomerizes to form a multimeric protein complex with Gemins 2-8 and Unrip (SMN Complex). SMN and other components of the SMN complex (gemin6, gemin7, gemin2, and gemin3) have been observed in the axons and dendrites of neurons, independent of splicing-related proteins (Sm proteins) (Sharma et al., 2005; H. Zhang et al., 2006). Multiple studies have demonstrated a role of SMN in the development, function, and maintenance of motor neuron axons and NMJs (Farrar et al., 2017). The extensive axons and specialized nature of the NMJs may render spinal motor neurons particularly susceptible to defects in RNP assembly and trafficking. In addition to SMN-dependent alterations in splicing, inhibition of RNP assembly and axonal localization may contribute to downstream pathogenic consequences. In the following section we will discuss evidence for the hypothesis that in addition to snRNP assembly, SMN also mediates assembly of mRNPs and their targeting for axonal transport.

1.6.4 SMN acts as a molecular chaperone for the assembly of mRNPs

Accumulating evidence shows that besides facilitating snRNP assembly, SMN plays also a role in the assembly of other RNPs with a role in histone mRNA processing, mRNA decay, and mRNA localization (D. K. Li, Tisdale, Lotti, & Pellizzoni, 2014a). The first evidence for its involvement in mRNA localization was the observation of reduced *β-actin* mRNA levels in axons of motor neurons from a severe SMA mouse model (Rossoll et al., 2003). In support of a role in mRNP assembly and trafficking,

numerous studies demonstrated that SMN localizes also to axons and growth cones *in vitro* (Dombert et al., 2014; Hao le et al., 2015; Jablonka et al., 2001; Pagliardini et al., 2000; Rossoll et al., 2003; Sharma et al., 2005; H. Zhang et al., 2006; H. L. Zhang et al., 2003). In addition, SMN was also shown to localize in Zebrafish motor axons during the period of robust axonal development, as well as in NMJs from E18 mouse embryos (Dombert et al., 2014; Hao le et al., 2015). Moreover, in axons SMN is localized to granules that are actively transported along cytoskeletal elements (Fallini et al., 2010; H. L. Zhang et al., 2003).

Another line of evidence comes from studies on SMN-interacting mRBPs, and their involvement in SMA-related phenotypes. SMN was found to associate with several mRBPs, including hnRNP-U, hnRNP-R, hnRNP-Q, KSRP/ZBP2/FBP2, IMP1/ZBP1, HuD/ELAVL4, FMRP, TIAR, SBP2 and FUS/TLS (Akten et al., 2011; Fallini et al., 2014; Fallini et al., 2011; Hua & Zhou, 2004; Hubers et al., 2011; Q. Liu & Dreyfuss, 1996; Mourelatos, Abel, Yong, Kataoka, & Dreyfuss, 2001; Piazzon et al., 2008; Rossoll et al., 2002; Tadesse, Deschenes-Furry, Boisvenue, & Cote, 2008; Wurth et al., 2014; Yamazaki et al., 2012), and SMN depletion results in reduced axonal localization of mRBPs and their associated mRNAs in cultured motor and sensory neurons (Fallini et al., 2016; Jablonka et al., 2006; Rossoll et al., 2003). One of those mRBPs, HuD/ELAVL4, is a neuron-specific member of the Hu protein family of mRBPs that is known to stabilize its mRNA targets upon binding (Bronicki & Jasmin, 2013). SMN associates with HuD in the context of an mRNP complex and is required for its localization into RNP granules and its transport in axons of cultured motor neurons (Fallini et al., 2011; Hubers et al., 2011). Knockout of HuD in Zebrafish results in axonal defects in motor neurons, similar to the effect of SMN knockdown (Hao et al., 2017; McWhorter, Monani, Burghes, & Beattie, 2003), whereas HuD expression in motor neurons rescues axonal defects caused by SMN deficiency (Hao et al., 2017). SMN also interacts and co-localizes in actively transported axonal granules with the RBP IMP1/ZBP1 in primary motor neuron cultures (Fallini et al., 2014). HuD and IMP1 interact in an mRNA-dependent manner, and in primary dorsal root ganglion (DRG) neurons both are required for axonal localization of

Gap43 mRNA, which encodes a protein with an important role in the regulation of presynaptic terminal function and axonal growth and plasticity (Yoo et al., 2013). In motor neurons from a severe SMA mouse model, *Gap43* mRNA is mislocalized from axons and growth cones, and overexpression of either HuD or IMP1 rescues this defect (Fallini et al., 2016).

SMN was also shown to associate with *Nrn1* mRNA which is one of the target mRNAs for HuD (Akten et al., 2011). In primary motor neuron cultures *Nrn1* co-localizes with SMN in motor neuron axons, and SMN knockdown leads to decrease of *Nrn1* in both soma and neurites of cultured cortical neurons (Akten et al., 2011). 3'UTR of *Nrn1* is able to provide axonal localization and translation for its transcript in cortical neurons (Akten et al., 2011). Interestingly, expression of *Nrn1* in zebrafish could rescue the axonal defects resulted from *Smn* knockdown (Akten et al., 2011). The *Nrn1* gene encodes a GPI-anchored protein that is involved in neuronal and synaptic development, regeneration and survival (S. Zhou & Zhou, 2014). As one of its functions, NRN1 was shown to promote axonal branching and neuromuscular synaptogenesis in motor neurons in *Xenopus* (Javaherian & Cline, 2005). Thus, SMN depletion can potentially lead to deficiency in *Nrn1* transport and translation in motor axons that would possibly result in defects in axonal growth and maturation of synapses formed by such axons. Intriguingly, *Nrn1* mRNA is localized to axons in both hippocampal and DRG neurons (Taylor et al., 2009; D. E. Willis et al., 2007), but the mechanisms of its axonal transport differ in these cells since in hippocampal neurons axonal localization of *Nrn1* depends on its 3'UTR, while in DRG neurons it is driven by motifs present in its 5'UTR (T. T. Merianda, Gomes, Yoo, Vuppalanchi, & Twiss, 2013). This difference is likely to be caused by competition for binding of limiting amounts of HuD between 3'UTRs of *Nrn1* and *Gap43* in DRG neurons, which results in *Nrn1* displacement from HuD by the more abundant *Gap43* transcripts despite the two-fold higher affinity of HuD for *Nrn1* (Gomes et al., 2017). This competition does not occur in embryonic hippocampal neurons that contain ~4-fold more HuD protein than adult DRG neurons (Gomes et al., 2017). These data also suggest the existence of additional mechanisms of *Nrn1* axonal transport that are HuD-independent (Gomes et al., 2017). Whether *Nrn1*

axonal transport is impaired in SMA patients, and how SMN contributes to HuD-dependent and HuD-independent *Nrn1* transport in motor neurons, are yet to be revealed.

Another SMN-interacting mRBP is hnRNP R that is required for axonal translocation of *β-actin* mRNA (Glinka et al., 2010; Rossoll et al., 2002). Knockdown of hnRNP R in motor neurons results in axonal defects and defective clustering of voltage-gated Ca^{2+} channels in growth cones, resembling the phenotypes observed in motor neurons from severe SMA mouse models (Glinka et al., 2010; Jablonka, Beck, Lechner, Mayer, & Sendtner, 2007; Rossoll et al., 2003). Local axonal translation of *β-actin* is impaired in motor neurons from severe SMA mouse model (Rathod, Havlicek, Frank, Blum, & Sendtner, 2012), while it is known that it is required for axonal development (Leung et al., 2006; Wong et al., 2017; J. Yao et al., 2006). *β-actin* mRNA is reduced in motor neuron axons from SMA severe mouse model, suggesting that impairment of *β-actin* local translation results from mRNA axonal transport defects (Rossoll et al., 2003). Interestingly, SMN knockdown in differentiated NSC-34 cells results in reduction of neuritic localization of *Anxa2* mRNA (Rage et al., 2013). *Anxa2* encodes protein Ca^{2+}-binding protein Annexin A2 that was shown to regulate actin remodeling (Hayes, Shao, Bailly, & Moss, 2006). Thus, it can be anticipated that lack of *β-actin* and *Anxa2* axonal transport can result in perturbation of local actin concentration and dynamics in growth cone.

Although currently little is known about the exact molecular mechanisms leading to mRNA transport defects in SMA pathology, several findings indicate that similar to the known snRNP assembly defects, mRNP assembly is also deficient in SMA. IMP1-containing mRNP granules isolated from cultured fibroblasts from SMA patients are reduced in size comparing to healthy controls, and motor neurons from an SMA mouse model and SMA patient fibroblasts show diminished binding of the mRBP IMP1/ZBP1 to *β-actin* zipcode region in reporter assays. Moreover, mRNP granules from patient fibroblasts show decreased association with both microtubules and microfilaments (Donlin-Asp et al., 2017). Taken together, these data support a role of SMN as a molecular chaperone for mRNP assembly that is required for the assembly of mRNP transport granules and their association with the cytoskeleton (Donlin-Asp et

al., 2017). Metabolic labeling in compartmentalized cultures of SMN-deficient primary neurons shows a reduction of global mRNA translation in axonal growth cones (Fallini et al., 2016). This defect may result from insufficiency in mRNP assembly and axonal transport, although a direct role of SMN in translational regulation has also been postulated (Bernabo et al., 2017; Sanchez et al., 2013). Another mechanism of how SMN-deficiency can affect local translation involves miRNAs and the mTOR pathway. In particular, upregulation of miRNA-183 in response to SMN deficiency caused subsequent downregulation of mTOR via direct binding to its 3'UTR (Kye et al., 2014).

According to current models, mRNA is transported along the cytoskeleton in a form of membrane-less mRNP granules that are driven by associated molecular motors (K. C. Martin & Ephrussi, 2009). Emerging evidence suggests that mRNA can also be co-transported in association with membranous vesicles (Jansen, Niessing, Baumann, & Feldbrugge, 2014). SMN interacts with the α-subunit of vesicle coat protein COPI (α-COP), and co-localizes with this protein in axonal growth cones of murine primary motor neurons (Peter et al., 2011). Moreover, these proteins are co-transported in differentiated PC12 cells (Peter et al., 2011). Expression of α-COP in SMN mutant Zebrafish was able to rescue axonal defects observed in this model (H. X. Li et al., 2015). Since COPI is present in axons and was shown to associate with a set of mRNAs, including *β-actin* mRNA (Bi, Tsai, Lu, Loh, & Wei, 2007; Peter et al., 2011; Todd, Lin, Ebert, Liu, & Androphy, 2013), it is plausible that the interaction between SMN and COPI mediates a vesicle-associated mode of axonal mRNA transport.

Currently, a direct link between observed insufficiency in axonal mRNA transport in SMA models and molecular mechanisms of the disease development has not been established. However, axonal defects that have been observed in multiple SMA animal models (Carrel et al., 2006; McWhorter et al., 2003; Rossoll et al., 2003; Ymlahi-Ouazzani et al., 2010) may result from inefficient axonal transport of SMN target mRNAs. Of note, local axonal translation of both *β-actin* and *Gap43* mRNAs, but not their expression in the cell soma, are required for axonal growth in primary DRG neurons, supporting branching and elongation of the axon, respectively (Donnelly et al., 2013). Both elongation and branching

defects were observed in zebrafish upon SMN depletion (McWhorter et al., 2003), which could be explained by a direct effect of *β-actin* and *Gap43* axonal transport failure. Importantly, transgenic expression of HuD in motoneurons of SMN mutants restored *Gap43* mRNA levels and rescued the observed motor axon and locomotor defects (Hao et al., 2017). SMN depletion has been shown to lead to a widespread reduction of mRNA content in axons (Fallini et al., 2011; Saal et al., 2014). While *β-actin*, *Nrn1*, and *Gap43* mRNAs encode proteins with important functions in regulating axon outgrowth and presynaptic terminal function and maintenance and may play a role in SMA pathophysiology, it is likely that additional mRNAs are mislocalized in response to SMN-deficiency. It will be important to gain a better understanding of the full repertoire of affected transcripts in SMA models *in vivo*, the effect of these changes on the local proteome, and how these defects are linked to axonal and synaptic pathology in SMA.

1.7 Other mRNA-processing functions of SMN

Aside from its role in snRNP and mRNP assembly, there is evidence for the involvement of SMN at other stages of the mRNA life cycle, including transcription and translation (**Figure 2**). The first evidence for the involvement of SMN in transcription came from the finding that it interacts with bovine papillomavirus transcriptional activator E2 and stimulates E2-dependent transcription (Strasswimmer et al., 1999). Subsequent studies identified additional SMN interactors involved in transcription, including the tumor suppressor and transcriptional activator p53 (Young et al., 2002) and the transcription corepressor mSin3A (Zou et al., 2004). Moreover, artificial recruitment of SMN to promotor regions resulted in repression of transcription (Zou et al., 2004). SMN was found to associate with key components of transcription machinery, such as RNA helicase A and RNA polymerase II. Overexpression of truncated SMNΔN27 results in transcription inhibition and accumulation of these components in gems and coiled bodies (L. Pellizzoni, Charroux, Rappsilber, Mann, & Dreyfuss, 2001). SMN was also shown to interact with the DNA/RNA helicase senataxin (Suraweera et al., 2009) and facilitate the association of senataxin and the C-terminal domain (CTD) of RNA polymerase II in a manner that was dependent on

CTD symmetric dimethylation (Zhao et al., 2016). Formation of this complex is required for resolving DNA-RNA loops (R-loops) and proper transcription termination (Zhao et al., 2016). It has been shown that SMN knockdown in SH-SY5Y cells leads to increased R-loop formation and DNA damage (Jangi et al., 2017). Of note, senataxin mutations are a rare cause of proximal spinal muscular atrophy (Rudnik-Schoneborn, Arning, Epplen, & Zerres, 2012), juvenile amyotrophic lateral sclerosis (Chen et al., 2004) and ataxia-ocular apraxia 2 (Moreira et al., 2004), indicating a possible link between the regulation of transcription termination via R-loops and neurodegeneration across different neurodegenerative diseases (for reviews, see (Salvi & Mekhail, 2015) and (Gama-Carvalho et al., 2017)).

The role of SMN in mRNP assembly and transport implies that *SMN* mutations can cause defects in local translation due to inefficient localization of mRNA to their destination sites. Indeed, defects in axonal translation in motor neurons from a mouse model of severe SMA (*Smn*$^{-/-}$;*Smn2*) and in cortical neurons upon SMN knockdown were reported (Fallini et al., 2016; Rathod et al., 2012). In addition, there is accumulating evidence that SMN can directly regulate translation. SMN can associate with the translation machinery, and it has been found in polyribosome fractions purified by ultracentrifugation from MN-1 cells (Sanchez et al., 2013). Moreover, SMN can anchor ribosomes to the plasma membrane, since the ribosomal content in plasma membrane fractions was depleted in SMA patient-derived fibroblasts or normal fibroblasts upon SMN knockdown (Gabanella et al., 2016). SMN deficiency in MN-1 cells does not affect overall translation rates but leads to increased translation of CARM1 arginine methyltransferase mRNA, and possibly other specific mRNAs, via currently unknown mechanism (Sanchez et al., 2013). Another study demonstrated that low amounts of SMN in cortical neurons reduce protein synthesis by upregulation of miR-183 microRNA and downregulation of mTOR pathway (Kye et al., 2014). In contrast, there were no significant differences in mTOR activation status and protein synthesis rate upon SMN knockdown in human fibroblasts under steady-state conditions. However, when membrane protrusion formation was stimulated in these cells, a decrease of translation rate was observed in *SMN* knockdown fibroblasts, and this difference was mTOR-dependent (Gabanella et al., 2016).

An SMN-dependent defect in translation has also been suggested by polysome profiling experiments, which indicated a reduction in the polysome peak of profiles from late-symptomatic SMA mouse tissue (Bernabò et al., 2017). RNA-seq data analysis identified genes associated with translation-related processes as significantly dysregulated in SMA motor neurons, providing evidence for a role of SMN in the regulation of ribosome biogenesis and translational activity (Bernabò et al., 2017). SMN-mediated regulation of translation is an emerging field that needs further studies to elucidate its molecular mechanism and potential role in SMA pathology, but it may very well be related to a function for SMN in assembling mRNA and associated proteins, similar to its role in snRNP and mRNP assembly.

1.8 Rationale, Hypothesis, and Objectives

1.8.1 Rationale

While diverse functions of SMN in regulating different aspects of mRNA processing are well established, the relative contributions of these SMN-dependent pathways and molecular mechanisms that result in disease pathology remain unclear. As a regulator of snRNP complex assembly, SMN deficiency is known to cause widespread changes in splicing and gene expression in various cellular and animal models of SMA. However, the question if and how a defect in the canonical housekeeping function of SMN in snRNP assembly directly cause the neurodevelopmental and neurodegenerative processes that lead to SMA pathogenesis remains. A thorough characterization of aberrant pre-mRNA processing in motor neurons of SMA mouse models and patients will continue to facilitate the identification of SMN-dependent isoforms (**Table 1**) and advance our understanding of the down-stream consequences of SMN deficiency that may explain motor neuron susceptibility and disease pathology.

Axonal localization and protein synthesis defects are prevalent in multiple neurological disorders including amyotrophic lateral sclerosis (ALS), Alzheimer disease (AD), Huntington disease (Twiss et al.), and Fragile X Syndrome (FXS), with recent studies emphasizing the role of local protein synthesis in regulating synaptic transmission and axon maintenance, and its relevance for human disease (Batista &

Hengst, 2016; Costa & Willis, 2017; Coyne, Zaepfel, & Zarnescu, 2017; Toshiaki Shigeoka et al., 2016). Research from several groups has begun to elucidate the non-canonical functions of SMN in regulating mRNP assembly and trafficking, as well as local and general translation (Donlin-Asp et al., 2016; Donlin-Asp, Rossoll, & Bassell, 2017). Furthermore, SMN has been shown to associate with mRBPs known to regulate the axonal localization and synthesis of growth-promoting mRNAs. A recent transcriptomic study identified a large number of mRNAs that are mislocalized in axons upon SMN knockdown in cultured mouse motor neurons *in vitro* (Saal et al., 2014). Additional studies to discern the molecular components and pathways critically affected in SMA motor neurons *in vivo* will be crucial to our understanding of SMN biology and SMA pathology, and may lead to the identification of additional targets for therapy.

1.8.2 Hypothesis

In light of the mounting evidence linking SMN deficiency to abnormal mRNA localization and metabolism, we hypothesized that SMN-deficiency leads to mRNA processing defects that underlie disease pathology and contributes to motor neuron susceptibility *in vivo.*

1.8.3 Objectives

We undertook the studies described in subsequent chapters with the following objectives: **1)** to identify novel mRNA targets and pathways dysregulated in the somatodendritic and axonal compartment of SMA spinal motor neurons at an early symptomatic stage *in vivo*, **2)** to identify novel mRNA targets and pathways dysregulated in the somatodendritic and axonal compartment of SMA spinal motor neurons at a late symptomatic stage *in vivo*, **3)** to identify and classify aberrant splicing events present in SMA motor neurons at early and late stages of disease, **4)** to determine whether SMA-specific dysregulated mRNA candidates identified by RNA-seq exhibit changes in protein expression *in vivo*.

1.9 Tables and Figures

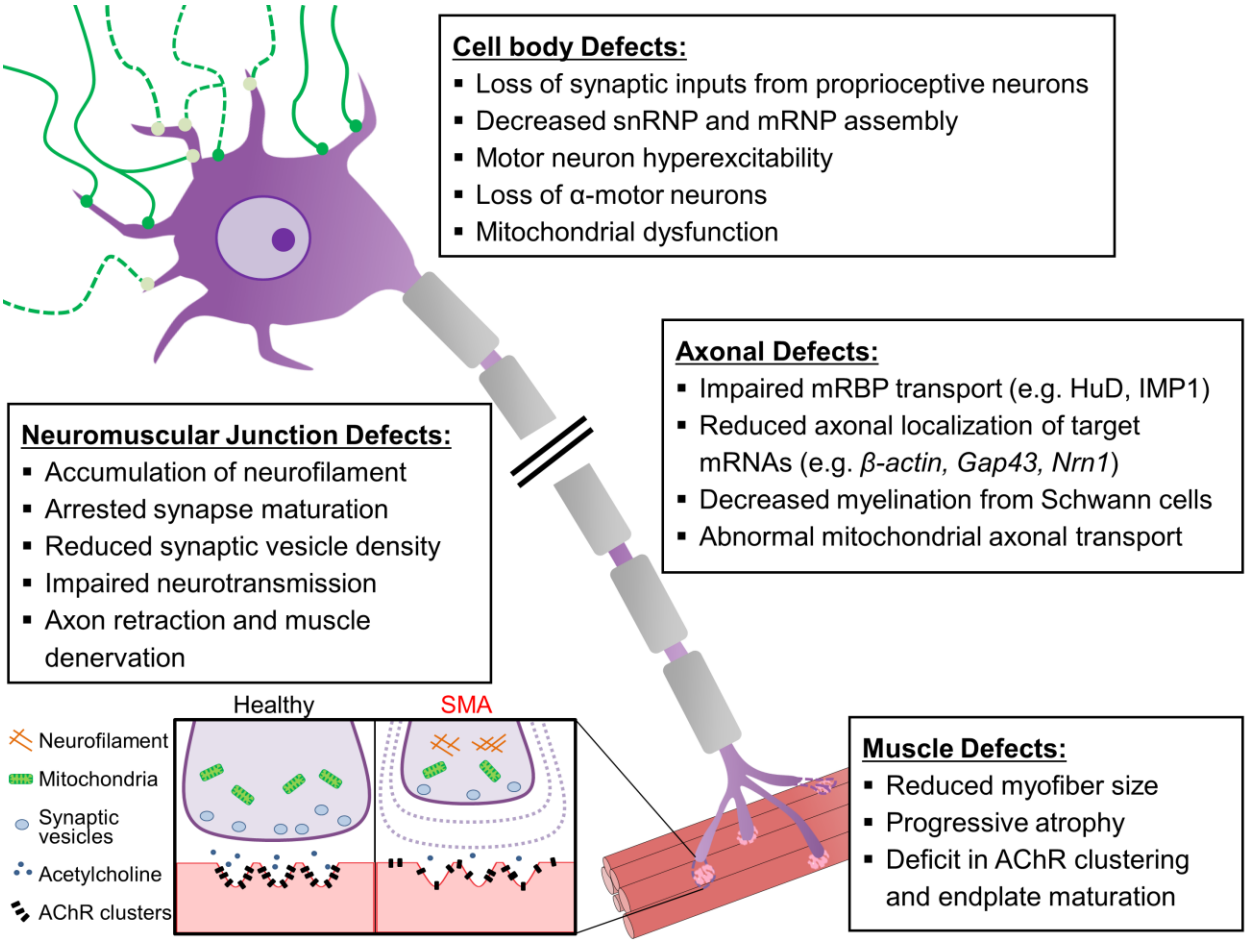

Figure 1. Cellular Defects in SMA motor neurons. Schematic of a spinal motor neuron, highlighting morphological and molecular defects within different cellular compartments.

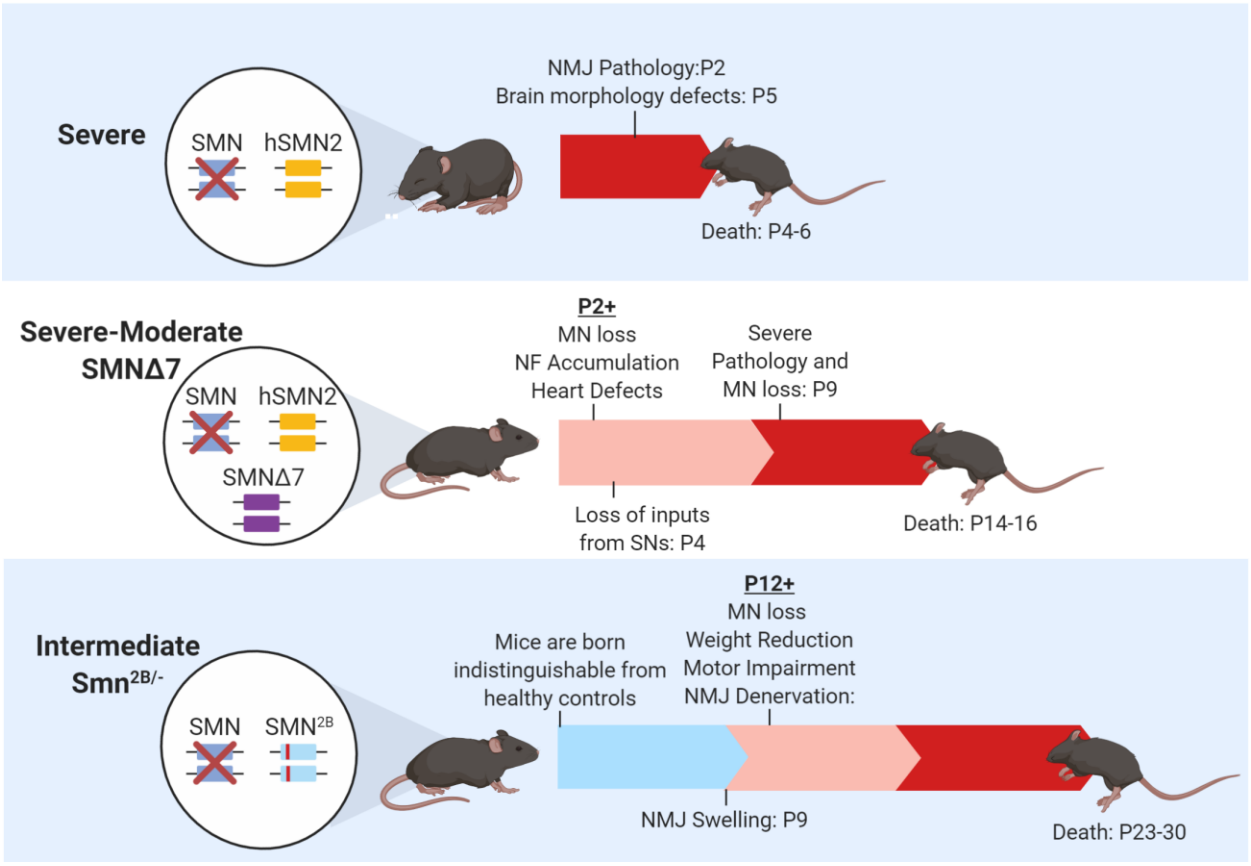

Figure 2. Timeline illustrating SMA pathogenesis and progression in common mouse models of SMA including severe (*Smn−/−; SMN2+/+*), SMNΔ7 (*Smn−/−; SMN2+/+; SMNΔ7+/+*), and intermediate (*Smn2B/-*) mouse models of SMA. MN, motor neuron; NMJ, neuromuscular junction

Table 1. Mis-spliced transcripts in SMA patients and animal models

Gene	Description	Species	Experimental condition	Cell line/tissue	Reference	Relevance to SMA pathology
SMN2	Splicing-deficient gene copy encoding SMN	human	SMA patient	iPS cells	(Jodelka, Ebert, Duelli, & Hastings, 2010)	SMN-deficiency affects *SMN2* splicing in a feedback loop that further reduces SMN protein levels(Jodelka et al., 2010)
			SMN1 knockdown	HEK293		
TMEM41b/ Stasimon	Transmembrane protein	*Drosophila*	*smn* loss-of-function	Larvae	(Lotti et al., 2012)	*Stasimon* knockdown in cholinergic increases EPSP amplitude in NMJ, restored by *Stasimon* expression. *Stasimon* expression rescues motor neuron defects in Zebrafish SMA model (Lotti et al., 2012)
		mouse	*SMN1* knockdown	NIH3T3		
			Moderate SMA model (*SMN2*$^{+/+}$;*SMNΔ7*$^{+/+}$;*Smn*$^{-/-}$) (Le et al., 2005)	Spinal cord, L1 DRG		
Nrxn2a	pre-synaptic membrane protein	zebrafish	*SMN* knockdown	Embryo	(See et al., 2014)	Knock-down of *Nrxn2a* results in motor axon defects (See et al., 2014)
		mouse	Severe SMA model (*Smn*$^{-/-}$/*SMN2*) (U. R. Monani et al., 2000)	Spinal cord		
Rit1	G protein	mouse	*SMN1* knockdown	NSC-34	(Custer et al., 2016)	Alternatively spliced *Rit1* isoform reduces neuritic length in NSC-34 cells (Custer et al., 2016)
			Severe SMA model (*Smn*$^{-/-}$/*SMN2*) (Hsieh-Li et al., 2000)	Lumbar spinal cord		
Agrn	heparin sulfate proteoglycan; organizer of the NMJ	mouse	Moderate SMA model (*SMN2*$^{+/+}$;*SMNΔ7*$^{+/+}$;*Smn*$^{-/-}$) (Le et al., 2005)	Motor neurons	(Z. Zhang et al., 2013)	SMN depletion leads to exclusion of Z exons from *Agrn* mRNA (Z. Zhang et al., 2013). It was previously established that *Agrn Z*$^{+}$ isoforms are important for maturation of postsynaptic termini in NMJ (Burgess, Nguyen, Son, Lichtman, & Sanes, 1999). Expression of *Agrn Z*$^{+}$ in motor neurons of SMA mice mitigates NMJ defects (J. K. Kim et al., 2017)
			Moderate SMA model (*SMN2*$^{+/+}$;*SMNΔ7*$^{+/+}$;*Smn*$^{-/-}$) (Le et al., 2005)	Motor neurons	(J. K. Kim et al., 2017)	
Uba1	Ubiquitin-like modifier activating enzyme 1	mouse	Severe SMA model (*Smn*$^{-/-}$/*SMN2*) (Hsieh-Li	Spinal cord	(T. M. Wishart et al., 2014)	*Uba1* knockdown disrupts axonal growth and branching in and leads to increase in β-catenin level

			et al., 2000; U. R. Monani et al., 2000)			Zebrafish. β-catenin level is also increased in SMA models, and inhibition of β-catenin signalling ameliorates neuromuscular pathology in SMA mice (T. M. Wishart et al., 2014).
Anxa2	Ca^{2+}-binding actin regulating protein	mouse	Moderate SMA model (*SMN2*$^{+/+}$;*SMNΔ7*$^{+/+}$;*Smn*$^{-/-}$) (Le et al., 2005)	Spinal cord	(Z. Zhang et al., 2008)	In addition to reported alterations in splicing, *Anxa2* was also shown to be overexpressed in SMA in several proteomic studies (Fuller et al., 2016; Mutsaers, Lamont, Hunter, Wishart, & Gillingwater, 2013; Sarvestany et al., 2014), and *Anxa2* mRNA is mislocalized from neurites in SMN-deficient NSC-34 cells (Rage et al., 2013).
Cacna1a *Cacna1b* *Cacna1c* *Cacna1e* *Cacna1h*	Voltage-gated Ca^{2+} channel subunits	mouse	Severe SMA model (*Smn*$^{-/-}$/*SMN2*) (Hsieh-Li et al., 2000)	Spinal cord	(Doktor et al., 2017)	In addition to reported alterations in splicing, reduction of Cacna1b in axonal growth cones of motor neurons from another severe SMA model mice (U. R. Monani et al., 2000), accompanied with alterations in their excitability, has been shown (Jablonka et al., 2007)

Table 2. mRNA components of SMN-dependent mRNPs

Transcript	**Species**	**Experimental Condition**	**Cell line/tissue**	**Reference**	**Type of evidence**
β-actin	mouse	Severe SMA mouse model (U. R. Monani et al., 2000)	Motor neurons	(Rossoll et al., 2003)	SMN knockout *reduces β-actin* mRNA localization in distal axons
		Severe SMA mouse model [22]	DRG sensory neurons	(Jablonka et al., 2006)	*β-actin* mRNA is reduced in growth cones of cultured sensory neurons from Smn-deficient embryos
		Smn knockdown	MN-1	(Hubers et al., 2011)	SMN is required for *β-actin* mRNA targeting to RNA granules
	rat	Transfection with hnRNP R expression constructs	PC12	(Rossoll et al., 2003)	Interaction between SMN and hnRNP R modulate *β-actin* mRNA localization in neuritic growth cones
		____	Cortical neurons	(Akten et al., 2011)	Co-precipitates with SMN
Gap43	mouse	Severe SMA mouse model [22]	Motor neurons	(Fallini et al., 2016)	*Gap43* mRNA is reduced in axons and growth cones
		Smn knockdown	Motor neurons	(Fallini et al., 2016)	*Gap43* mRNA is reduced in axons and growth cones
		Smn knockdown	MN-1	(Hubers et al., 2011)	SMN is required for recruitment of *Gap43* mRNA to RNA granules
	zebrafish	*SMN* and *HuD* mutants	Motor neurons	(Hao et al., 2017)	*Gap43* mRNA levels are decreased in motor neurons in HuD-dependent manner
	rat	____	Cortical neurons	(Akten et al., 2011)	Co-precipitates with SMN
Nrn1/Cpg15	rat	*Smn* knockdown	Cortical neurons	(Akten et al., 2011)	Co-precipitates with SMN. SMN knockdown affects *Nrn1* mRNA levels in both soma and neurites.
Anxa2	mouse	*Smn* knockdown	NSC-34	(Rage et al., 2013)	Associates with SMN complex and is reduced in axons upon SMN knockdown
Cox4i2	mouse	*Smn* knockdown	NSC-34	(Rage et al., 2013)	Associates with SMN complex and is reduced in axons upon SMN knockdown
Tau	mouse	*Smn* knockdown	MN-1	(Hubers et al., 2011)	SMN is required for recruitment of *Tau* mRNA to RNA granules
p21	mouse	Mild SMA mouse model (Jablonka, Schrank, Kralewski, Rossoll, & Sendtner, 2000)	Spinal cord	(Tadesse et al., 2008)	SMN depletion increases *p21* transcript stability

Table 3. mRBP components of SMN-dependent mRNPs

Name	Species	Tissue/Cell line	Reference	Other Supporting Evidence
hnRNP R	human	HEK293	(Rossoll et al., 2002)	Interaction with SMN is required for association between *β-actin* mRNA and hnRNP R (Rossoll et al., 2003)
	mouse	Motor neurons, spinal cord extracts	(Dombert et al., 2014)	
KSRP/FBP2/ MARTA1	mouse	N2a, spinal cord	(Tadesse et al., 2008)	p21 mRNA that is targeted for degradation by KSRP is upregulated in SMA tissues (Tadesse et al., 2008)
HuD/ELAVL4	rat	Cortical neurons	(Akten et al., 2011)	SMN is required for HuD targeting into RNA granules (Hubers et al., 2011). The HuD target mRNA *Gap43* is decreased in motor neurons from Smn mutant zebrafish (Hao et al., 2017)
	mouse	Spinal cord		
		Motor neurons	(Fallini et al., 2011)	
		MN-1	(Hubers et al., 2011)	
	zebrafish	Motor neurons	(Hao et al., 2017)	
IMP1/ZBP1	rat	Brain	(Fallini et al., 2014)	SMN facilitates association of IMP1 with *β-actin* mRNA (Donlin-Asp et al., 2017)
	mouse	Motor neurons		
SBP2	human	HEK293	(Wurth et al., 2014)	Levels of several SBP2-dependent selenoprotein mRNAs are reduced in spinal cords from SMA mice (Gribling-Burrer et al., 2017)

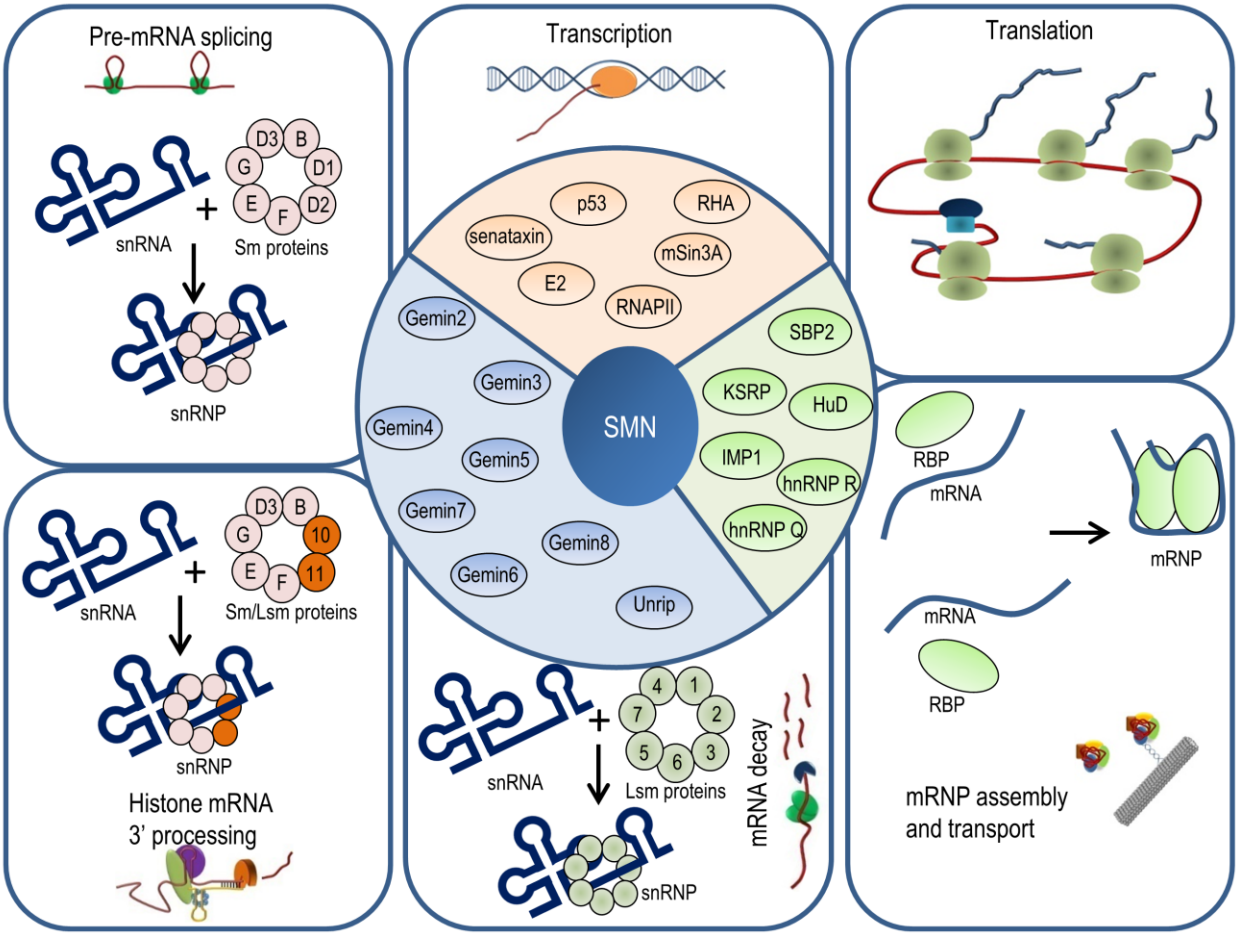

Figure 2. Molecular Functions of SMN in mRNA processing. SMN can associate with a large selection of proteins to regulate snRNP assembly (splicing, histone mRNA processing, mRNA decay), transcription, translation, and mRNP assembly (mRNA transport and local translation).

Chapter 2

Characterization of Motor Neuron-specific mRNA processing defects in a mouse model of Spinal Muscular Atrophy

Price, PL., Tsai, C., Engel, K., Taliaferro, M., DiDonato, C., Bassell, G., Rossoll, W. (2019).

Abstract

Spinal Muscular Atrophy (SMA) is a neuromuscular disease characterized by a progressive loss of spinal motor neurons and consequently, a loss of locomotor abilities. SMA is directly caused by reduced levels of the ubiquitously expressed survival of motor neuron (SMN) protein, yet the molecular mechanisms by which reduced levels of SMN cause the dysfunction and degeneration of motor neurons remain elusive. By combining motor neuron–specific tagging of ribosomes with affinity purification of translating ribosomes, we have performed a comprehensive RNA-seq study to establish the profile of ribosome-bound mRNAs, or "translatome", in spinal motor neurons at pivotal time points in an intermediate mouse model of SMA. At postnatal day 9 (P9), before the onset of any disease-associated symptoms, we observed an early and persistent upregulation of transcripts involved in p53-mediated signaling pathway. We also observed a reduction of several markers of motor neuron subpopulations, including Matrix Metalloproteinase-9 (MMP9) at P9 and P19, suggesting that fast fatigable motor neurons may be more vulnerable to SMN depletion. Further analysis of splicing alterations present in our SMA translatome profiles identify novel SMN-dependent splicing perturbations that may influence SMA pathology in motor neurons. Taken together, data from these studies provide a comprehensive assessment of motor neuron-specific changes in mRNA processing at early and severe time-points in disease *in vivo*, revealing novel targets that may contribute to motor neuron degeneration in SMA.

2.1 Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive neurodegenerative disease, predominantly characterized by progressive muscle denervation and loss of spinal motor neurons (Lunn & Wang, 2008). The majority of patients with SMA are affected by a severe, infant-onset form of the disease that results in a median life expectancy of 12-18 months, making SMA the leading genetic cause of infant mortality. SMA has an incidence rate of 1:10,000 live births and a carrier rate of 1:40 (Pearn, 1978; Thomas W. Prior et al., 2010; Sugarman et al., 2012). SMA is caused by homozygous deletions or inactivating mutations in the survival of motor neuron 1 (*SMN1*) gene (Lorson, Hahnen, Androphy, & Wirth, 1999). Humans possess a second centromeric copy of the gene, *Smn2*, containing a single C to T nucleotide substitution in exon 7. This mutation results in an alternative splicing event that skips exon 7 and compromises the stability of the SMN protein product, leaving only a small fraction (~10-20%) of full-length SMN that is unable to adequately compensate for the absence of Smn1 (Lorson et al., 1999; Umrao R. Monani et al., 1999). Therefore, SMA is directly caused by low levels of SMN protein.

The ubiquitously expressed SMN protein is essential for normal cellular function and organismal development, with complete loss leading to embryonic lethality in mice (Schrank et al., 1997). The best characterized role of the SMN protein is as part of the SMN-Gemin multiprotein complex, which facilitates the assembly of spliceosomal small nuclear ribonucleoproteins (snRNPs) (U. Fischer et al., 1997; L. Pellizzoni et al., 2001) and therefore pre-mRNA splicing (Stephen J. Kolb, Battle, & Dreyfuss, 2007). Consistent with this housekeeping role of SMN, defects in RNA splicing have been observed in numerous SMA models (Baumer et al., 2009; Custer et al., 2016; Francesca Gabanella et al., 2007; Garcia et al., 2016; Praveen et al., 2012; See et al., 2014). Nevertheless, these splicing defects are present in all cell types, and fail to explain the increased susceptibility of motor neurons to SMN deficiency (Gogliotti et al., 2012; V. L. McGovern et al., 2015; Park, Maeno-Hikichi, Awano, Landmesser, & Monani, 2010). This discrepancy in function and phenotype suggests additional roles of SMN may contribute to SMA pathophysiology. Work from our lab and others has shown SMN also interacts with mRNA binding

proteins (mRBPs) and facilitates the formation of higher-order mRNP granules, a process crucial to the regulation of mRNA stability, localization, and local translation (Kiebler & Bassell, 2006; Rossoll & Bassell, 2009b) (Donlin-Asp et al., 2014; Fallini et al., 2016). Importantly, SMN-deficient motor neurons were shown to have reduced levels of poly(A) mRNA present in axons and growth cones (Akten et al., 2011; Fallini et al., 2011; Rossoll et al., 2003). Although the elucidation of these non-canonical roles of SMN may partially contribute to the neuronal bias of SMA pathology, the specific defects in molecular pathways and cellular functions that are responsible for selective motor neuron degeneration remain unclear.

Mouse models of SMA have played an invaluable role to our current understanding and treatment of the disease. In contrast to humans, mice possess only one copy of the SMN gene that does not undergo alternative splicing. The addition of human SMN2 transgenes into the background of a homozygous deletion of murine Smn1 has allowed for the creation of multiple severe mouse models of SMA (U. R. Monani et al., 2000). As the most cited and best characterized mouse model of SMA, SMNΔ7 mice carry transgenic constructs of human *SMN2* with and without exon 7 on the SMN knockout background to recapitulate phenotypes observed in SMA patients (Le et al., 2005). These mice have an average life expectancy of 14 days or less, and tend to at the severe end of the disease spectrum. The use of intermediate mouse models of this disease would offer a unique opportunity to study intricate aspects of SMA pathology previously masked by the rapid progression observed in more severe models. Increased temporal resolution of cell-type specific expression and RNA processing patterns at the disease onset can reveal disease mechanisms, clinically relevant biomarkers, and converging pathways that underlie motor neuron sensitivity in SMA. Prior studies have primarily relied on two approaches to study SMN-dependent dysregulation of mRNA processing: profiling of homogenized bulk tissue and *in vitro* cellular models. Homogenization of whole spinal cord tissue often fails to capture subtle yet pivotal changes a relatively scarce population such as motor neurons may undergo during degeneration. Alternatively, *in vitro* models permit precise examination and convenient experimental manipulation of a specific cell type,

but fail to recapitulate the complex and dynamic environment of complex tissues and multiple cell types present in the nervous system. Establishing mechanistic links between SMN-dependent changes in RNA processing and metabolism and SMA motor neuron pathology *in vivo* is crucial to our understanding of disease.

The advent of methods such as RiboTag, TRAP, and ribosome profiling has introduced a new aspect to assess changes in gene expression. Despite several advances in our ability to accurately identify and quantify diverse genetic profiles, the targeting of cell transcriptomes using modern methods can introduce several extraneous variables that confound results and obscure critical differences between experimental conditions. The use of epitope-tagged ribosomal proteins facilitates the isolation and quantification of gene expression within specific cell populations *in vivo*, circumventing numerous limitations associated with performing cell type-specific quantitative genomics in complex tissues. As one of the most common methods of isolating a unique cell type from a complex mixture, fluorescence-activated cell sorting (FACS) relies on transgenic expression or immunocytochemistry to fluorescently label and subsequently sort the cell population of interest (Gross et al., 2015). Obtaining a single cell suspension of labeled cells necessary for FACS often requires mechanical and enzymatic dissociation that may induce changes in gene and protein expression (Haimon et al., 2018; Rossner et al., 2006; Elisenda Sanz et al., 2009; Sugino et al., 2006; F. Yao et al., 2005). Alternatively, laser capture microdissection (LCM) alleviates the issue of enzymatic dissociation by using a high-precision laser and microscope to visually identify and isolate specific cell types in sectioned tissue samples. Nevertheless, tissue must be processed and sectioned prior to capture, and the highly-specific microdissection of cell somas can result in the exclusion of dendrites and axons. Despite the multiple disadvantages associated with procuring a specific population from a complex organ, the most significant weakness to identifying meaningful changes in gene expression may lie in targeting the transcriptome itself. Mounting evidence is revealing the limited efficacy of transcriptomic approaches to reflect changes in protein expression (Bogeat-Triboulot et al., 2007; Gygi, Rochon, Franza, & Aebersold, 1999; Haimon et al., 2018; Smircich et al.,

2015). Studies have also shown proteomic data better correlates with translatomic data than with the transcriptome (Y.-F. Li et al., 2018) (Smircich et al., 2015). Thus, evaluating cell type-specific translatomes via RiboTag is a powerful approach that employs the sensitivity of modern sequencing technology with cell type-specific targeting of mRNA populations that better represent changes in protein levels.

To investigate factors influencing motor neuron susceptibility in a mouse model of SMA, we employ RiboTag technology to examine motor neuron-specific changes in gene expression at key time points of SMA pathology *in vivo*. The intermediate $Smn^{2B/-}$ mouse model of SMA allows for detailed examinations of pathology over a prolonged time course of 21-30 days (M. Bowerman et al., 2012; DiDonato et al., 2001). By combining the spatial resolution of cell-type specific mRNA isolation with the temporal resolution of the $Smn^{2B/-}$ model, we established a system conducive to studying complex, cell-type specific alterations in gene expression *in vivo*. Dysregulated gene expression and processing could be a cause and a consequence of SMN-dependent motor neuron degeneration in the spinal cord. Therefore, we aimed to capture changes in motor neuron gene expression at early (P9-10) and late (P19-20) symptomatic stages of disease. $Smn^{2B/-}$ pups are born indistinguishable from healthy $Smn^{2B/+}$ littermates, and do not display disease phenotypes until P10 (**Chapter 1, Fig. 2**). Ages P9-10 in $Smn^{2B/-}$ mice have been well-characterized as an early or pre-symptomatic stage of disease, in which changes in motor neuron number and NMJ occupancy are also not yet present (M. Bowerman et al., 2012; Boyer et al., 2013; Eshraghi, McFall, Gibeault, & Kothary, 2016). The rapid progression of disease pathology results in the appearance of reduced weight gain and a steady decline in locomotor abilities starting at P12. By P19-20, $Smn^{2B/-}$ mice have lost approximately 75% of their lumbar spinal motor neurons (Cerveró et al., 2018) and display dramatic decrease in body weight and motor performance (M. Bowerman et al., 2012; Cerveró et al., 2018; Courtney, Mole, Thomson, & Murray, 2019).

We applied a stringent analytical approach to our RNA-seq results to identify differentially expressed genes and established expression profiles of dysregulated transcripts at each time point. We

then expanded our analysis to examine alternative splicing events present within our motor neuron-derived RNA-seq data sets at P9 and P19. The extent of gene dysregulation reflected phenotypes at each time point. Evaluation of differential gene expression revealed several known and novel dysregulated transcripts. Biochemical and immunohistological methods used to validate down-regulated targets identified in the expression profiles on the protein level revealed a reduction in Arhgap9, Bag3, and MMP9 in SMA spinal neurons. Furthermore, we observed a selective reduction of motor neurons expressing MMP9 present even at the early symptomatic time point, suggesting a shift in motor neuron subpopulations rather than a general down regulation. A deeper examination of our motor neuron-specific RNA-seq data allowed us to quantify and characterize changes in splicing. Lastly, by targeting motor axons innervating highly susceptible muscle groups, we aimed implement our motor neuron-specific translatome strategy to identify changes in the axonal translatome between SMA and WT motor axons *in vivo*. Overall, this work combines strategic animals, powerful bioinformatic approaches, and reliable biochemical methods to reveal novel changes in RNA processing and protein expression that may contribute to the specific motor neuron degeneration in SMA.

2.2 Results

2.2.1 Translatome profiling of SMA motor neurons at early symptomatic and severe time points

RiboTag technology permits the *in vivo* tagging of ribosomes in a cell-type specific manner through Cre-mediated expression of the hemagglutinin (HA) epitope-tagged 60S subunit ribosomal protein L22 (RPL22-HA) (Elisenda Sanz et al., 2009). As motor neurons rely on the constitutive expression of choline acetyltransferase (ChAT) for the biosynthesis of acetylcholine, ChAT is considered a definitive marker of motor neurons in the spinal cord (D. Wu & Hersh, 1994). We crossed RiboTag mice expressing Cre recombinase driven by ChAT (RC) into the $SMN^{2B/2B}$ intermediate mouse model of SMA to achieve RC::$Smn^{2B/+}$ and RC::$Smn^{2B/-}$ mice (**Figure 1A**). $Smn^{2B/-}$ mice are born indistinguishable

from healthy *SMN*$^{2B/+}$ littermates and develop progressive muscle weakness over an extended time course have a median lifespan of 28 days (M. Bowerman et al., 2012(Cerveró, 2018 #305).

SMN-deficiency has been shown to cause alterations to RNA processing in multiple models of SMA, yet studies conducting whole transcriptome or bulk tissue sequencing have been unable to link processing defects to specific motor neuron degeneration. Therefore, we first sought to characterize spinal motor neuron-specific changes to ribosome-associated mRNA present at pivotal time points in disease pathology, before the onset of obvious symptoms and at a late stage of disease progression. We confirmed Cre-dependent expression of RPL22-HA in spinal motor neurons using immunohistochemistry to visualize HA and ChAT immuno-positive cells predominantly located in the ventral horn (**Figure 1B**). Once target genotypes were achieved, a representative cohort of *RC::Smn*$^{2B/+}$ (n=4) and *RC::Smn2B/-* (n=4) were monitored to verify comparable phenotypes previously described in the *Smn2B* mouse model. No deviations in phenotype or survival were observed in the RC2B mice. Comparable to previously published examination of *Smn*$^{2B/-}$ pathology, *RC::Smn*$^{2B/-}$ mice displayed significant reductions in weight by P12, locomotor deficits by P16, and survived a median of 25 days. Spinal cord collection, RPL22-HA affinity purification, and RNA-seq were performed for 3-4 biological replicates for each of 4 unique experimental groups: P9 *RC::Smn*$^{2B/+}$ (early wild-type), P9 *RC::Smn*$^{2B/-}$ (early SMA), P19 *RC::Smn*$^{2B/+}$ (late wild-type), and P19 *RC::Smn*$^{2B/-}$ (late SMA). Tissue homogenization and immunoprecipitation of the HA-tag confirmed our ability to highly enrich for HA-RPL22 within a heterogenous tissue population (**Figure 1C**). Following immunoprecipitation, ribosome-associated mRNA was isolated and RNA integrity number (RIN) evaluated. Only samples displaying a RIN of 7 or greater were selected. Sample mRNA was then amplified using random priming, converted into cDNA, linearly amplified, fragmented, and prepared as a sequencing library. RNA-sequencing was performed using an Illumina Hiseq5000 platform.

To evaluate levels of unspecific background, negative control samples (*RiboTag*$^{+/+}$*;Cre*$^{-/-}$) were collected for each genotype and time point and run in parallel. The total number of mapped reads per

sample ranged between 42,763,880 and 44,570,352; between 70% and 87% of reads were uniquely mapped to the reference genome. Transcripts were assembled and transcript abundance measured in transcripts per million (TPM). Differentially expressed genes (DEGs) were determined across replicate samples by applying a cutoff of Log2Fold >1 and p value < 0.05 using DEseq. Expression profiles were compared between wild-type and SMA cohorts at P9 and P19, time points coinciding with early and late stage disease pathology (M. Bowerman et al., 2012) (Cerveró et al., 2018). Principle component analysis (PCA) displayed appropriate sample segregations based on age (PC 1) in relation to genotype (PC 2) (**Figure 2A**). A noticeable increase in variance could be observed between SMA and WT P19 samples on PC2, suggestive of profound disease-related changes to the motor neuron translatome. Hierarchical clustering between WT and SMA translatomes displayed distinct groups at P9 and P19 time points (**Figure 2B**). The extent of translatome dysregulation between SMA and control mice was less pronounced at the early-symptomatic (P9) with only 85 genes changed, respectively (**Figure 2C, D**). Importantly, this finding provides further evidence that SMN-dependent alterations in splicing do not lead to widespread changes in mRNA expression in early-symptomatic motor neurons (Baumer et al., 2009). By P19, the number of significantly dysregulated transcripts had increased to 1,051, likely as a consequence rather than the cause of neurodegeneration.

2.2.2 Upregulation of p53-mediated signaling precedes the onset of NMJ denervation and motor neuron loss

At P9, we identified 67 genes upregulated within SMA motor neurons (**Table 1**; **Figure 2D**). The number of upregulated genes increased to 471 by P19, 40 of which displayed continued upregulation from P9. Gene ontology (GO)-analysis of upregulated transcripts at P9 and P19 showed a strong enrichment of genes involved in p53-mediated signaling (**Figure 3A, B**). Similar observations have been noted in motor neuron transcriptomes from multiple models of SMA, including embryonic stem cell-derived motor neurons and from motor neurons in intermediate (Murray, Beauvais, Gibeault, Courtney, & Kothary, 2015) and severe mouse models of SMA (Baumer et al., 2009). Genes in the GO category “Signal

transduction as mediated by p53" and showing a robust upregulation in P9 SMA motor neurons included, but were not limited to, Fas, Perp, Gste1, and Cdkn1a (**Table 1**). Of note, Cdkn1a showed the highest up-regulation (3.83 Log2FC) in P9 SMA motor neurons compared to WT. At P19, GO-term analysis for biological processes identified 51 enriched pathways among the up-regulated transcripts (**Fig 3C**), including a further enrichment of up-regulated transcripts related to p53 signal transduction. Of note, Fas, Car12, and Perp were highly upregulated, with the p53-mediated pro-apoptotic protein Pmaip1 (Log2 FC 2.9) being the most up-regulated transcript in P19 SMA motor neurons (**Table 1**).

2.2.3 Down-regulated transcripts suggests SMN-deficiency selectively affects specific motor neuron subtypes

Although the 18 transcripts down-regulated at P9 did not produce a significant GO-term pathway, the contents of this list have notable associations to motor neuron development and disease. Here, we observed a significant 1.12 Log2 fold down-regulation of Chondrolectin (Chodl) at P9 that progresses to a 3.2 Log2FC down-regulation by P19 (**Table 2**). Layilin (Layn), a hyaluronan receptor and Chodl ortholog, was also shown to be down-regulated at P9 (1.1 Log2FC) and P19 (2.5 Log2FC). Previously, Chondrolectin (Chodl) has been found to be highly expressed in the mouse and human spinal cord, and has been implicated in motor neuron axon growth during development (Zhong et al., 2012). Additionally, Chodl has been shown to affect cell survival and rescue motor neuron outgrowth defects in zebrafish with reduced levels of SMN (Sleigh et al., 2014). Defects in Chodl splicing and expression have also been observed in multiple models of SMA (Baumer et al., 2009; Murray et al., 2015; Sleigh et al., 2014). Glutamate ionotropic receptor NMDA type subunit 3B (Grin3b) encodes for the protein NR3B, and displayed a 1.38 Log2FC down-regulation at P9 and a further reduction to 2.64 Log2FC at P19 (**Table 2**). It is primarily found in motor neurons and increases in expression during early postnatal development to influence cell excitability. Altered expression of NR3B has been implicated in motor neuron disease, including SMA (Fukaya, Hayashi, & Watanabe, 2005) BCL2 Associated Athanogene 3 (Bag3), Transmembrane Protein 175 (Tmem175), and Solute Carrier Family 35 Member D3 (Slc35d3) show an

early down-regulation at P9, and have all been linked to the regulation of autophagy in neurons(Gamerdinger, Kaya, Wolfrum, Clement, & Behl, 2011; Jinn et al., 2017; Wei et al., 2016).

The drastic increase in the amount of differentially expressed genes at P19 reflects the phenotypic severity of disease at this stage. Of the 18 down-regulated transcripts identified at P9, 14 remained down-regulated at P19 (**Figure 2D**). The total number of down-regulated transcripts increased dramatically to 561 at P19. R-spondin 2 (Rspo2; Log2FC 2.99) promotes the clustering of acetylcholine receptors and NMJ function and emerged as one of the top 10 down-regulated transcripts at P19 (Li, 2018). Potassium inwardly-rectifying channel, subfamily J, members 12 and 14 (Kcnj12 and Kcnj14) also appeared highly down-regulated at P19 (Log2FC 2.6 and 2.7) (**Table 2**), and participate in controlling MN excitability (Murata, Yasaka, Takano, & Ishihara, 2016; Töpert et al., 1998).

Using GO-term analysis to determine pathway enrichment by biological processes (BP), we identified pathways involved in mitochondrial assembly and function, and cation transport enriched within P19 down-regulated transcripts (**Figure 3C**). Such pathways include mitochondrial respiratory chain complex assembly, mitochondrion organization, and NADH hydrogenase complex assembly. Although mitochondrial defects have been described in animal models of SMA and human patient tissue, the late emergence of down-regulated mitochondrial components suggests mitochondrial impairment is likely not a cause of disease onset but rather a consequence of SMN depletion and motor neuron degeneration (Acsadi et al., 2009; Nimrod Miller et al., 2016; Ripolone et al., 2015; C. C. Xu et al., 2016). KEGG pathway analysis of the P19 down-regulated translatome (**Figure 3D**) revealed an enrichment of transcripts involved in amyotrophic lateral sclerosis (ALS), Alzheimer's disease, and ribosomes, suggesting many of the DEGs identified at this late time point may represent a broad signature of neurodegeneration.

2.2.4 Translatome Findings Reflect Changes in Protein Expression

In order to validate our translatome data and further investigate the motor neuron populations affected by dysregulated RNA processing, we evaluated protein expression of target candidates within spinal cord lysates from P19 mice. The absence of a significant difference in candidate protein expression in total spinal cord lysate (**Figure 4A-C, Figure 5A**) suggested the prominent down-regulation we observed in our translatome data may be unique to motor neurons, and not a ubiquitous effect of SMN deficiency in the spinal cord. To test this hypothesis, we performed immunofluorescent labeling in the L1-L3 region of P19 RPL22-HA spinal cord. BAG3 and MMP9 presented as highly dysregulated ribosome-associated transcripts at P9 and P19, therefore, we sought to examine protein expression in SMA motor neurons. Though present in multiple cell types in the spinal cord, BAG3 and MMP9 displayed the highest expression in laminae VIII and IX motor neurons.

Given its role in regulating autophagy and cell survival, we performed a similar analysis of Bag3 protein expression. We observed high levels of expression in wild-type motor neurons and a significant reduction in expression when compared to P19 SMA motor neurons (**Fig 4D, E**). Recent studies have suggested MMP9 is selectively expressed by fast motor neurons (Kaplan, 2014 #341). SMA motor neurons displayed a significant reduction in MMP9 immuno-reactivity at P9 and P19 (**Figure 5B, C**). While evaluating MMP9 expression within P19 spinal motor neurons, it became evident that MMP9 expression was absent in a higher percentage of SMA motor neurons as compared to wild-type. Indeed, quantification of the total number of MMP9-immunoreactive motor neurons between genotypes at P19 revealed 90.5% of P19 SMA wild-type spinal motor neurons expressed MMP9 as compared to the 69.5% in P19 wild-type animals (**Figure 5D**). This finding suggests MMP9 expression may represent a population of motor neurons more susceptible to degeneration in SMA.

2.2.5 Aberrant Splicing Can be Detected in the Translatome of Early Symptomatic and Late Symptomatic Motor Neurons

Given the well-documented role of SMN in spliceosomal snRNP levels, assembly, and function, we sought to identify cell type-specific changes to alternative splicing present in P9 and P19 SMA motor

neuron translatomes. Therefore, we used rMATs software to evaluate changes in mRNA isoform structure based on 5 annotated categories of alternative splicing: Skipped Exons (SE), Retained Introns (RI), Mutually Exclusive Exons (MXE), Alternative 5' Splice Site (5'SS), and Alternative 3' Splice Site (3'SS) (**Figure 6A**) (Katz, Wang, Airoldi, & Burge, 2010). SMN deficiency led to 1,168 (#genes) events at P9 and 1,139 events (genes) by P19, respectively (l ΔPsi l > 0.1, Bayes Factor > 20, FDR<=0.05) (**Figure 6B**). Of the splicing categories assessed, the largest number of events belonged to the skipped exons (SE) category (926 significant changes at P9 and 788 at P19). An overlap of 259 genes with skipped exons were identified across P9 and P19 samples (**Figure 7)**. At both time-points, Mdm2 appeared as the gene undergoing the highest levels of exon skipping in SMA motor neurons (**Table 3**). SMN-dependent Mdm2 exon skipping has been demonstrated in multiple cellular models, and been shown to play a direct role in p53-mediated motor neuron death in a mouse model of SMA (Van Alstyne et al., 2018). Other transcripts that have previously been identified as undergoing exon-skipping in SMN-deficient models include, but are not limited to: Chodl, slc30a9, Snrpa1, Castf2, Uspl1, and Agrn.

We also identified numerous novel SMN-dependent alternatively spliced transcripts in the SE category. Pathways related to protein translation and ubiquitination were found to be enriched within P9 SE transcripts. Performing GO-term analysis of P19 SE genes, we identified several affected pathways enriched with genes involved in, but not limited to, cellular protein localization, cytoskeleton organization, intracellular transport, protein transport, and cellular modification (**Table 4**). Mutually exclusive exons (MXE) were the second most observed alternative splicing event, with 110 events identified at P9 and 165 at P19 (**Figure 6B**). Work from our lab and others have shown defects in the axonal localization of mRNA in SMN-deficient cellular models (Fallini et al., 2016). Localization signals are often housed in 5' and 3' untranslated regions (UTRs) of mRNA, and are necessary for interaction with RNA binding proteins. In our analysis, we identified 72 3' UTR alternative splice site events (3'SS) at P9 and 52 events at P19 (**Figure 6B**). Within these events, 13 genes displayed alternative splicing at both P9 and P19 (Figure). GO-term analysis performed on the list of 3'SS transcripts at P19 revealed a

significant enrichment of genes involved in the regulation of RNA splicing. Retained intron (RI) events were the least observed of the alternative splicing categories, with 10 events (10 genes) observed at P9 and 31 events (30 genes) at P19 (**Figure 6B**). Six genes undergoing RI events were identified at both time points (**Figure 7**). Of the 1,079 DEGs identified in the P9 and P19 translatome analysis, only 68 genes (6.3%) appeared to undergo alternative splicing events, suggesting that altered gene expression found in our translatome analyses are primarily not a direct consequence of aberrant splicing.

2.3 Discussion

While many neurodegenerative diseases are caused by genetic defects in widely expressed genes with general cellular functions, they present with a selective degeneration of specific neuronal cell types (Fu, 2018 #412). Understanding the cell-type specific susceptibility has remained an unanswered question that is essential for understanding the molecular of basis of pathology in neurodegenerative diseases. With the advent of novel methods for the cell-type specific profiling in intact animals, it has become possible to perform spatially restricted omics studies *in vivo* (Jung & Jung, 2016). Here, we combined cell-type specific expression and isolation of ribosome-associated RNA to perform quantitative transcriptomics and reveal novel changes in RNA expression and splicing within motor neurons from an intermediate mouse model of SMA at early and late stages of pathology. The RiboTag methodology has been used to reveal novel and crucial changes in wide range of cell-types and diseases (Ceolin et al., 2017; Kang et al., 2018; Elisenda Sanz et al., 2009) Although ChAT is also expressed in a small population of interneurons in the spinal cord (Rozani et al., 2019), its robust and predominant expression in spinal motor neurons make it an ideal promoter to designate our neuronal population of interest. Moreover, ChAT-Cre mice have shown better recombination efficiency than Hb9-Cre driver mice, leading to better rescue of the SMA phenotype with conditional SMN expression in motor neurons (Paez-Colasante et al., 2013).

Characterizing the motor neuron translatome at the P9 early symptomatic stage prior to widespread pathology and any obvious phenotypic abnormalities permitted the identification of dysregulated components and pathways closely associated with disease onset and pathomechanisms,

rather than downstream consequences of neurodegeneration. With rapid onset and disease progression resulting in significant reductions in weight and locomotor abilities by P14, the evaluation of the motor neuron translatome at P19 provides a critical snapshot of gene dysregulation coinciding with severe disease pathology, but may also identify protective factors for remaining motor neuron subpopulations. The findings in this study validate and expand on previously described candidates with the identification of novel genes and molecular pathways whose dysregulation may contribute to the pathological mechanisms by which SMN depletion leads to the degeneration of motor neurons.

The extent of gene dysregulation observed coincided with disease phenotypes at P9 and P19, and affected mRNAs of functionally diverse genes pathways. The identification of only 85 dysregulated genes at P9 supports the modest differential gene expression observed at early/pre-symptomatic time-points in more severe mouse models of SMA, and further suggests SMN-deficiency does not cause extensive changes to gene expression early in the disease (Baumer et al., 2009; Murray et al., 2015; Z. Zhang et al., 2013). Although GO-term analysis could not identify any enrichment for a particular pathway within P9 down-regulated, several of these genes have been linked to neuronal function and survival. There is increasing evidence that motor neuron susceptibility may be dictated by motor neuron subtype, with fast-fatiguable (FF) α-motor neurons function and survival often being the most affected in motor neuron diseases (Hedlund, Karlsson, Osborn, Ludwig, & Isacson, 2010; Kanning, Kaplan, & Henderson, 2010). Electrophysiological recordings coupled to cell-type specific transcriptomics have facilitated the characterization of unique gene expression profiles across motor neuron subtypes. Calcitonin gene-related peptide (CGRP)/*calca*, *chodl*, *mmp9*, and *spp1* (Osteopontin) have been described in multiple studies as potential markers of Fast-type MNs (Kaplan, 2014 #341)(Enjin, 2010 #410)(Manuel, 2019 #411). Our finding of several of these transcripts being down-regulated at an early symptomatic time-point suggests either a global reduction across motor neuron subtypes, or an early and selective loss of these subtypes. Rho GTPase activating protein 9 (Arhgap9) emerged as the one of the most down-regulated ribosome-associated RNAs in SMA motor neurons at both P9 and P19. Arhgap9 is a member of the Rho-GAP

family of GTPase activating proteins, and has activity towards Cdc42 and Rac1 (Sakakibara, Nemoto, Nukiwa, & Takeshima, 2004). Little is known about the function of Arhgap9 in neuronal populations. However, in a study to determine factors that influence motor neuron vulnerability and resistance in a SOD1 mouse model of amyotrophic lateral sclerosis (ALS), both Arhgap9 and MMP9 were identified as genes that are selectively expressed in motor neurons with increased susceptibility to degeneration (Kaplan et al., 2014). Reducing MMP9 levels in adult ALS mice preserved muscle innervation, motor neuron number and lifespan, yet complete loss of MMP9 (*MMP9-/-*) in developing ALS mice led to unwanted side effects including an increase in premature deaths (Spiller et al., 2019). Despite a noticeable inability to run as fast as ALS;MMP9+/+ mice, MMP9 knockout mice retained more motor neurons. It should also be noted that using a Chodl-Lacz mouse model, the authors observed 96% of MMP-9 positive motor neurons also expressed Chodl. As both Chodl and MMP9 have been linked to motor neuron survival in diseases, further characterization of motor neuron populations co-expressing these markers could offer insight into the molecular mechanisms. Here, the MMP9 downregulation observed in our SMA translatomic data appears to be driven by a selective loss of MMP9-positive motor neurons, rather than a global reduction of MMP9 expression in all spinal motor neurons. This may be due to a difference in development or selective loss of the FF subtype. Taken together, MMP9 appears to represent a convergent mediator of cell death in response to pathological changes in motor neurons. Future studies aimed at reducing MMP9 expression in motor neurons through pharmacological inhibitors or AAV-mediated shRNA would provide invaluable insight into the molecular profiles that underlie motor neuron vulnerability in SMA.

Multiple studies have described impairment in mitochondrial morphology, function, and axonal transport, including increased oxidative stress levels and organelle fragmentation in mouse models of SMA (Nimrod Miller et al., 2016; C. C. Xu et al., 2016). Upon examining mitochondrial ultrastructure at a presymptomatic stage of disease in the SMNΔ7 mouse model, researchers observed structural abnormalities present in 25% of mitochondria in SMA motor neurons compared to only 3% in littermate

controls. Morphological abnormalities in size and cristae density have been linked to impaired ATP synthesis. The results of our translatomic studies identified pathways associated with mitochondrial structure and function as being significantly dysregulated at a severe time point in disease, and provide further support for the involvement of mitochondria in the pathology of SMA. Nevertheless, we did not observe significant changes in genes related to mitochondria at our early-symptomatic time point, suggesting mitochondrial abnormalities may be a downstream consequence of accumulating disease pathology.

Dysregulated autophagy has gained increasing attention as a pivotal contributor to neurodegenerative diseases. Increased autophagy has previously reported in several cellular and animal models of SMN-deficiency(Custer & Androphy, 2014; Deguise et al., 2016; Piras et al., 2017), and pharmacological inhibition of autophagy delayed motor neuron degeneration and slightly extended survival in a severe mouse model of SMA (Piras et al., 2017). In alignment with the translatome findings, Bag3 displayed a significant reduction in protein expression in SMA spinal motor neurons. Unlike MMP9, we did not observe a meaningful change in the overall percentage of Bag3-positive motor neurons between P9 SMA and WT, and concluded Bag3 down-regulation is a more ubiquitous feature in SMA spinal motor neurons. In its best-characterized role, Bag3 interacts with HSP70 and HSPB8 to mediate chaperone-assisted selective autophagy (Carra, Seguin, Lambert, & Landry, 2008). Bag3 has been implicated in the clearance of protein aggregates associated with multiple age-related neurodegenerative diseases including ALS, Huntington's disease, and Alzheimer's disease (Gamerdinger et al., 2011). As a multifunctional protein, Bag3 has also been shown to participate in regulating cytoskeleton organization and apoptosis (Gamerdinger et al., 2009; Gamerdinger et al., 2011). Additional P9 down-regulated transcripts linked several to the regulation of autophagy in neurons, including Hspb1 (Haidar et al., 2019), Tmem175 (Jinn et al., 2017), and Slc35d3 (Wei et al., 2016). Interestingly, a large genome-wide association study (GWAS) recently identified Tmem175 as a risk factor for Parkinson disease. Tmem175 is potassium channel located in late endosomes and lysosomes, and has been shown to

mediate lysosomal degradation, lysosome-mediated autophagosome clearance, and mitochondrial respiration (Jinn et al., 2017). Autophagosome accumulation and deficits in mitochondrial respiration have been described in cultured motor neurons and SMA mice (N. Miller, H. Shi, A. S. Zelikovich, & Y. C. Ma, 2016; Piras et al., 2017). The significant down-regulation of Tmem175, Bag3, and slc35d3 we observe early in disease pathology may represent central components of disease pathogenesis in motor neurons *in vivo*, coupling the dysregulation of autophagy to mitochondrial dysfunction.

The RNA-seq data sets obtained from spinal motor neurons provided a unique opportunity to also assess splicing alterations. Although hundreds of alternative splicing events were observed at P9 and P19, only ~6% of the aberrantly spliced genes also displayed changes in gene expression, suggesting alternative splicing does not result in widespread changes in gene expression. Given that SMN-deficiency decreases the amount of mRNA trafficked to axons in culture primary motor neurons (Fallini et al., 2016), the novel transcripts identified as having alternative 5' and 3' splice sites in SMA may severe as valuable candidates in further elucidating the effects of SMN depletion to axonal localization, translation, and NMJ maturation. While the identification of exon skipping being the predominant alternative splicing event in SMN deficient tissue is in alignment with previous studies (Custer et al., 2016; Z. Zhang et al., 2013), we observed comparable numbers of alternative splicing present at P9 and persisting until P19. In performing exon array-analysis on whole spinal cord tissue from the severe SMNΔ7 mouse model at varying disease time-points, Baumer et al., did not observe widespread splicing abnormalities until the onset of pathology, and therefore concluded alternative splicing is unlikely to contribute to disease pathogenesis (Baumer et al., 2009). Although significant motor neuron loss and phenotypic abnormalities are not readily observed until after P9, the high degree of alternative splicing present in our data set may be indicative of disease onset and the molecular alterations that precede more overt phenotypes. Of note, changes in neuromuscular morphology and occupancy have been observed in P10 mice, and may suggest NMJ dysfunction is a major contributor to dysregulated splicing in SMA motor neurons (Courtney 2019). Alternatively, given the dependence of SMN expression to motor neuron function and survival, it remains

plausible that SMN-dependent splicing undergoes the greatest level of dysregulation specifically within motor neurons *in vivo*. Identifying cell-type specific splicing dysregulation early in disease may be precluded by bulk tissue homogenization approaches, particularly in neonatal mice in which alternative splicing is paramount to organismal development and maturation. Despite the abundance of alternative splicing present P9 and P19, the overlap between affected genes is low. Heterogeneity between the time points may be the result of altered neonatal development in SMN-deficient motor neurons early in disease, and reflect a developmental delay and maturation defect observed in SMA motor neurons. Subsequently, the population of alternatively spliced genes at P19 may largely be driven by widespread gene dysregulation and cellular degeneration. Studying the aberrantly spliced transcripts present at both time-points and in comparison with previous splicing assessments in various SMA models can advance our understanding of the direct and indirect effects of SMN depletion to RNA processing. In this study we observed a small degree of overlap in aberrant splicing identified in 3 other studies of SMN deficiency in cell and animal models including but not limited to, Mdm2, Uspl1, Agrn, Chodl, Tesc, Snrpa1, Nup88, and Dcxr (Supplemental Table) (Custer et al., 2016; Huo et al., 2014; Jangi et al., 2017). Alternatively spliced transcripts present at P9 and P19 may serve as the best candidates for further examination of SMN-dependent RNA processing. It should be noted that differences in the number and type of alternative splicing events identified are likely influenced by our experimental model. The RiboTag system relies on cell-type specific expression and purification of a tagged ribosomal protein to selectively isolate ribosome-associated mRNA. Thus, the necessary association with a ribosome could largely exclude alternative splicing events that effect transcript stability or translation. Alternatively, splicing perturbations identified by this method may have a higher likelihood of undergoing translation and producing protein isoforms with divergent functions. It will be important to evaluate if splicing alterations identified by transcriptomic data can be observed on the protein level, and whether these modifications influence disease pathology.

The mechanisms by which upregulated p53 signalling affects SMA pathology remain unclear. In response to stress stimuli or injury, cells initiate a p53-mediated signaling cascade which upregulates pro-apoptotic factors (Nag, Qin, Srivenugopal, Wang, & Zhang, 2013). In healthy cells, p53 is maintained at nearly undetectable levels by the E3 ligase MDM2, which targets p53 for proteasomal degradation (Kulikov et al., 2010; Nag et al., 2013). In addition to direct inhibition by p53 downstream targets, SMN-dependent splicing alterations to MDM2 mRNA leads to the production of an MDM2 isoform missing exon 3 and thus its ability to interact with p53 and facilitate its degradation (Van Alstyne et al., 2018). This MDM2 isoform excluding exon 3 was abundant in our P9 and P19 data set, undergoing the highest level of exon skipping of all alternatively spliced transcripts identified at both time points. Notably, we observed an early and significant upregulation of Cdkn1a which encodes the stress-response protein p21. SMN depletion has been shown to increases p21 transcript stability (Tadesse et al., 2008). Upregulation of p21 is a cellular response to stress, often favoring survival but resulting in cell senescence (Dotto, 2000). While p53 has been shown to induce expression of p21 (He et al., 2005), the association between p21 and p53 in SMA remains unclear. Upregulation of p53 signaling has been observed in multiple models of SMN-deficiency, yet studies attempting to link p53 directly to motor neuron death in SMA have yielded conflicting results. In one study expression and phosphorylation-dependent activation of p53 was shown to occur early in vulnerable motor neuron populations of the *SMNΔ7* mouse model, extending into more resistant motor neuron populations late in disease pathology (Simon et al., 2017). Pharmacological inhibition or targeted knockdown of p53 significantly improved motor neuron survival and suggested p53 activity is the key function driving motor neuron degeneration in SMA (Simon et al., 2017). Moreover, phosphorylation of serine 18 on p53 was shown to be a distinctive event restricted to particular motor neuron populations in disease and underlying cell susceptibility (Simon et al., 2017). To further investigate the link between p53 and SMA pathology further, a second group crossed a p53 inducible knockout mouse line with the $Smn^{2B/-}$ intermediate mouse model of SMA to generate an SMA mouse model lacking p53 expression. Here, post-natal homozygous deletion of p53 increased in the preservation and function of NJMs but was unable to alter the time course and extent of motor neuron loss

in *Smn*$^{2B/-}$ mice (Courtney, 2019 #339). Similarly, an early study crossed the severe mouse model of SMA (*Smn*$^{-/-}$; *hSmn2*$^{+/-}$) with p53 knockout mice and observed no change in disease severity or lifespan (Tsai, Chiu, Wang, Hsieh-Li, & Li, 2006). While p53 may have a role in regulating axonal degeneration in spinal motor neurons, these findings suggest motor neuron loss is p53 independent. Redundancies between p53 and other apoptotic pathways could exist and explain motor neuron cell death even in the absence of p53. The death receptor Fas has largely been implicated as a key component of p53 activation and apoptosis. Nevertheless, induction of Fas upregulation, activation, and cell apoptosis has been observed in the absence of p53 (S. G. Kim et al., 2002). In our hands, we observed an early and persistent upregulation of Fas in the SMA motor neuron translatome. It remains a possibility that Fas activation could lead to motor neuron death in a p53 independent manner. Further examination of the functional contributions of dysregulated candidates involved in apoptosis may reveal the missing link between p53-mediated signaling and motor neuron death.

Our findings provide a comprehensive overview of changes in RNA expression and processing present in motor neurons throughout the spinal cord during key stages in SMA pathology. It should be noted that motor neuron susceptibility in SMA varies between spinal cord segments and between subtypes. The multiple markers associated with FF motor neurons we observed down-regulated at P9 and P19 may serve as identifiers for vulnerable populations of spinal motor neurons. Performing a comparison between these highly degenerative populations and resistant populations can further elucidate targets underlying motor neuron loss in SMA. Additionally, evaluating the functional role of the dysregulated or alternatively spliced targets identified in this study would further reveal novel underlying and/or contributing mediators of motor neuron vulnerability and could serve as valuable therapeutic targets for motor neuron preservation in SMA, as well as other motor neuron diseases.

2.4 Tables and Figures

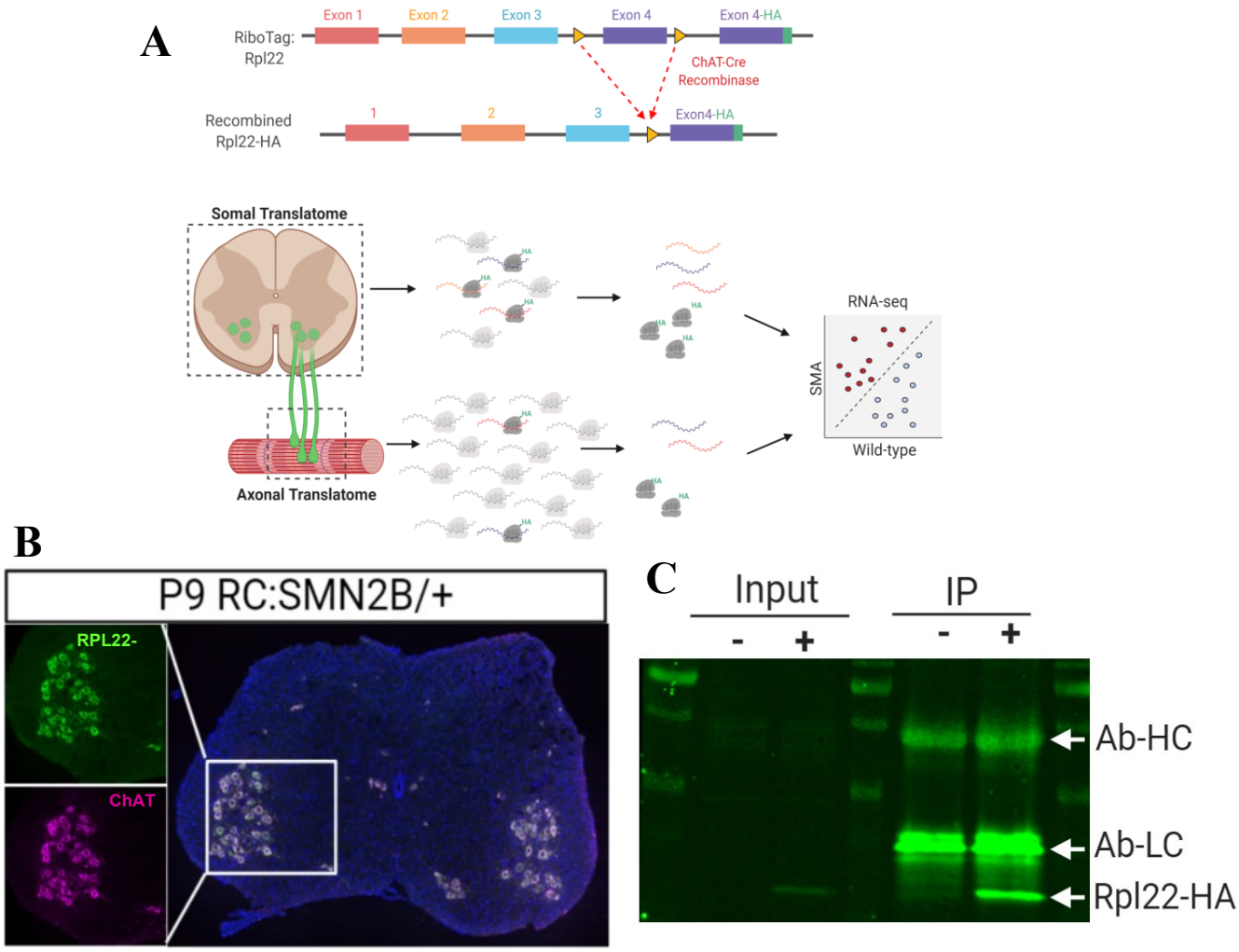

Figure 1. Illustration of RiboTag strategy to achieve motor neuron-specific ribosome tagging and "translatome" analysis *in vivo*. A) The RiboTag mouse is crossed to mouse line expressing Cre-recombinase driven by choline acetyltransferase (ChAT), results in the deletion of wild-type exon 4 and replacement with the HA-tagged exon 4 only in cholinergic neurons. **B)** Motor neurons are the predominant cholinergic population in the mouse spinal cord, and will now contain RPL22-HA-tagged ribosomes. Tagged ribosomes are present in the motor neuron soma as well as the presynaptic terminal innervating muscles. **C)** Once tissue is harvested and homogenized, tagged ribosomes can be immunoprecipitated from the heterogeneous tissue lysate. Associated mRNA can then be isolated for RNA-sequencing.

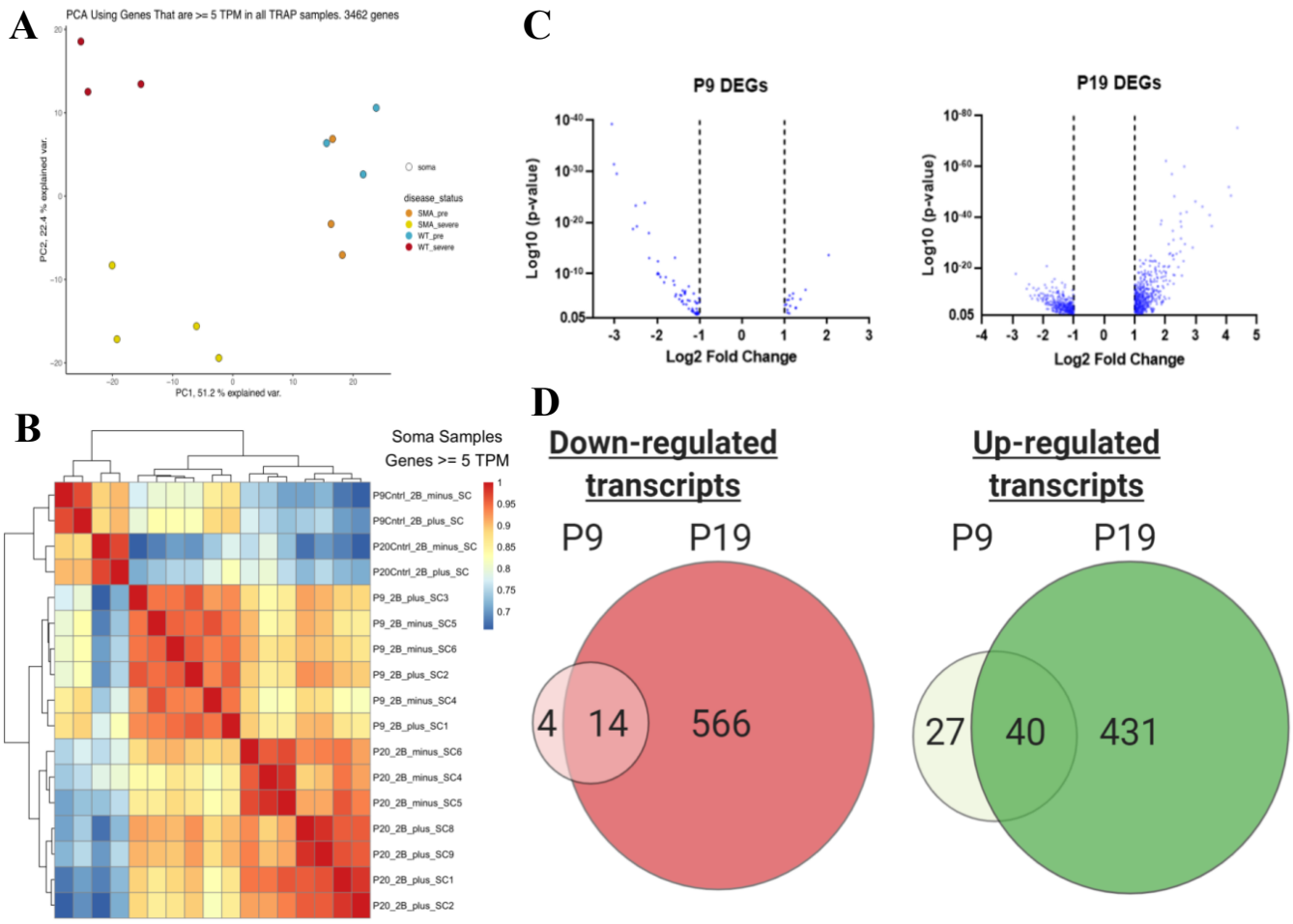

Figure 2. Dysregulation of the motor neuron translatome in SMA coincides with disease severity
A) PCA of P9 and P19 motor neuron RNA-seq transcriptomes. *n* = 3–4 biological replicates per group. B) Heatmap of top expressed genes present in experimental samples (RC+) and control samples from P19. Volcano plots of DEGs (Log2FC > 1 or <-1) at P9 and P19 SMA motor neurons. D) Venn diagram illustrating similarities and differences of DEGs identified at early and late symptomatic time points.

Table 1. Top 20 statistically upregulated transcripts in P9 and P19 SMA motor neurons

P9 Upregulated Transcripts (Top 20)			
Gene Symbol	**Gene name**	**log2FC**	**p-value adj.**
Cdkn1a	cyclin dependent kinase inhibitor 1a	-3.83117	2.24e-78
Dcxr	dicarbonyl and l-xylulose reductase	-3.06712	5.25e-40
Plek2	pleckstrin 2	-3.01629	3.80e-32
R3hdml	r3h domain containing like	-2.95434	2.72e-30
Krt13	keratin 13	-2.57143	1.67e-19
1700007K13Rik		-2.50383	4.41e-24
Fas	fas cell surface death receptor	-2.48026	5.19e-20
Zfp87	zinc finger protein 87	-2.29083	1.32e-24
Abhd11os	(long noncoding rna)	-2.19554	1.10e-18
Trim47	tripartite motif containing 47	-2.18892	8.12e-14
C130080G10Rik	nc rna	-1.99456	1.16e-10
Spc25	spc25, ndc80 kinetochore complex component	-1.98245	3.55e-13
A530058N18Rik	ncrna	-1.98165	7.02e-11
Rhod	ras homolog family member d	-1.96222	1.16e-10
Csrp3	cysteine and glycine rich protein 3	-1.92498	4.20e-10
Gdf15	growth differentiation factor 15	-1.83532	4.83e-09
Pidd1	p53-induced death domain protein 1	-1.79658	4.42e-10
Hbb-bt	hemoglobin subunit beta	-1.61785	2.37e-09
Ddit4l	dna damage inducible transcript 4 like	-1.59426	1.50e-08
Crip2	cysteine rich protein 2	-1.5789	7.12e-14
P19 Upregulated Transcripts (Top 20)			
Pmaip1	phorbol-12-myristate-13-acetate-induced protein 1	-2.89703	1.6584E-18
Lamc2	laminin subunit gamma 2	-2.53515	1.3643E-12
Lgals3	galectin 3	-2.46069	2.6652E-14
Alox5	arachidonate 5-lipoxygenase	-2.41775	6.9923E-10
Prr15l	proline rich 15 like	-2.36483	9.5194E-09
C130080G10Rik	nc rna	-2.31799	5.7686E-13
Prss56	serine protease 56	-2.2774	4.7385E-10
Itgad	integrin subunit alpha d	-2.22139	4.9409E-12
Sbsn	suprabasin	-2.194	1.0132E-08
Xdh	xanthine dehydrogenase	-2.16782	5.3835E-08
Rhod	ras homolog family member d	-2.15092	3.8951E-10
Slc22a7	solute carrier family 22 member 7	-2.15036	1.0371E-12
Perp	perp, tp53 apoptosis effector	-2.13276	6.803E-10
Rgs12	regulator of g protein signaling 12	-2.07852	9.2996E-11
Spc25	spc25, ndc80 kinetochore complex component	-2.07568	7.3737E-09
Tfcp2l1	transcription factor cp2 like 1	-2.01681	3.3343E-08
Car12	carbonic anhydrase 12	-2.01413	1.0496E-07

Fam159b	shisa like 2b	-2.01112	9.5667E-11
Crh	corticotropin releasing hormone	-1.9962	1.1947E-08
Bpifc	bpi fold containing family c	-1.98567	3.0861E-09

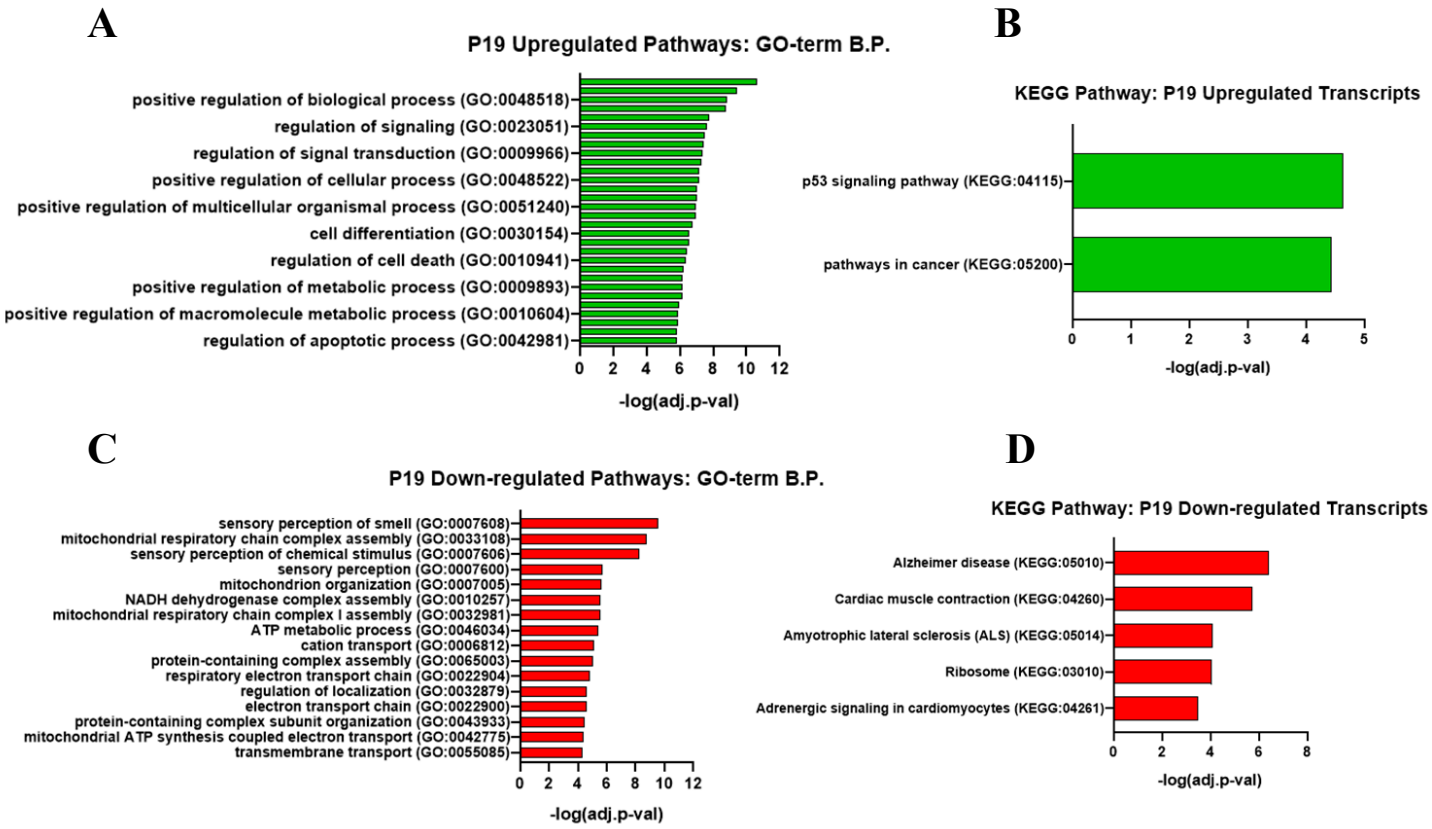

Figure 3. GO-term analysis identifies biological pathways affected in P9 and P19 SMA motor neurons.

Table 2. Top 20 statistically down-regulated transcripts in P9 and P19 SMA motor neurons

P9 Down-regulated MN Somal Transcripts			
Gene Symbol	**Gene Name**	**log2FC**	**p-val adj.**
Arhgap9	Rho GTPase activating protein 9	2.041867	2.21E-14
Cpeb2	cytoplasmic polyadenylation element binding protein 2	1.494543	1.38E-07
Grin3b	glutamate ionotropic receptor NMDA type subunit 3B	1.3778	9.11E-06
Gm4117		1.270851	0.000459
Ighm	immunoglobulin heavy constant mu	1.262107	0.000459
Calcb	calcitonin related polypeptide beta	1.202552	7.26E-07
Susd2	sushi domain containing 2	1.188414	6.37E-06
Layn	Layilin	1.135261	0.000181
Hspb1	heat shock protein family B (small) member 1	1.121312	1.69E-06
Chodl	Chondrolectin	1.121282	0.000153
Gm10684		1.112249	0.005179
Ptgis	prostaglandin I2 synthase	1.105231	0.005281
Me3	malic enzyme 3	1.09899	1.22E-05
Slc35d3	solute carrier family 35 member D3	1.070582	0.003829
Tmem175	transmembrane protein 175	1.060196	1.28E-05
Mapk11	mitogen-activated protein kinase 11	1.044834	9.49E-05
Mmp9	matrix metallopeptidase 9	1.06985	0.001112
Bag3	BCL2 associated athanogene 3	1.0096	0.000127
P19 Down-regulated MN Somal Transcripts (Top 20)			
Gm5868		4.362887	4.8E-76
Arhgap9	Rho GTPase activating protein 9	4.153598	2.98E-49
Mmp9	matrix metallopeptidase 9	4.084203	1.17E-52
Ighm	immunoglobulin heavy constant mu	3.521421	2.68E-37
Gm43398		3.451362	1.2E-41
Chodl	Chondrolectin	3.215522	5.72E-45
Rspo2	R-spondin 2	2.986367	5.13E-47
Gm10684		2.897195	8.45E-29
Kcnj14	potassium voltage-gated channel subfamily J member 14	2.696895	6.94E-43
Grin3b	glutamate ionotropic receptor NMDA type subunit 3B	2.64211	3.42E-30
Kcnj12	potassium voltage-gated channel subfamily J member 12	2.623519	9.23E-61
Gm37168		2.595679	1.67E-23
Grid2ip	Grid2 interacting protein	2.542024	8.97E-18
Layn	Layilin	2.524072	4.32E-37
Slc35d3	solute carrier family 35 member D3	2.51034	2.31E-32
Hspb6	heat shock protein family B (small) member 6	2.502932	4.81E-39
Cd24a		2.497302	3.52E-42
Tesc	Tescalcin	2.493389	2.85E-27
Wnk4	WNK lysine deficient protein kinase 4	2.429835	3.54E-21
Ptgis	prostaglandin I2 synthase	2.367056	2.09E-26

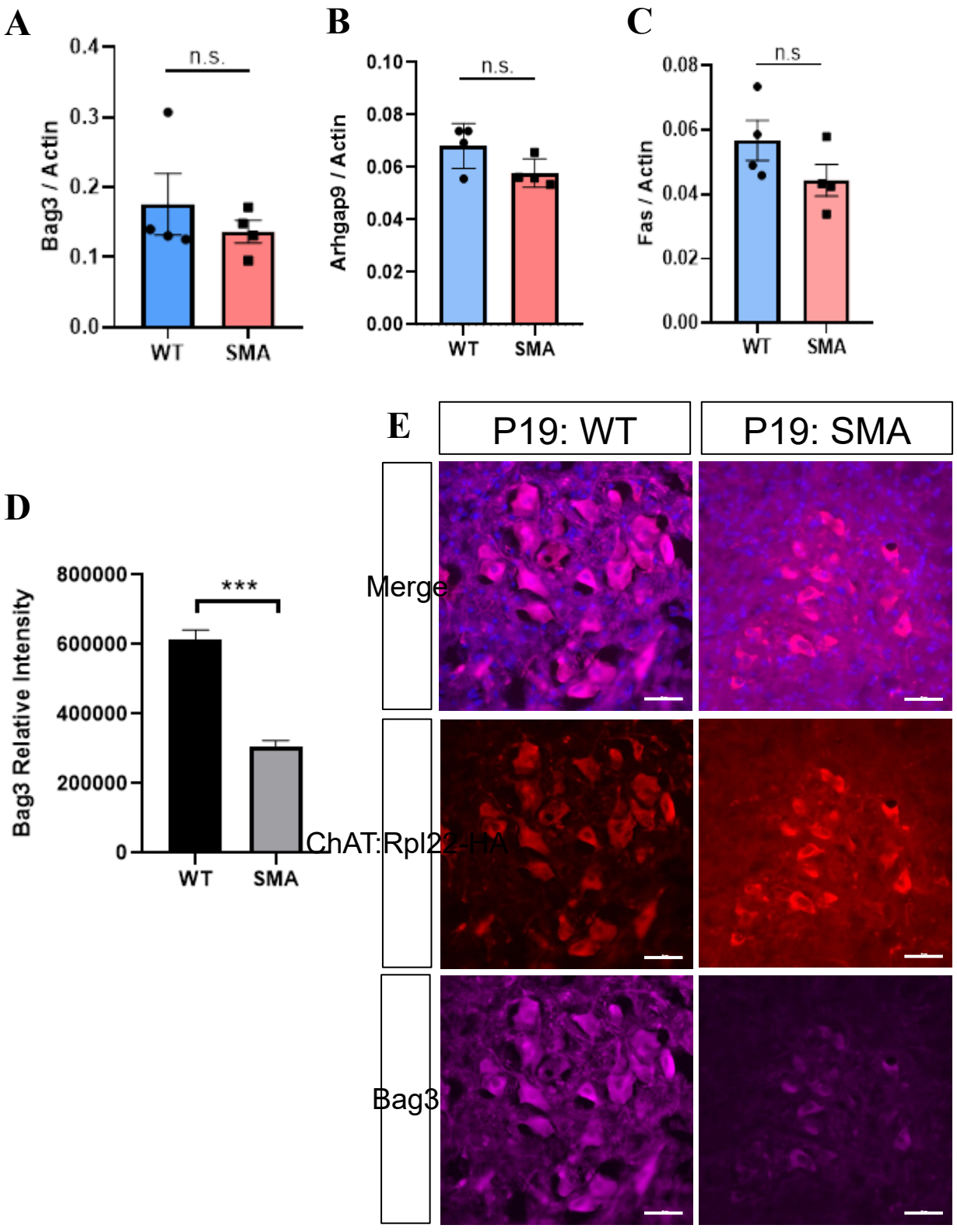

Figure 4. Bag3 protein expression is reduced in P19 SMA motor neurons. A) A difference in Bag3, Arhgap9, and Fas protein expression was not detectable in full spinal cord lysate fromP19 SMA and WT mice. Despite observing no difference in Bag3 protein expression, **B)** Quantification of Bag3 (magenta) in P19 spinal motor neurons (red) revealed a significant reduction in protein expression.

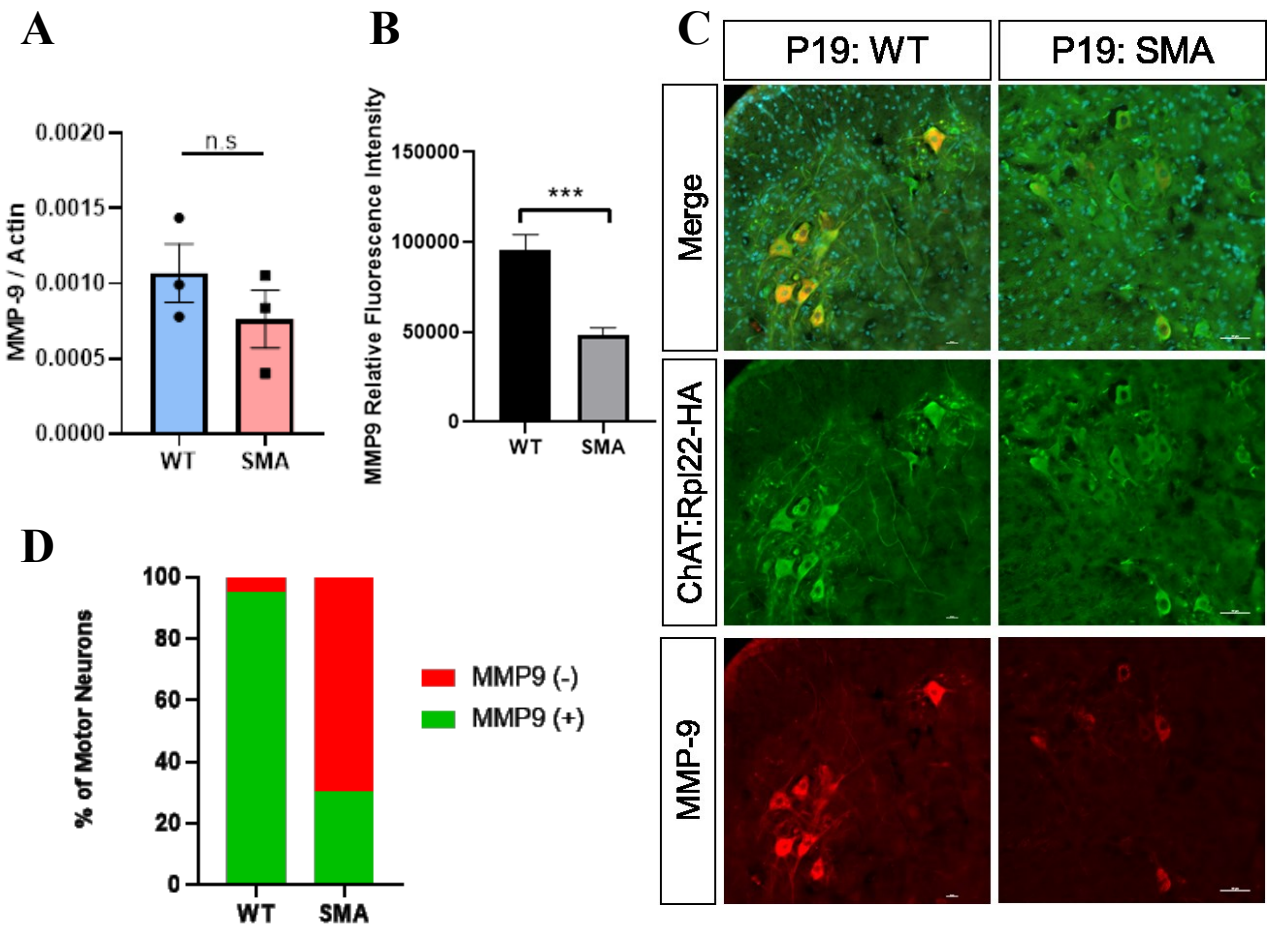

Figure 5. MMP9 protein expression is reduced in P19 SMA motor neurons. A) Despite observing no difference in MMP9 protein expression levels between WT and SMA P19 total spinal cord lysate, **B)** Quantification of MMP9 (magenta) in P19 spinal motor neurons (red) revealed a significant reduction in protein expression.

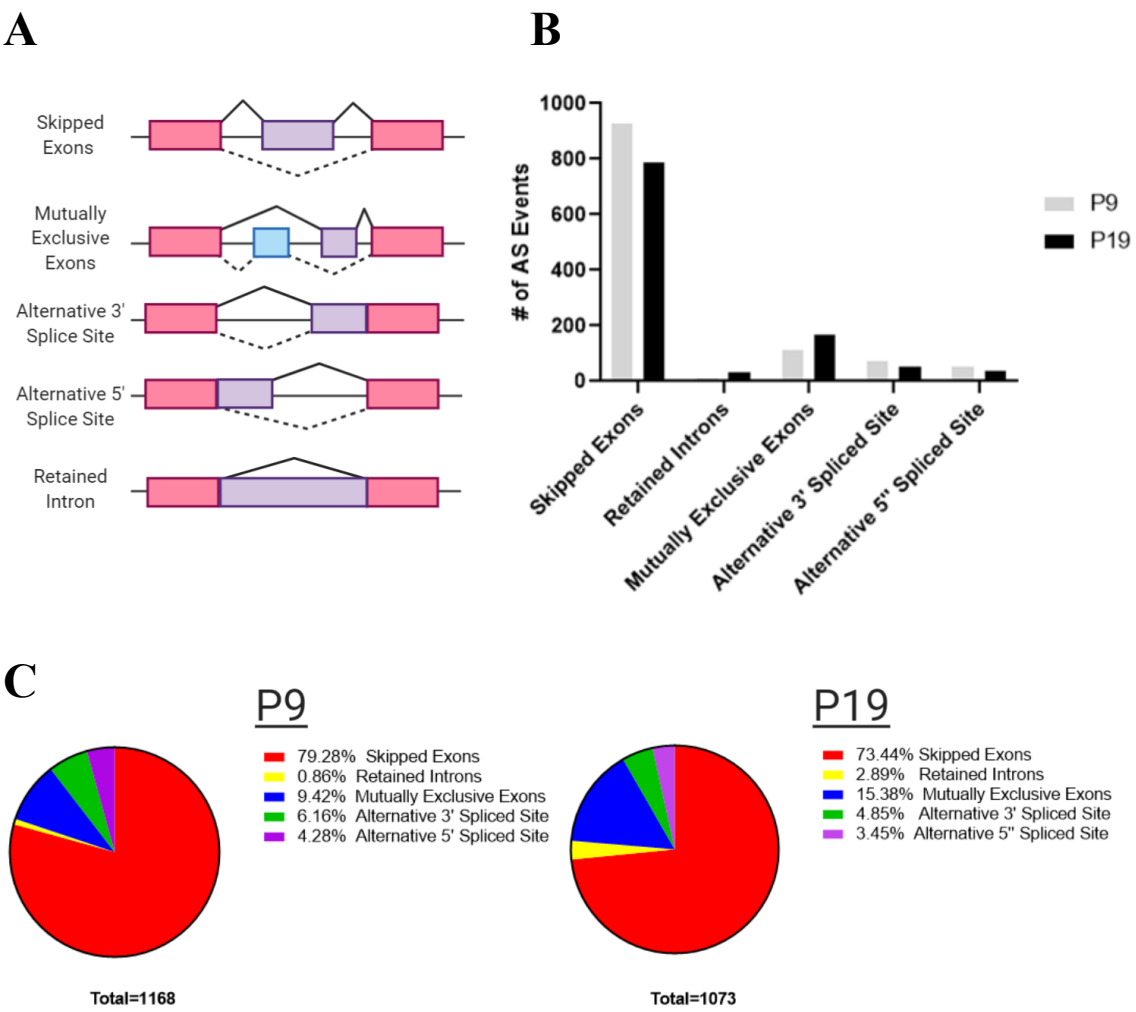

Figure 6. Aberrant splicing is present in SMA motor neurons at early and late stage of disease. A) Illustration of the alternative splicing events categorized in our analysis. **B)** Quantification of the number and type of unique alternative splicing events present at P9 and P19. **C)** Pie chart depicting the abundance of each alternative splicing event by percentage.

Table 4. Top 5 GO term: Biological Process pathways affected by skipped exons in P19 SMA motor neurons.

GOTerm	Term P-Value Corrected with Bonferroni step down	Group P-Value Corrected with Bonferroni step down	Nr. Genes
paranodal junction assembly	7.471E-04	3.097E-06	5.00
regulation of cytoplasmic translation	3.269E-02	5.500E-05	6.00
proteasomal protein catabolic process	4.921E-02	4.142E-05	29.00
cellular component assembly involved in morphogenesis	1.890E-02	1.461E-05	117.00
organelle organization	3.684E-05	2.126E-07	154.00

Top 20 Alternative Splicing Events

	Mdm2	Rab14	Tpd52l1	Neto2	Bdp1
	Nrk3	Arhgef40	Alg3	Nacad	Mmadhc
	RP23-434K24.1	Tesc	Ntrk3	Eed	Tm2d3
	Usp11	Ufsp2	Lrrc20	Gas5	Cntn4
	Iscu	Fxr1	Tfdp2	Glt8d1	Meis2
	Dpp7	Pts	Prkag2	Rbbp5	Tmem55b
	Scrib	Ppp1cc	P2rx6	Zfp13	Slc15a4
	Tpd52l1	Fam133b	Lingo2	Htra2	Rabep2
	Slc30a9	Polr2i	Mllt10	Gas5	Tmem255a
	Prkag2		Tlk2	Dda1	Vldlr
	Hs6st2		Zeb1	Gnpat	Ipmk
	Tspan17		43160	Stx5a	Eci2
	Emc9		Porcn	Mau2	Nup88
	Ythdf2		Atp9b	Htra2	Reps1
	Brsk2		Csrnp3	Tmem80	Zfp612
	RP24-309H3.4		Cacna1a	Clasp1	Mfsd10
	Tsen2		Brinp2	Nae1	Miip
	Zufsp		Syt16	Gapdh	Unc119
	Ifi27		Aifm3	AC099860.1	Sltm
P19	Mdm2	Tcea2	Tpd52l1	Mrps17	Kcnq2
	Enox2	Arhgdig	Alg3	Tmem80	Rcan3
	Cdc37l1	Nrbp2	Mia2	Nacad	Nptn
	Mdm2	Tesc	Mef2a	Paip1	Mus81
	Slc30a9	Snrpa1	Cd38	Tcea2	Reps1
	Pmp22	Faim2	Enthd2	Mrps17	Magi1
	Tpd52l1	Dusp22	Aifm3	Kif2a	Ivns1abp
	RP24-309H3.4	Fxr1	Mtg2	Diablo	Supt4a
	Mical2	Phf24	Cetn4	Zmynd11	Acin1
	RP24-309H3.4	Arhgef40	RP23-146D13.2	Agrn	Hnrnpk
	Smn1	Mapk11	Cadm1	Hnrnpc	Abcd4
	Lgals8	Wdr73	Scn9a	Zfp740	Cep170b
	Dgat1	Wdr83	Gria4	Cul5	Tmem55b
	Fhl1	Taz	Slc30a9	Scg3	Fam98c
	Kctd9	Rsrp1	Zfand3	Ctu2	Dyrk1a
	U2af1	Fibp	Tmem147	Actr6	Palm3
	Armcx5	Galt	Guf1	Adgrb1	Arhgef40
	Pmp22	Tecr	Actn4	Hmgn3	Eif4g3
	Pnkp	Polr2i	Lrrfip2	Smox	Unc119
	Tpd52l1	Plekhj1	Rgs11	Dcaf11	Cox16

Table 3. Top 20 alternatively spliced transcripts from each category. Transcripts that appear multiple times represent multiple isoforms identified.

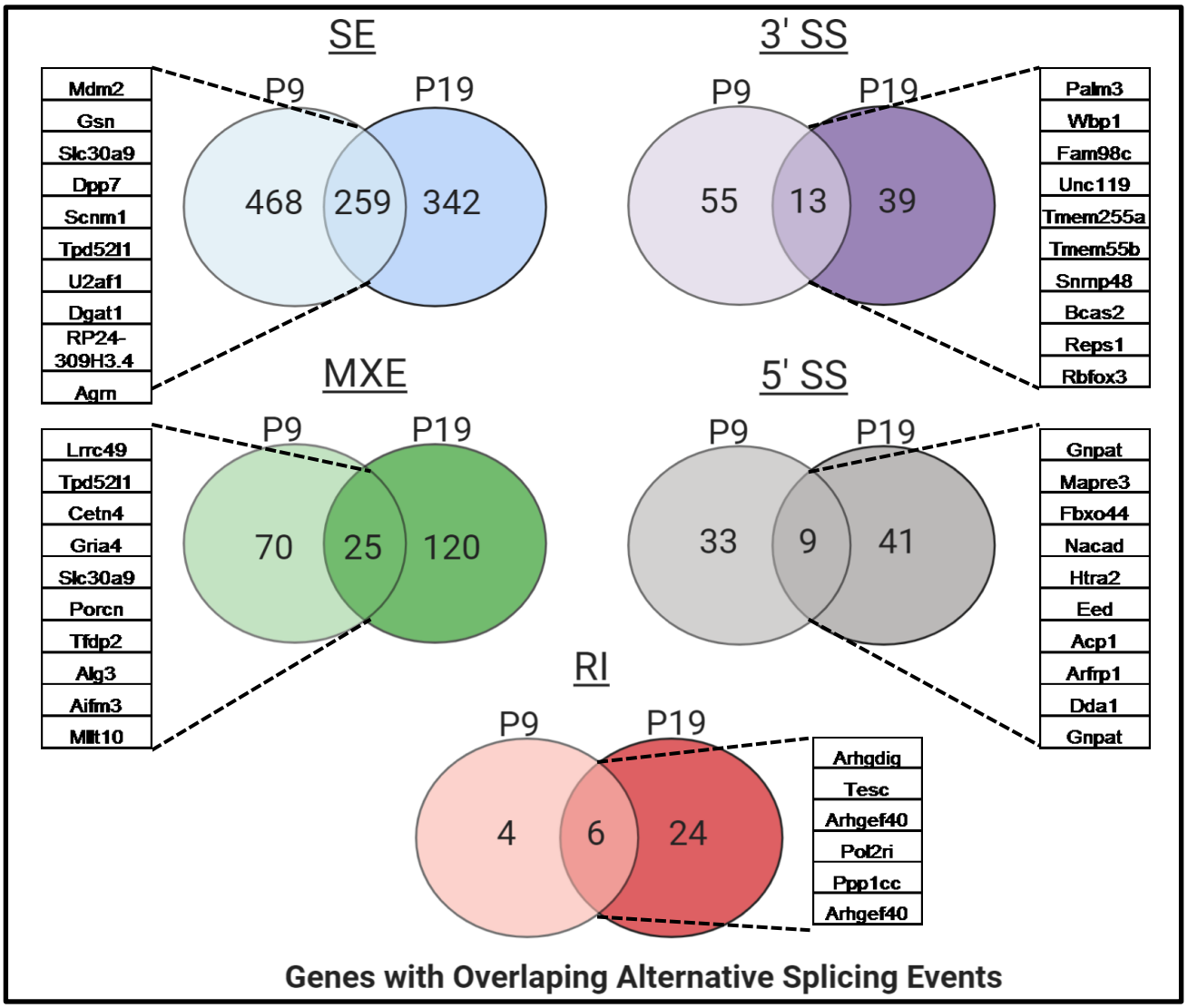

Figure 7. Venn diagram depicting similarities and changes between alternatively spliced genes identified at P9 and P19. Diagrams were constructed for skipped exons (SE), mutually exclusive exons (MXE), retained introns (RI), alternative 5' spliced site (5'SS), and alternative 3' spliced site (3'SS). An abbreviated list of transcripts present at P9 and P19 is provided for each category.

2.5 Characterizing the Axonal Translatome in SMA Motor Neurons *in vivo*

Characterizing the *in vivo* changes to mRNA processing in motor neuron axons is a crucial aspect to understanding the pathological basis of SMN-deficiency. Previous research from our lab and others has shown a reduction in mRNA transport and translation in multiple *in vitro* models of SMA, however, it remains unclear whether these defects occur *in vivo*. To address if mRNA profiles are dysregulated in motor neuron axons *in vivo*, we sought to isolate HA-tagged ribosomes from motor axons innervating susceptible muscle groups. The transversus abdominus (T), rectus abdominis (R), and external oblique (E), are large muscle groups that help maintain thoracic and pelvic stability (**Figure 8A**). A previous study using the *Smn*2B mouse model observed significant denervation of these muscles groups in $Smn^{2B/-}$ P21 mice (Kline et al., 2017; Murray et al., 2015). Therefore, we elected these significantly affected muscle groups, collectively termed (TRE), for the characterization of wild-type versus SMA motor axon translatomes *in vivo*.

2.5.1 Results

A pilot study performed to assess feasibility revealed a faint HA+ band in the input fraction lysate of TREs pooled from 4 mice (TRE-In) (**Figure 8B**). Subsequent immunoprecipitation demonstrated our ability to enrich axon-derived RPL22-HA from motor neurons innervating axons (TRE-IP), thus depleting RPL22-HA from the remaining supernatant (TRE-Sup). From these promising results, we increased our pooled sample number per experimental replicate and proceeded with an optimized protocol for tissue homogenization, immunoprecipitation, mRNA isolation of n=3-4 samples for wild-type and SMA TREs at P9 and P19. Corresponding negative controls ($Smn^{2B/+}$::RC-/-; $Smn^{2B/-}$::RC -/-) were processed in parallel. Samples were then sent to the Mayo Clinic Genomics Core for quality assessment, processing, and RNA-seq. Due to the minute amount of starting material, the RIN for each sample could not be evaluated by the Bioanalyzer reliably. Therefore, we evaluated sample quality following RNA amplification and cDNA library generation. Samples with adequate scores were selected for RNA-seq.

RNA-seq was conducted using the Illumina4000 platform. Following alignment and QC, we identified over 20,000 genes across our samples. We performed a principal component analysis (PCA) to assess variance between our WT, SMA, experimental and control groups. Groups appeared to segregate appropriately between PC1 (age) and PC2 (genotype) (**Figure 8C**). Similar to the somal MN translatome, we observed an increase in variance between our P9 and P19 SMA and WT samples, suggesting a marked change in the RNA populations related to age and disease progression. Using DEseq2 or Partek Genomic Suite, we applied stringent filtering parameters to further excluded RNA contaminants derived from muscle (TPM count > 5; Fold Change > 1.5; p-value <0.05). We identified 3,270 DEGs at P9 and at P19. At P9, 358 genes were significantly dysregulated in SMA MN axons. By P19 the extent of transcript dysregulation had increased to 2,912 genes.

At P9, we identified 245 down-regulated genes and 113 upregulated genes in SMA MN axons A GO-term analysis to identify affected biological processes across our P9 down-regulated axonal transcripts revealed a high percentage of genes per group related to cell respiration, ribosome biogenesis, apoptotic signaling, and mitochondrial function (**Fig 9A**). Interestingly, mitochondrial organization, cellular respiration, and translation were the top 3 most affected pathway according to corrected group p-values. Previous research has shown mitochondrial and ribosomal transcripts comprise a large amount of the axonal transcriptome in cultured neurons (Saal, 2014 #127)(Bigler, Kamande, Dumitru, Niedringhaus, & Taylor, 2017; Dianna E. Willis et al., 2007), sustaining our *in vivo* findings. Transcripts upregulated in P9 SMA MN axons displayed higher percentages of terms per group in several categories, the highest related to positive regulation of viral genome replication, vacuolar transport, protein heterotrimerization, musculoskeletal movement, membrane protein ectodomain proteolysis, and regulation of alternative mRNA splicing via splicesome (**Figure 9B**).

By P19, 2,380 genes were down-regulated and 532 genes were upregulated in SMA MN axons. GO-term analysis identified 517 enriched groups within the P19 down-regulated transcripts. The large list of down-regulated genes affected pathways of diverse biological processes. Regulation of cellular

component assembly, macromolecule process, and G protein-coupled receptor signaling pathway displayed the lowest group corrected p-values within the list of identified groups (**Table 5**). Evaluation of the 532 upregulated genes in P19 axons by GO-term analysis revealed 5 enriched groups. The 3 groups displaying the lowest group p-value consisted of gene expression, macromolecule metabolic process, and RNA processing (**Table 6**).

Although the majority of genes identified in our analysis are expressed by both muscle and neurons, we observed a high degree of muscle-related transcripts overrepresented in our lists of P9 and P19 axonal DEGs. The actin cytoskeleton plays a critical role in the formation and stabilization of the NMJ on both the presynaptic and postsynaptic side. Further investigation into the transcripts comprising this GO group revealed a high association with smooth and skeletal muscle functions. In order to achieve the highest level of accuracy from our axonal translatome RNA-seq data, we set out to establish stringent parameters that would allow us to effectively identify axon-derived versus muscle-derived transcripts. We first aimed to set an expression cutoff that genes must pass in all samples (SC and TRE) in order to be considered true signal. This exclusion process significantly reduced the number of DEGs, yet did not remove enough transcripts highly associated with muscle function. Despite our efforts to filter out muscle-derived transcripts, we were unable to establish an unbiased system to discern our axonal "signal" from muscle-derived "noise". The negative controls accompanying our samples provided insight into the highly variable nature of background binding in our muscle samples. Without a method to segregate noise from signal, we cannot conclude whether the differences in RNA populations we observe by PCA are a result of differences in the axonal translatomes between disease or if the sample genotypes effects background binding of non-specific mRNAs.

We next set out to optimize the axonal IP method, ultimately reducing the background binding of non-specific RNAs. As a way to determine the effectiveness of our optimization efforts, we identified troponin C2, Fast Skeletal Type (Tnnc2) as a muscle-derived mRNA whose high TPM values in our RNA-seq data further indicated substantial contributions from muscle-derived mRNA. We first tested

different immunoprecipitation beads and HA antibodies to determine levels of non-specific protein binding in our samples. We observed comparable IP of HA-RPL22 between conditions, yet slightly less background binding with Protein G Dynabeads (Thermo) when evaluated by silver stain. We then attempted to reduce non-specific bindings of RNA by blocking the immunoprecipitation beads prior to pull-down. After subjecting beads to various blocking conditions (e.g. BSA, tRNA, and/or sperm salmon, etc.) then incubating the blocked beads with TRE muscle lysate, we isolated any bound mRNA on the beads and assessed effectiveness by evaluating which method displayed the lowest levels of *Tnnc2* binding. Using this method, blocking beads with a mixture BSA and tRNA resulted in the lowest amount of non-specific binding by *Tnnc2*. In repeating our P19 axonal translatome experiment using these optimized conditions, we were unable to isolate RNA of sufficient quality to proceed to RNA-seq. Future experiments should consider performing all steps prior to RNA isolation at 4°C and using higher amounts of RNase inhibitor to prevent RNA degradation.

As a last measure to obtain candidates from our axonal translatome data, we created a curated list of axonal transcripts highly expressed or uniquely expressed specifically within neuronal populations (**Tables 7, 8**). This list resulted in the identification of 21 dysregulated transcripts at P9 and 18 dysregulated at P19. At P9, 15 transcripts were significantly downregulated while 6 were upregulated. We then identified 9 downregulated and 9 upregulated transcripts in the P19 neuro-specific axonal translatome.

2.5.2 Discussion

Neurons are quintessential examples of cells that heavily rely on local translation to drive functional and morphological polarity and establish elaborate branched axonal and dendritic compartments. Observations of local protein synthesis in presynaptic preparations (Autilio et al., 1968; Morgan & Austin, 1969) and axons (Edstrom & Sjostrand, 1969; Koenig, 1967) date back to the 1960s. Nevertheless, local protein synthesis remained controversial for decades. Recent advances in technology

have facilitated our ability to examine protein synthesis in a spatially restricted fashion. The accumulation of numerous studies have substantiated the phenomenon of local translation, demonstrating axonal protein synthesis is present in developing axons where it plays an essential role in regulating pathfinding, axon elongation, and branching. To date, much of the evidence for axonal translation has been provided by *in vitro* models utilizing chambers or microfluidic devices to separate axons from somas (Michael Briese et al., 2016; Rotem et al., 2017; Saal et al., 2014). While these studies have elucidated the extent and diversity of axonally localized mRNA *in vitro*, direct evidence of axonal localization and translation *in vivo* remains scarce. In 2016, Shigeoka et al., demonstrated abundant and diverse populations of ribosome-associated mRNA could be isolated and quantified from adult retinal ganglion cell bodies (somal translatome) and axons (axonal translatome) *in vivo* using the RiboTag mouse model (Toshiaki Shigeoka et al., 2016; Shigeoka et al., 2018). Thus, we adapted this method to accurately investigate the axonal translatome composition and its modulation in affected motor neurons in an intermediate mouse model of SMA *in vivo*.

The isolation of motor neuron polysomes from whole muscle dissections posed unique and intricate challenges to obtaining a selective population of mRNA. Although ribosomes and polysomes have been observed in axons, information regarding their abundance in mature axons is lacking. Using the aforementioned method, ribosomes and mRNA originating muscle vastly outnumber the tagged ribosomes originating from motor axons. In the 2016 paper from Shigeoka et al., researchers examined the cell body translatome in retinal ganglion cells (RGCs) by harvesting eyes, and the axonal translatome by harvesting the terminal projections of RGCs located in superior colliculus (Toshiaki Shigeoka et al., 2016). The researchers apply strict criteria to their axonal RNA-seq analysis, yet the location of their samples makes it difficult to truly assess potential contributions from background. The exceedingly different genetic profiles of skeletal muscle and motor neuron axons offers a system to identify mRNAs highly associated with a particular cell type and thus indicative of potential contamination. Unlike our experimental model, the composition of the target population (RGC axons) and background populations

(superior colliculi) used by Shigeoka et al are highly similar, and establishing a clear line between axon derived signal and background derived from other neuronal populations unclear.

The PCA performed on P9 and P19 samples suggests populations of mRNA isolated using this method have similar and significant degree of variance between age and genotypes. Despite our inability to confirm the exact source of our mRNA populations, contributions, the differences we observe may reflect important changes in the muscle transcriptome of SMA mice. Performing validation of DEG candidates via western blot of whole muscle lysate could confirm the dysregulation identified in our RNA-seq data is largely representative of dysregulation in susceptible muscle populations in SMA.

It should be noted that characterization of the axonal translatome was also attempted in sciatic nerve. However, we were unable to obtain sufficient mRNA of high enough quality to proceed to library preparation and RNA-sequencing. The sciatic nerve is a heavily myelinated bundle of sensory and motor axons that innervate the lower limbs. Although isolation of these nerves greatly reduces the contributions of non-neuronal cell types in the lysate, the high degree of myelination makes complete homogenization difficult. Moreover, the high lipid content can interfere with protein immunoprecipitation and RNA stability. Subsequent studies aiming to observe the axonal translatome should consider selecting smaller muscle groups with higher innervation ratios of motor neurons to muscles. The lumbrical muscles of the mouse are exceptionally small spindle-shaped muscles that flex the metacarpophalangeal joint in the forepaw (Russell, Ng, Faulkner, Claflin, & Mendias, 2015). Similar to the TRE muscles, the lumbricals were shown to be significantly denervated in the $Smn^{2B/-}$ mouse model at P21 (Murray et al., 2015). Reducing the size of the target muscle group may improve one's ability to isolate tagged ribosomes.

2.5.3 Tables and Figures

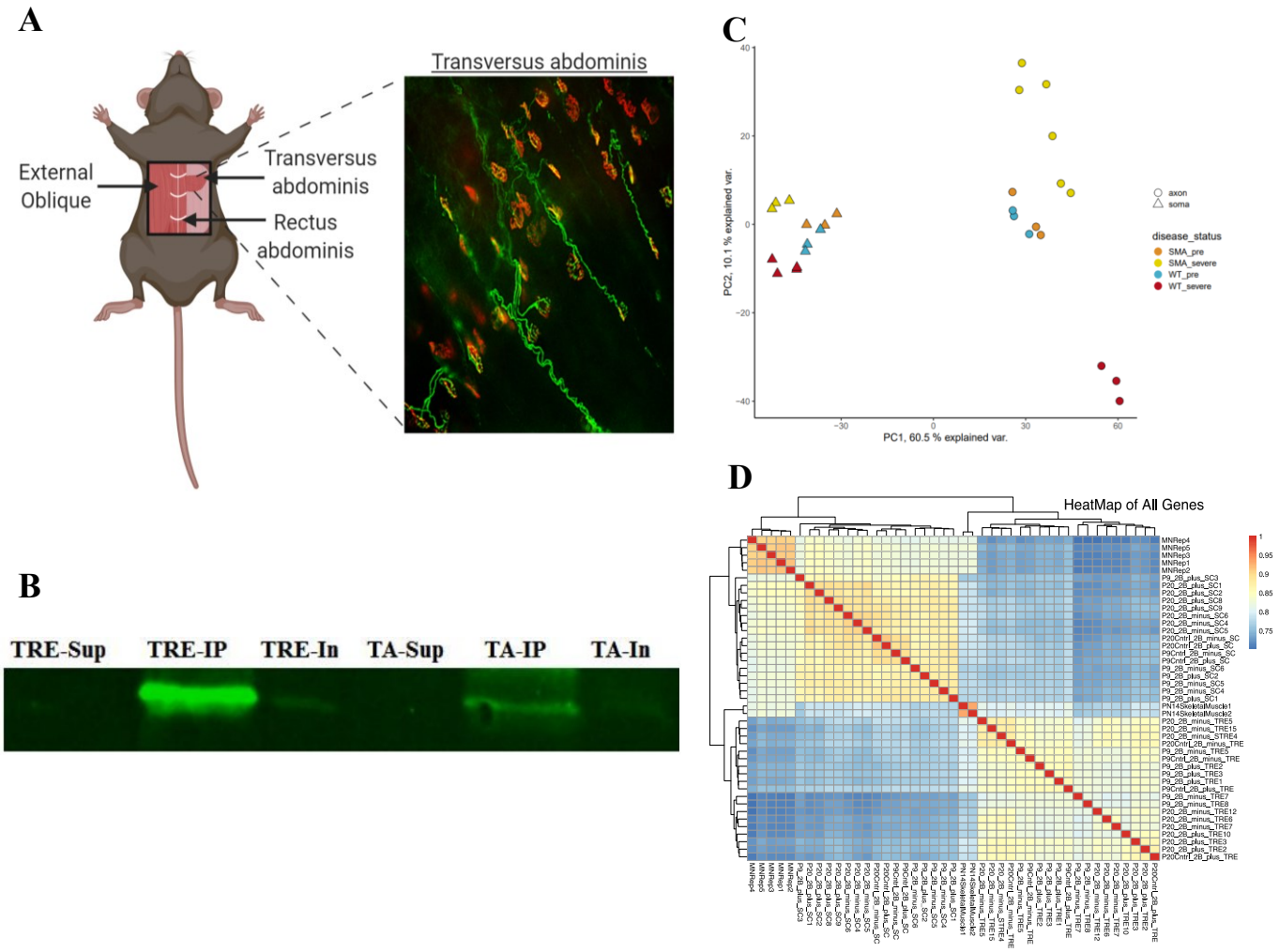

Figure 8. **Capturing the axonal Translatome in affected motor neuron axons *in vivo*. A)** Illustration of targeted abdominal muscle groups: transversus abodminus, rectus abdominus, and external oblique (TRE). **B)** Western blot analysis of HA-RPL22 TRE, and the tibialis anterior (TA) demonstrates our ability to detect a substantial enrichment of axonal HA-RPL22 following anti-HA immunoprecipitation (IP) when compared to supernatant (Sup) and input (In). **C)** Principal component analysis all samples (somal and axonal) reveals unique segregation patterns between translatome source and disease severity. D) Heatmap clustering of all samples, including controls.

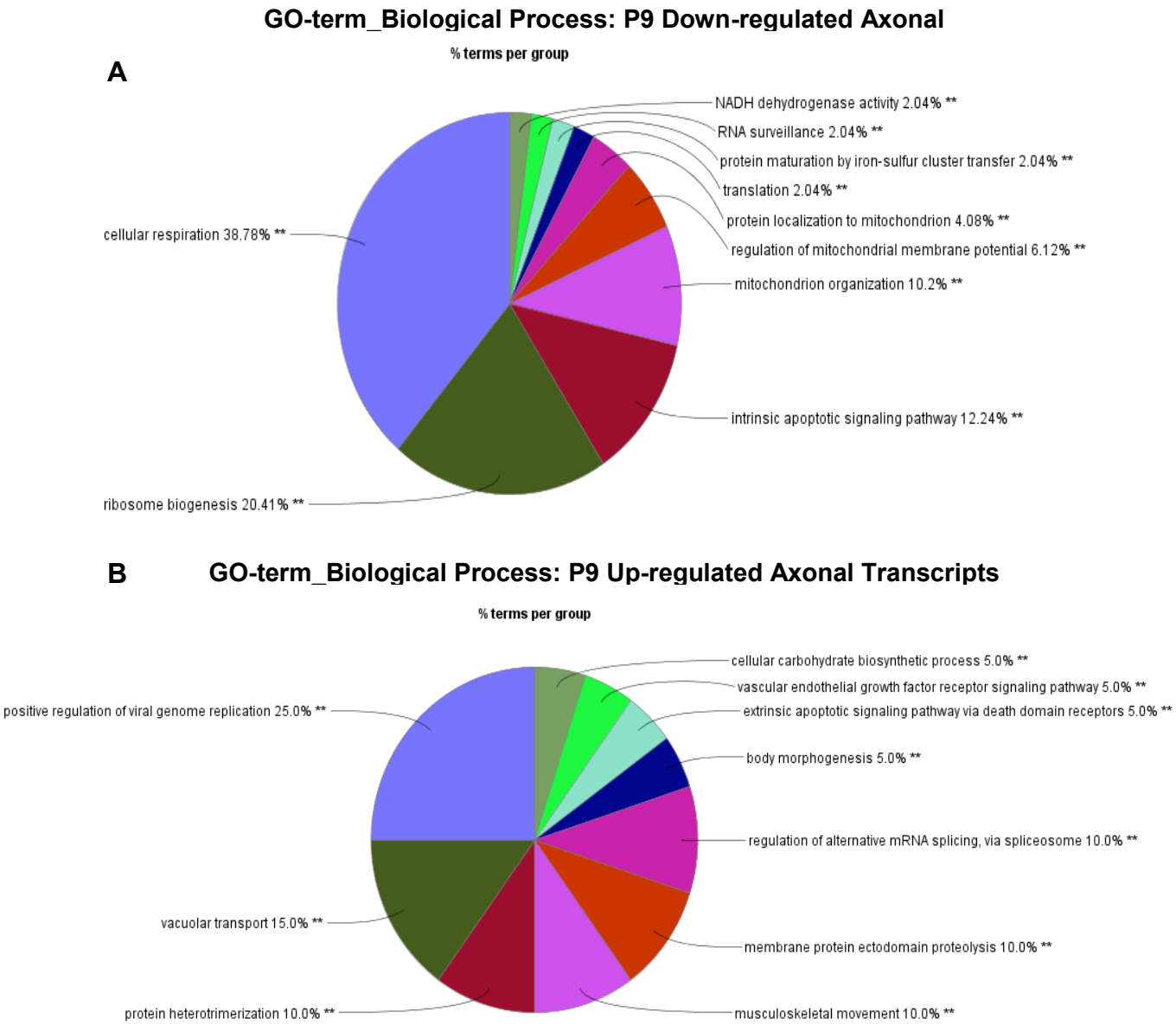

Figure 9. **Capturing the axonal Translatome in affected motor neuron axons *in vivo*.** A) Illustration of targeted abdominal muscle groups: transversus abodminus, rectus abdominus, and external oblique (TRE). B) Western blot analysis of HA-RPL22 TRE, and the tibialis anterior (TA) demonstrates my ability to detect a substantial enrichment of axonal HA-RPL22 following anti-HA immunoprecipitation (IP) when compared to supernatant (Sup) and input (In).

Table 5. Top 5 Biological Processes Enriched in P19 down-regulated transcripts in SMA axons

GOTerm	Term P-Value Corrected with Bonferroni step down	Group P-Value Corrected with Bonferroni step down	Nr. Genes
cellular component assembly	1.205898E-21	1.5280E-43	491.00
macromolecule metabolic process	1.462505E-20	4.9470E-42	1202.00
G protein-coupled receptor signaling pathway	7.111359E-40	1.1361E-41	80.00
organelle organization	1.787599E-26	6.3329E-36	614.00
positive regulation of cellular process	9.427347E-21	2.0327E-34	815.00

Table 6. Top 5 Biological Processes Enriched in P19 up-regulated transcripts in SMA axons

GOTerm	Term PValue Corrected with Bonferroni step down	Group PValue Corrected with Bonferroni step down	Nr. Genes
gene expression	7.757E-05	4.171E-08	169.00
macromolecule metabolic process	8.622E-06	4.514E-08	264.00
RNA processing	1.533E-05	6.027E-08	47.00
cellular catabolic process	2.562E-05	3.018E-07	86.00
cellular protein metabolic process	1.073E-04	3.884E-07	164.00

Table 7. Curated list of neuro-specific transcripts dysregulated in P9 SMA axonal translatome

P9 Motor Neuron Axonal Translatome: Down-regulated DEGs		
Gene Symbol	**Gene name**	**log2FoldChange**
Miat	Myocardial Infarction Associated Transcript	2.14
Kcna2	Potassium Voltage-Gated Channel Subfamily A Member 2	1.84
Rgs7bp	Regulator Of G Protein Signaling 7 Binding Protein	1.83
Adam22	ADAM Metallopeptidase Domain 22	1.82
Gm3373		1.8
Wdr31	WD Repeat Domain 31	1.76
Elovl6	ELOVL Fatty Acid Elongase 6	1.72
Ankrd35	Ankyrin Repeat Domain-Containing Protein 35	1.69
Cttnbp2	Cortactin Binding Protein 2	1.55
Gria3	Glutamate Ionotropic Receptor AMPA Type Subunit 3	1.33
Tro	Trophinin	1.22
Snhg11	Small Nucleolar RNA Host Gene 11	1.19
Tmem181b-ps	Tmem181b-ps transmembrane protein 181B, pseudogene	1.09
Sapcd2	Suppressor APC Domain Containing 2	1.09
Gm37376		1.04
P9 Motor Neuron Axonal Translatome: Up-regulated DEGs		
Ntng1	Netrin G1	-1
Nkain4	Sodium/Potassium Transporting ATPase Interacting 4	-1.2
Gpm6a	Glycoprotein M6A	-1.46
Tmem254b	Transmembrane protein 254	-1.48
Crnde	Colorectal Neoplasia Differentially Expressed	-1.81
Pak3	P21 (RAC1) Activated Kinase 3	-2.43

Table 8. Curated list of neuro-specific transcripts dysregulated in P19 SMA axonal translatome

P19 Motor Neuron Axonal Translatome: Down-regulated DEGs		
Gene Symbol	**Gene name**	**log2FoldChange**
Rpl15-ps2	ribosomal protein L15, pseudogene 2	2.348913
Bicd1	BICD Cargo Adaptor 1	2.239074
Ntng1	Netrin G1	1.800063
Gm14305		1.564467
Gm47428		1.265151
Cttnbp2	Cortactin Binding Protein 2	1.261788
Pkib	CAMP-Dependent Protein Kinase Inhibitor Beta	1.186421
Tmem181b-ps	Tmem181b-ps transmembrane protein 181B, pseudogene	1.174887
Slc1a1	Solute Carrier Family 1 Member 1	1.097602
P19 Motor Neuron Axonal Translatome: Up-regulated DEGs		
Cspg5	Chondroitin Sulfate Proteoglycan 5	-1.02219
Cntn1	Contactin 1	-1.049158
Mdga2	MAM Domain Containing Glycosylphosphatidylinositol Anchor 2	-1.067803
Celf3	Trinucleotide Repeat-Containing Gene 4 Protein	-1.159647
Shank1	SH3 And Multiple Ankyrin Repeat Domains 1	-1.262095
Ccdc30	Coiled-Coil Domain-Containing Protein 30	-1.787
Gm20659		-2.102913
Crnde	Colorectal Neoplasia Differentially Expressed	-2.444556
Ntrk3	Neurotrophic Tyrosine Kinase, Receptor, Type 3	-2.574372

2.6 Materials and Methods

Animals

All mouse experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Mayo Clinic Department of Comparative Medicine and the Institutional Animal Care and Use Committee (IACUC Protocol #A00002916-17). ChAT-Cre (B6;129S6-Chattm2(cre)Lowl/J; Jackson Laboratory No. 006410), Rpl22-HA/+ mice (B6N.129-Rpl22tm1.1Psam/J; Jackson Laboratory No. 011029), B6.Smn$^{2B/2B}$, and mice were housed in ventilated cages under standard laboratory conditions and maintained under a 12hr light: 12hr dark with *ad lib* access to breeder chow and water. To generate our wild-type (Smn$^{2B/+}$;ChAT-Cre/+;Rpl22-HA+/+) and SMA (*Smn*$^{2B/-}$;ChAT-Cre+/-;Rpl22-HA+/+)

Mice homozygous for the 2B mutation (*Smn*$^{2B/2B}$; C57BL/6 background) were obtained as a generous gift from Dr. Christine DiDonato (Northwestern University). The 2B mutation introduces a substitution of 3 nucleotides in the exon splicing enhancer in exon 7 of the *Smn* gene, resulting in a 64% reduction of full-length SMN protein. Nevertheless, these mice are viable, fertile, and do not develop any observable phenotypes. Our Smn$^{2B/+}$ (WT) and Smn$^{2B/-}$ (SMA) experimental models were generated by crossing heterozygote knockout for the *Smn* gene (*Smn*$^{+/-}$ [B6.Cg-Smn1tm2Mrph/J, stock 007963], with *Smn*$^{2B/2B}$ mice. To generate our experimental wild-type (*Smn*$^{2B/+}$;ChAT-Cre +/-;Rpl22-HA+/+), SMA (*Smn*$^{2B/-}$;ChAT-Cre+/-;Rpl22+/+), and corresponding negative controls models, *Smn*$^{2B/2B}$ mice were crossed with Rpl22-HA+/+; ChAT-Cre+/- mice and bred to achieve *Smn*$^{2B/2B}$; ChAT-Cre+/-;Rpl22-HA+/+ (2B;RC) mice. In parallel, *Smn*$^{+/-}$ mice were bred with Rpl22-HA+/+ to produce *Smn*+/-; Rpl22-HA+/+ (SmnKO-R) mice. Thus, by crossing 2B:RC and *Smn*KO-R mice we are able to generate our wild-type (*Smn*$^{2B/+}$; Rpl22-HA+/+, ChAT-Cre+/-) SMA (*Smn*$^{2B/-}$;ChAT-Cre+/-;Rpl22+/+), and corresponding negative controls (*Smn*$^{2B+/-}$; Rpl22-HA+/+;Chat-Cre-/-).

Experimental and maintenance cages were kept in the same room and were inspected every morning for new litters, death, or endpoint criteria. During the survival study, humane endpoints were

followed according to the Institutional animal care and use (IACUC) and Department of Comparative Medicine at Mayo Clinic (severe dehydration, hypothermia, significant weight loss, or dystocia). At endpoints, mice were euthanized using a CO2 chamber followed by cervical dislocation. The exact date and cause of death of any pup was recorded. The euthanized mice were excluded from the survival study.

Tissue Collection

Since SMA and mice are collected prior to sexual maturity, samples consist of male and female mice. At P9-10 and P19-20 time-points, mice were anesthetized with Isoflurane (Henry Schein) until respiratory arrest was observed. We harvested TRE samples by following published protocols to access the TVA (Au - Murray, Au - Gillingwater, & Au - Kothary, 2014) , yet took caution to collect the TVA, RA, and EO. The TRE was dissected as a whole, washed briefly in cold, RNase free 1x PBS, and snap frozen in liquid nitrogen. Spinal cords were then isolated by cutting the vertebrate column with a strait scissor in front of the back legs and before the medulla oblongata and extruded using a syringe filled with cold 1x PBS with 30G 0.5 inch needle (BD Biosciences) into cold RNase-free 1x PBS. Spinal cords were quickly washed in 1xPBS, transferred to an Eppendorf tube, snap frozen in liquid nitrogen, and stored at -80°C until processing.

RiboTag Immunoprecipitation

At selected time points, mice were euthanized with isoflurane inhalation in accordance with IACUC policy. Tissue samples consisting of sciatic nerves (bilateral), the TRE, diaphragm, and spinal cord were quickly removed, washed in ice cold 0.1M phosphate-buffered saline (PBS), snap-frozen in liquid nitrogen, and genotyped. Tissues from cre-positive and negative were pooled for RiboTag-IP(two spinal cords and 6 TRE for one group). Spinal cord tissue was homogenized in ice cold lysis buffer (50mM UltraPure Tris HCl pH7.4, 5mM MgCl2, 100mM KCl, 1mM DTT, 1mg/ml Heparin, 100U/ml SUPERase In (Thermo), 0.01 mg/ml Cycloheximide (Sigma) and Complete EDTA-free Protease Inhibitor Cocktail

(Thermo) 2-5% weight/volume using chilled glass douncers until the solution was homogenous. TRE samples were homogenized by grinding pooled tissue in liquid nitrogen using a mortar and pestle. Pulverized muscle was then transferred to lysis buffer and homogenized further using cold douncers. Lysates were centrifuged at 16,000 rcf for 10 minutes at 4°C and supernatant consisting of post-mitochondrial fractions were collected in fresh pre-chilled Eppendorf tubes using chilled RNase free pipet tips. If needed, 80ul can be collected and used as input for fraction analysis. Samples were then pre-cleared by incubation with washed unconjugated Dynabeads Protein G (Life Technologies 10004D) for 1 hour at 4°C with rotation. In our hands, the polyclonal HA antibody (9110, Abcam) and the monoclonal HA antibody used in the original RiboTag study (HA11, Covance) (Sanz 2009) yielded similar results. After 1 hour, pre-cleared supernatants were transferred to new chilled Eppendorf tubes and 8 µl of anti-HA antibody (Covance) was added to the supernatant, followed by 4 h of incubation with slow rotation at 4 °C. Meanwhile, Dynabeads Protein G (Thermo Fisher Scientific), 200 µl per sample lysate, were equilibrated in lysis buffer by washing three times. At the end of 4 hours, antibody containing supernatants were transferred to the prewashed beads and incubated in a 4 °C cold room for 12-16 hrs. Beads were washed three times for spinal cord and four times for TRE samples with high-salt buffer (50 mM Tris, 300 mM KCl, 12 mM MgCl2, 1% NP-40, 1 mM DTT, Superase In, 0.1 mg/ml cycloheximide (Sigma), 0.5 mg/ml Heparin (Sigma), and Complete EDTA-free Protease Inhibitor Cocktail (Roche) in Ultrapure Water (Invitrogen), 5 min per wash in a cold room on a rotator. After removing the final wash, ribosome complexes were dissociated from beads and RNA by adding 350 ul of Buffer RLT (Qiagen) supplemented with 2-bmercaptoethanol to each sample, high-speed vortexing for 3 mins, and incubating at room temperature for 5 mins. Samples were then vortexed for an additional 3 mins, Dynabeads pelleted, and supernatant transferred to LoBind DNA tubes (Eppendorf). 250ul of 100% Ethanol were added to each sample, gently pipetting up and down to mix, and transferred to RNAeasy micro kit (Qiagen) columns for in-column DNase I treatment and RNA purification following manufacturer's protocol.

RNA-seq

An Agilent Technologies Bioanalyzer Pico Chip was used to assess the integrity of purified RNA. The RNA integrity number (RIN) values for processed samples ranged from 7.3 to 9.8. RNA samples were amplified using v4 SMARTseq (Clonetech) according to manufacturer instructions, and template DNA library construction was performed with Nextra XT (Illumina). To generate high-resolution transcriptomic data for expression and splicing analysis, we sequenced 100-bp reads to a depth of ~50 M fragments per sample on and Illumina HiSeq 4000. Reads were mapped to the mouse mm10 assembly using Kallisto (v0.45.0) using the transcripts from Ensembl release 67 to guide mapping.

Statistical Methods

Statistical analysis was performed using GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA). Data are expressed as means ± standard error of the mean (SEM). For single comparisons (#MMP9 -positive MNs/ total MNs), either the Student's t test or Kolmogorov-Smirnov test was used. Differences were considered to be statistically significant if $p \leq 0.05$.

Alternative Splicing Analysis

Adapters were first trimmed off of the raw reads using cutadapt v1.16, then aligned to the mouse genome build GRCm38 using STAR v2.6.1. The BAM files obtained from STAR were sorted by coordinate, and used as the input to quantify isoform abundance and splicing patters using rMATS v4.0. Significant events were those that passed an FDR adjusted p-value cutoff of <0.05.

Data access

All RNA-seq data generated in this study will be deposited at the Gene Expression Omnibus (GEO) of the National Center for Biotechnology Information.

Immunohistochemistry

P9 and P19 $Smn^{2B/+}$ and $Smn^{2B/-}$ mice were anaesthetized and perfused transcardially with ice cold 0.1M PBS followed by cold 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Spinal cords were removed, postfixed in 4% paraformaldehyde 0.1M PBS (pH7.4) overnight at 4°C, washed twice in 0.1M PBS, then cryoprotected in 30% Sucrose in 0.1MPBS overnight at 4°C. Tissue samples were embedded in optimal cutting temperature medium (OCT; Fisher) and frozen at -20°C. Transverse serial cryostat sections (P9-16µm; P19-20µm thickness) were allowed to adhere to coverslips on a 37°C slide warmer for 20-30 minutes and stored at -80°C.

For visualization of protein expression in the spinal cords of P9 and P19 $Smn^{2B/+}$ and $Smn^{2B/-}$ mice, slides were removed from -20C, permeabilized in 0.05% Tween-20 in 0.1M PBS for 10mins at room temperature, and incubated in blocking buffer consisting of 10% Normal Goat Serum, 0.05% Tween-20, and 0.5% BSA in 0.1M PBS for 1 hour at room temperature. For analysis of motor neuron expression in spinal cord, the following primary antibodies were used: goat anti-ChAT 1:100 (Millipore), rabbit anti-Bag3 1:200 (Proteintech), rabbit anti-Arhgap9 1:100 (Proteintech), rabbit anti-MMP-9 1:4000 (Abcam), rabbit anti-GFP 1:1000 (Invitrogen), rabbit anti-Fas 1:1000 (Abcam), chicken anti-GFP 1:500 (Aves), mouse anti-HA 1:1000 (Biolegend), and mouse anti-mCherrry 1:500 (Novus). Secondary antibodies conjugated to Alexa fluor 488, 555, and 594, and 647 were generated in goat (Invitrogen). Images were acquired as z-stacks at 20x and 40x magnification on a Nikon Ti-2 or Zeiss confocal microscope. Images are presented as maximum intensity projections

Genotyping

Adult mice used for breeding and line maintenance were ear punched at weaning age (P19-21). For mice used in this study, ear tissue was collected during spinal cord and TRE tissue harvesting.

Primer Name	Primer Sequence (5'-3')
Cre-recombinase: Forward	GAA CCT GAT GGA CAT GTT CAG G
Cre-recombinase: Reverse	AGT GCG TTC GAA CGC TAG AGC CTG T
Myogenin: Forward	TTA CCT CCA TCG TGG ACA GC
Myogenin: Reverse	TGG GCT GGG TGT TAG CCT TA

Smn-KO allele: Forward	GCC TTC TTG ACG AGT TCT TCT G
Smn-KO allele: Reverse	TGT CAC CGT TCT TTA GAG CAT G
Smn2B: Forward	TCC CAG GCA GTT TTA GAC TCA
Smn2B: Reverse	GAG ACC GAG GCA GGC TAA C
RiboTag: Forward	GAG ACC GAG GCA GGC TAA C
RiboTag: Reverse	TTT CCA GAC ACA GGC TAA GTA
MetRS-L274G: Forward	GACCACTACCAGCAGAACACC
MetRS-L274G: Reverse	AGAAGAGGTAGTTGCCACTATCC
MetRS-WT: Forward	CTCTTCCCTCGTGATCTGCAACTCC
MetRS-WT: Reverse	CATGTCTTTAATCTACCTCGATGG
MetRs-Control: Forward	GAGACTCTGGCTACTCATCC
MetRs-Control: Reverse	CCTTCAGCAAGAGCTGGGGAC

Chapter 3

Examining the Motor Neuron-specific Proteome in a mouse model of SMA

Price, PL., Morderer, D., M., DiDonato, C., Bassell, G., Rossoll, W. (2019).

3.1. Characterization of the motor neuron-specific proteome in SMA

In the present study, we adopted RiboTag technology in order to evaluate changes in mRNA processing specific to motor neurons during key time points in SMA pathology. Isolation of MN transcripts is restricted to those currently associated with a HA-tagged ribosome, introducing a level of selectively that may better represent changes in protein expression. Nevertheless, association with a ribosome does not guarantee translation. Evaluating MN-specific changes to the proteome in SMA would provide powerful complementing research and further refine our understanding of the factors underlying motor neuron susceptibility. To date, identification of proteomic changes in SMA motor neurons has largely been restricted to whole spinal cords lysates, patient cells, and *in vitro* models. We will now explore model systems for examining changes to the MN proteome in response to SMN-deficiency *in vivo*.

Advances in protein labeling methods have made it possible to isolate and measure cell-type-specific proteomes from mixed cell populations *in vivo*. Evaluating similarities and differences between these specific cell populations can provide invaluable insight into biological function as well as disease-causing dysfunction in complex, multi-cellular organisms. Bio-orthogonal strategies based on modified amino acids permit the selective labeling of proteins in a cell-type specific manner. To determine cell-type specific changes to the motor neuron proteome at P9 and P19, we have adopted the MetRS L274G mouse model. In this model, Cre-mediated expression of a methionyl-tRNA synthetase (MetRS) with an expanded amino-acid binding site (MetRS L274G) allows methionine tRNA to be charged with the methionine surrogate azidonorleucine (ANL). The cell-type specific population of polypeptides possessing ANL can be tagged using CLICK chemistry and used for visualization by microscopy or proteome profiling via mass spectrometry. Thus, incorporation of the MetRS transgene into our SMA mouse model containing motor neuron-specific Cre expression has allowed us to tag, visualize, and identify disease-dependent changes in protein expression within spinal motor neurons.

To assess conditional expression and protein labeling in spinal motor neurons of *in vivo*, MetRS* mice were crossed with ChAT-Cre to induce GFP-2A-MetRS* expression in cholinergic neurons. Four days after delivering a new litter of *Smn*$^{2B/+}$:ChAT-Cre::MetRS* (wild-type) and *Smn*$^{2B/-}$:ChAT-Cre::MetRS* (SMA) pups, dams were supplied with drinking water supplemented with ANL (30-40mM) and maltose (to increase water intake). Concurrently, pups received 0.25mg/ g body weight intraperineal (IP) injections of ANL every 48 hours from P3-19, totaling 7-8 IP injections. Following tissue perfusion and dissection at P19, we clearly observed selective protein labeling restricted to spinal motor neurons of the dam and pups via FUNCAT. Tagging biotin to ANL-labeled proteins in P19 spinal cord lysates via CLICK chemistry and performing a western blot revealed a significant portion of biotin-tagged proteins spanning all molecular weights present in P19 spinal cord lysates. Subsequent affinity purification confirmed our ability to selectively enrich for biotin-tagged proteins.

Having established an effective method to perform cell-type specific proteome labeling in neonatal mice *in vivo*, we sought to utilize this method to identify disease-associated changes in the motor neuron proteome between P19 wild-type and SMA mice. We applied stringent parameters to our proteomic analysis, filtering for proteins exclusively detected in ChAT:Cre+ samples and those possessing a fold change of 1.5 or greater in a minimum of two biological ChAT:Cre+ replicates.

3.2 Conclusions and Future Directions

We identified over 9,000 proteins within our P19 motor neuron proteome samples from SMA and WT mice. Of these 9,000 proteins, 520 were significantly dysregulated in P19 SMA motor neuron. Subsequent comparisons between the P19 motor neuron translatome and proteome will provide insight into the ability of tagged-ribosome enrichment and mRNA isolation to reflect or potentially predict changes in protein expression.

3.3 Methods

Transgenic animals

The cassette - STOP^{FLOX} - GFP-2A-MetRS*- expressed under the control of the CAG actin-derived promoter knock-in in the mouse ROSA26 locus was developed and received as a gift from the Schuman lab at Max Planck. Our *Smn*$^{2B/+}$ (WT) and *Smn*$^{2B/-}$ (SMA) experimental models were generated as described in Chapter 2.7. To generate our experimental wild-type (Smn$^{2B/+;}$ChAT-Cre/+;MetRS+/+), SMA (*Smn*$^{2B/-}$;ChATcre+/-; MetRS+/+), and corresponding negative controls models, *Smn*$^{2B/2B}$; ChAT-Cre+/- mice were crossed with MetRS+/+ and bred to achieve *Smn*$^{2B/2B}$; ChAT-Cre+/-; MetRS+/+ (Smn2B;MC) mice. In parallel, *Smn*-KO+/- mice were bred with MetRS+/+ to produce *Smn*+/-; MetRS+/+ (*Smn*KO-M) mice. Thus, by crossing Smn2B:MC and *Smn*KO-M mice we are able to generate our wild-type (*Smn*$^{2B/+}$; MetRS+/+, ChAT-Cre+/-) SMA (*Smn*$^{2B/-}$;ChATcre+/-; MetRS+/+), and corresponding negative controls (*Smn*$^{2B+}$/-; MetRS+/+; ChatCre-/-). Genotyping was done by PCR (see Chapter 2). All the procedures involving animals were performed under protocols compliant with and approved by IACUC and Mayo Clinic Department of Comparative Medicine.

ANL administration in mice

Following parturition, dams were given 30mM ANL in drinking water supplemented with 3.5-7% D-maltose (Sigma) from pup P3-19. To achieve sufficient protein labeling for the generation of motor neuron-specific proteomes, pups received 0.3mg/g body weight intraperineal injections of ANL (pH 7.5) resuspended in water and sterile filtered every 48 hrs from P3-19.

Fluorescent non-canonical amino-acid tagging (FUNCAT) in tissue

After metabolic labeling *in vivo,* P19-21 mice were euthanized after anesthesia with isofluorane and transcardially perfused with cold 1x PBS supplemented with 20 mM methionine, followed by 4% PFA in PBS. Spinal cords were removed, post-fixed in 4% PFA overnight at 4°C, and allowed to sink in 30% sucrose in 1x PBS for 24 h at 4°C. L1-L3 segments of the spinal cord were mounted in OCT and cryosectioned at 20-30µm. Sections were then incubated overnight at 4 °C in blocking buffer consisting of 0.5% TritonX-100, 10% goat serum and 2% bovine albumin serum in 1x PBS. Samples were washed

two times in PBS pH 7.8 and “clicked” overnight with gentle agitation using biotin-alkyne (PEG4 carboxamide-propargyl biotin, Thermo) and the Click-iT™ Protein Reaction Buffer Kit (Thermo). After extensive washing with 0.5% TritonX-100 in 1x PBS, sections were incubated overnight at 4 °C with anti-GFP and anti-biotin primary antibodies in 1:1 dilution of the blocking buffer in 1x PBS, washed in 0.1% Triton in 1x PBS and incubated for 1h at RT with secondary antibodies and DAPI in diluted blocking buffer. Slides were then washed in 0.1% Triton in 1x PBS and then 3 times in 1x PBS and mounted with Prolong Glass including NucBlue (Thermo). Fluorescence imaging was performed with a laser scanning confocal microscope (Zeiss) with ×10, ×20, or ×40 objectives with appropriate excitation laser lines and spectral detection windows.

Antibodies

The following antibodies were used for immunofluorescence labeling (IF) and/or immunoblotting (IB) at the indicated dilutions: mouse anti-biotin (IF and WB, 1:500, Sigma) and/or chicken anti-GFP (IF, 1:500; IB: 1:1,000; Aves), goat anti-chicken Alexa 488 (IF, 1:500; Invitrogen), goat anti-mouse 555 (IF, 1:500, Invitrogen), goat anti-rabbit FITC (IF, 1:800, Jackson laboratory), goat anti-mouse or anti-rabbit IR680 or IR800 (IB, 1:10,000, Licor), Gp anti-MAP2 (1:1,000, SYSY).

Figures

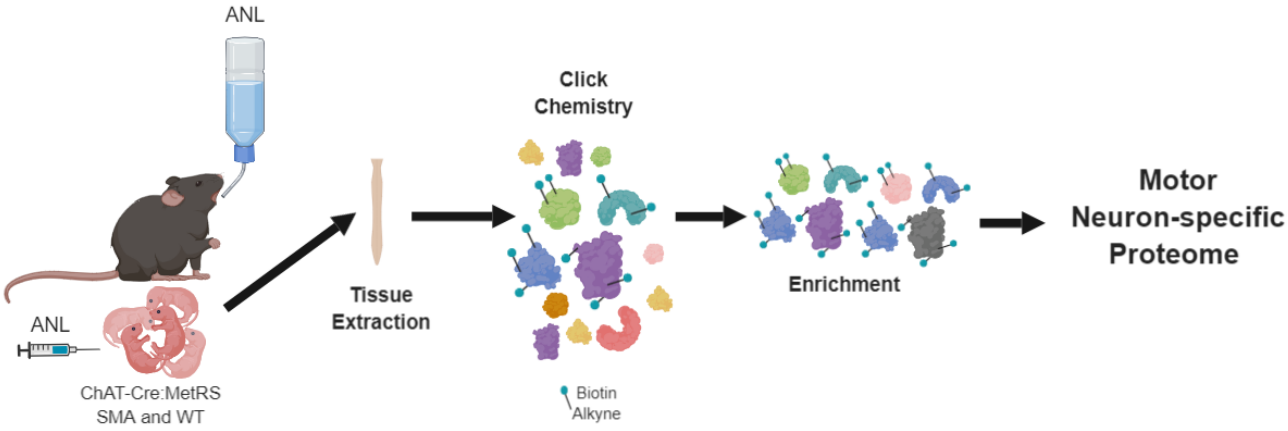

Figure 5. Illustration of ANL administration and cell-type specific protein labeling in vivo. A) ANL is administered to dams and pups via drinking water or intraperineal injection starting at P3. Only motor neurons expressing MetRSL274G under a ChAT promoter will be able to incorporate ANL into nascent proteins. B) Once harvested, ANL incorporated proteins can be tagged with biotin using CLICK chemistry and enriched by pull-down with streptavidin. Isolated proteins can then be evaluated by tandem mass spectrometry.

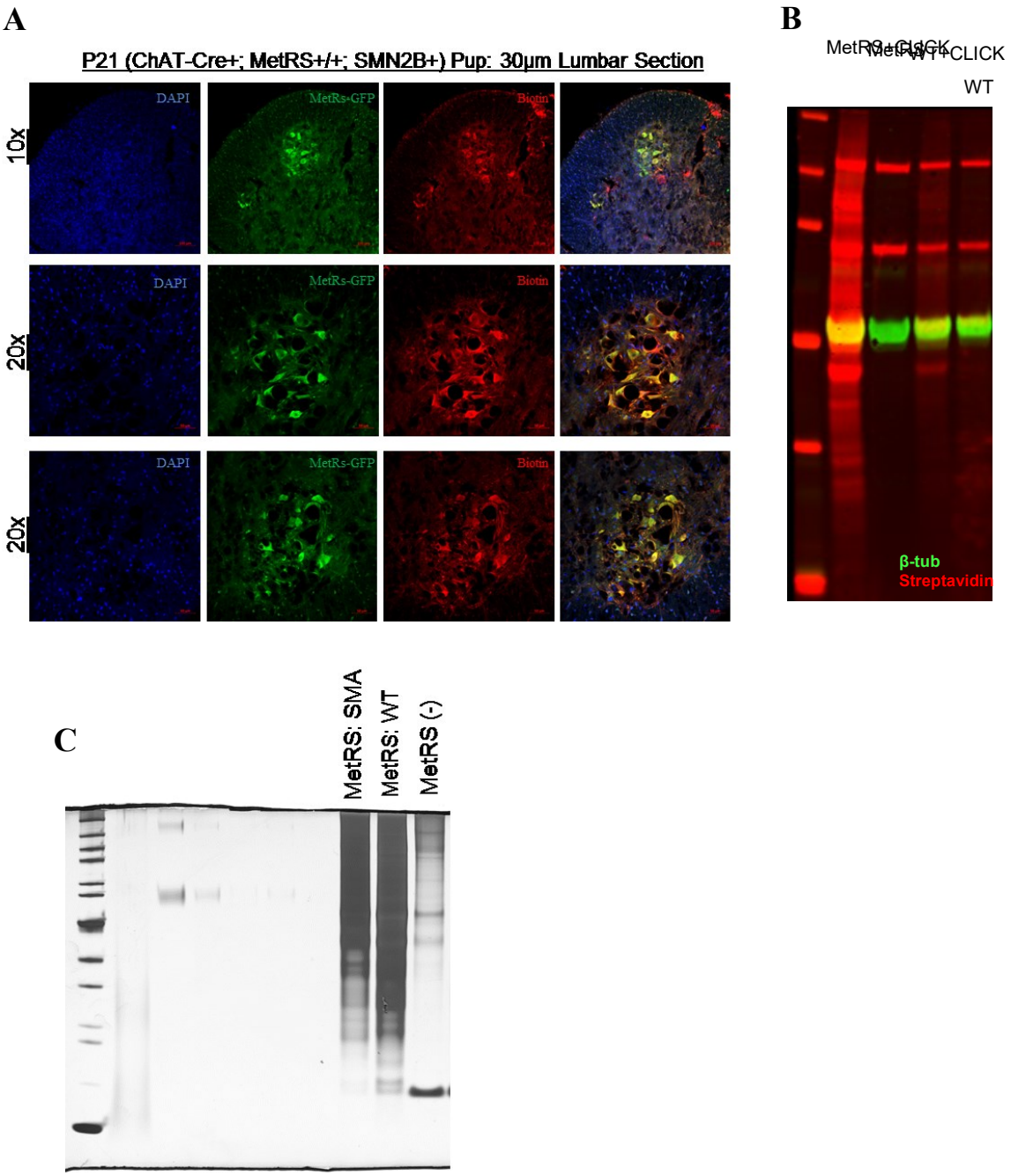

Figure 5. ANL incorporation in motor neuron proteins from P21 mice. A) After 18 days of ANL administration, P21 mice show ANL incorporation restricted to motor neurons in laminae VIII and IX using FUNCAT. **B)** BONCAT using biotin to label ANL incorporated proteins revealed a wide range of molecular weights. **C)** Subsequent pull-down and silver stain confirmed our ability to highly enrich for biotin-tagged proteins.

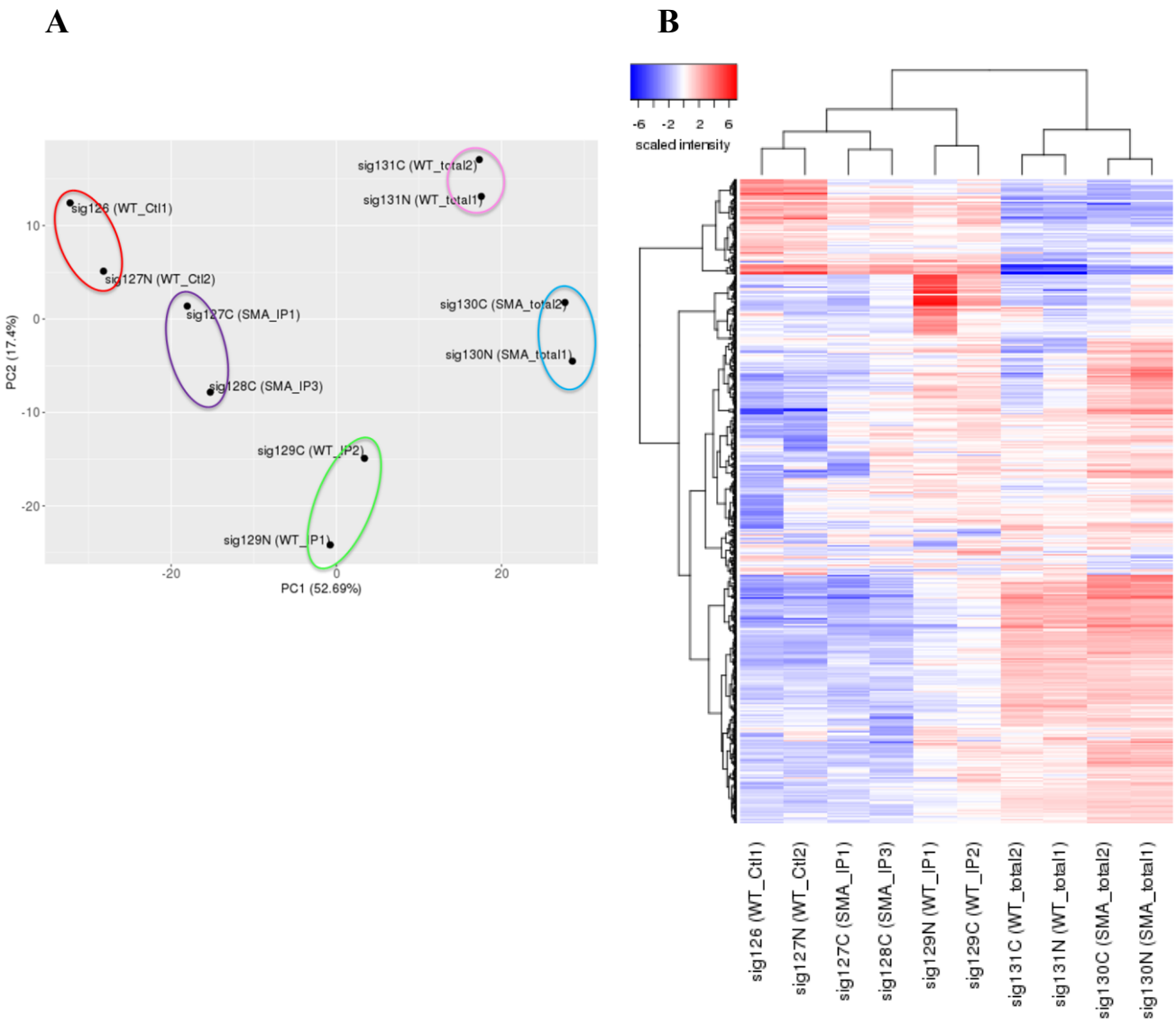

Figure 5. Motor neuron proteomics and profiles from P19 SMA and WT mice. A) PCA of samples sent for mass spectrometry reveals similar segregation patterns between proteomes specifically from motor neurons (WT_IP; SMA_IP), proteomes from the entire spinal cord (WT_total; SMA_total) and non-MetRS expressing negative controls (WT_Ctl). B) Hierarchical clustering highlighting similarities and differences between the abundance of proteins identified between conditions.

Chapter 4: Conclusions and future directions

Difference in susceptibility of specific cellular populations is a common feature of neurodegenerative diseases. Despite considerable progress in understanding the processes of mRNA localization and processing in various models of SMA, the molecular mechanisms that underlie motor neuron-specific degeneration remain poorly understood. Previous studies examining mRNA processing defects have been limited to *in vitro* modeling using patient tissue or SMN-deficient cell lines, or bulk tissue homogenization. Though valuable, these methods remove cells from the dynamic environment of a living organism or lack the sensitivity to capture changes in scare cell populations. Employing powerful technologies to examine cell-type specific changes in gene expression *in vivo* has facilitated our understanding of how particular populations are affected by neurological diseases (Ceolin et al., 2017; Kang et al., 2018; McKinley et al., 2019). The work presented in this dissertation furthers our understanding of motor neuron-specific mRNA processing *in vivo*. Here, I hypothesized that mRNA processing and axonal localization are altered in motor neurons of an intermediate mouse model of SMA *in vivo*. The primary findings that provide support for this hypothesis are as follows:

1. Dysregulation of the motor neuron translatome coincides with disease severity.
2. Aberrant splicing is present early in disease, but does not lead to widespread changes in gene expression.
3. Components of the p53-mediated signaling pathway are upregulated early in disease.
4. Down-regulation of mitochondrial components is a late feature of SMA disease pathology.
5. Fast-fatiguable motor neurons may constitute a highly vulnerable motor neuron population in SMA.

To understand why disease pathology in SMA may lead to spinal motor neuron loss, we adopted RiboTag technology to analyze gene expression profiles at pivotal time points in disease. Through strategic mouse crossing, we achieved motor neuron-specific expression of tagged ribosomes in our intermediate SMA mouse model. In this model dams give rise to mixed litters of indistinguishable healthy

($Smn^{2B/+}$) and SMA ($Smn^{2B/-}$) pups, offering ideal controls to evaluate disease-specific changes. Furthermore, the gradual development of SMA pathology in this mouse model allowed us capture translatome changes at disease-relevant time points. Employing the RiboTag method to characterize changes in translatome of SMA motor neurons across disease provides invaluable insight into defects in mRNA processing that may underlie motor neuron vulnerability. In this final chapter, I will discuss future directions aimed to expand and substantiate the data and analysis discussed in this work.

4.1 Comprehensive characterization of spinal motor neuron subtypes in SMA

As an alternative approach to characterize SMN-dependent changes to the motor neuron proteome *in vivo*, we have crossed our Smn2B mouse model with a line expressing the red fluorescent protein tdTomato. Driven by a ChAT promoter, tdTomato is selectively expressed within cholinergic motor neurons. Following tissue removal, digestion, and cell dissociation, tdTomato-positive cells within the spinal cord can be isolated by fluorescence-activated cell sorting (FACS). Once a highly pure population of motor neurons is obtained, these cells can undergo additional processing and used by mass spectrometry proteomic analysis.

Additionally, this model and technique can permit the characterization of motor neuron subpopulations in the spinal cord. Rather than sending FACS isolated cells for a collective evaluation of changes to the motor neuron proteome in SMA, individual motor neurons can be characterized using single-cell RNA-seq. Single-cell RNA-seq approaches circumvent the averaging artifacts associated with traditional bulk RNA-seq data, providing novel insights into the cellular and molecular diversity underlying superficially homogenous populations (S. Liu & Trapnell, 2016). The unique molecular signatures identified can denote subtle differences in cell cycle stage, differentiation, development, and response to stimuli or treatment. Furthermore, the use of computational suites (e.g. Monocle6) can calculate branching trajectories of specific motor neuron subpopulations, enabling identification of

lineage-specific gene expression and key genetic profiles that delineate disease susceptibility and resistance (S. Liu & Trapnell, 2016).

Previous studies have also demonstrated the ability to identify single alleles or single splice isoforms preferentially expressed by cell (Shalek et al., 2013). Such experiments could provide substantial evidence linking SMN expression levels to changes in molecular composition of motor neuron profiles that may directly or indirectly influence alternative splicing and the production of particular isoforms.

According to the results described in Chapter X, MMP9 expression may play a pivotal role in defining more susceptible populations of motor neurons.

4.2 Explore translatome differences between cholinergic neurons in the brain of SMA mice

The cholinergic system in the brain plays a crucial role in regulating a wide range of functions including sensory and motor processing (Minces, Pinto, Dan, & Chiba, 2017), arousal (Szymusiak, 1995), and wakefulness (M. Xu et al., 2015). Cholinergic neurons are distributed in an uneven and clustered manner across various brain regions, with subcortical areas and the brainstem containing the highest density of cholinergic neurons in the brain (X. Li et al., 2018). Although cognitive function is well preserved in chronic SMA, multiple studies have observed thalamic lesions and degeneration in the brains of SMA patients at the severe end of the disease spectrum (Ito et al., 2004; Shishikura, Hara, Sasaki, & Misugi, 1983).

In a severe mouse model of SMA, SMN deficiency resulted in regional selectivity in the brain with the hippocampus displaying reduced cell proliferation, impaired neurogenesis, and abnormal development (Thomas M. Wishart et al., 2010). The RC::$Smn^{2B/+}$ and RC::$Smn^{2B/-}$ mouse model using for our spinal cord translatome evaluation provides an ideal system to examine the effects of SMN depletion in cholinergic neurons in the brain. To date, these features have not been sufficiently explored in the intermediate $Smn^{2B/-}$ mouse model employed in the aforementioned experiments. Examining subcortical populations of cholinergic neurons for pathological abnormalities would increase our understanding of the

extent or selectivity of SMN reduction on spatially and functionally different populations of cholinergic neurons. Nevertheless, we would hypothesize SMN deficiency would have minimal effects on mRNA processing in the brains of P19 $Smn^{2B/-}$ mice. Failing to reject the null hypothesis would suggest a pathological reduction of SMN expression has little to no meaningful impact on modifying the expression profile of subcortical populations of cholinergic neurons in the brain. This result would support the absence of abnormalities in brain development and cognition observed in the majority of patients. Alternatively, rejecting the null hypothesis would suggest SMN deficiency does significantly impair mRNA processing in cholinergic neurons in the brain. Defining what features present or absent in subcortical cholinergic neurons in SMA brains offer resistance to degeneration and performing subsequent comparison studies between the dysregulated translatome of brain-derived cholinergic neurons versus spinal motor neurons could elucidate modifiers that contribute to susceptibility or resistance.

4.3 AAV-mediated Rescue of Translatome Candidates in SMA Mice

Adeno-associated virus (AAV) is currently one of the most actively investigated vehicles for gene therapy, already showing remarkable results in patients suffering from congenital blindness, hemophilia, and SMA. Recombinant AAV (rAAV), which lacks viral DNA and thus the ability to replicate and assemble, is essentially a protein-based nanoparticle engineered to traverse the cell membrane and deliver its DNA cargo into the nucleus of a cell. Once delivered the necessity for the deposited single-stranded DNA to be converted to double-stranded DNA presents a limiting step in the onset of transgene expression (McCarty, Monahan, & Samulski, 2001). Self-complementary AAV (sc-AAV) offers an alternative approach to improve transgene activation. At the cost of reducing the packaging capacity of the transgene to approximately 3.3 kb, sc-AAV contains a single-stranded packaged genome that complements itself to form double-stranded DNA, thus bypassing this conversion step and greatly reducing the time until transgene onset (McCarty, 2008; McCarty et al., 2001). Collectively, the selection

of AAV serotypes, capsids, and promoters play a vital role in the success of AAV as an effective gene therapy.

The dysregulated targets identified in our SMA motor neuron translatome study offers several novel candidates to further explore the functional contributions of these factors to disease pathology and motor neuron susceptibility. As described in Chapter X, we observed a significant reduction in Mmp9 expression in our SMA Translatome data at P9 and P19. Immunohistochemical analysis of MMP9 protein expression in SMA mice revealed a reduction in the percentage of MMP9-positive motor neurons present at P9 and further reduced by P19. Interestingly, researchers studying factors that influence motor neuron susceptibility in ALS identified MMP9 and Arhgap9 among the top candidates. Inhibiting expression of MMP9 pharmacologically or through AAV-mediated knockdown resulted in a significant delay in motor phenotypes and a 20% increase in lifespan (Kaplan et al., 2014). This study also revealed a strong overlap between MMP9 and Chodl expression in motor neurons. In addition to our translatome analysis, down-regulation of Chodl has been observed in several studies and models of SMA (Baumer et al., 2009; Z. Zhang et al., 2008; Zhong et al., 2012). Studies conducted in zebrafish have demonstrated Chodl facilitates axon growth, branching, NMJ morphology and synaptic transmission (Oprişoreanu et al., 2019). Moreover, overexpression of Chodl was shown to partially rescue axonal defects and motor neuron loss in a zebrafish model of SMA (Sleigh et al., 2014). Together, these findings raise questions including, 1) Are Mmp9, Arhgap9, and Chodl co-expressed in a subpopulation of motor neurons? 2) Can altering MMP9, CHODL, or ARHGAP9 expression attenuate SMA phenotypes and improve survival?

4.4 Concluding Remarks

Our results demonstrate the wealth of data that can be derived from cell type specific longitudinal translatomic studies performed in mouse models of disease. The data presented in this thesis indicated SMN depletion leads to dysregulated mRNA processing in motor neurons *in vivo*. Although aberrant splicing events are abundant early in disease pathology, widespread changes in gene expression were not observed until a late stage in disease. Nevertheless, dysregulated genes at the P9 time point represent

potential drivers of motor neuron pathology in disease. The upregulation of p53-mediated signaling pathways is a common observation among SMN-deficient models, yet the link between p53 activity and motor neuron cell death remains unclear. Likewise, the identification of an early and selective loss of Mmp9 expressing motor neurons offers a new and exciting direction to examine factors that influence motor neuron susceptibility. Our data provide several novel dysregulated components in SMA motor neurons that may serve as novel targets for therapeutic intervention. Taken together, this work has important implications for expanding our understanding of motor neuron susceptibility in motor neuron diseases.

References

. (!!! INVALID CITATION !!! (M.-O. Deguise et al.)).

Acsadi, G., Lee, I., Li, X., Khaidakov, M., Pecinova, A., Parker, G. C., & Huttemann, M. (2009). Mitochondrial dysfunction in a neural cell model of spinal muscular atrophy. *J Neurosci Res, 87*(12), 2748-2756. doi:10.1002/jnr.22106

Akten, B., Kye, M. J., Hao le, T., Wertz, M. H., Singh, S., Nie, D., . . . Sahin, M. (2011). Interaction of survival of motor neuron (SMN) and HuD proteins with mRNA cpg15 rescues motor neuron axonal deficits. *Proc Natl Acad Sci U S A, 108*(25), 10337-10342. doi:10.1073/pnas.1104928108

Al-Zaidy, S. A., & Mendell, J. R. (2019). From Clinical Trials to Clinical Practice: Practical Considerations for Gene Replacement Therapy in SMA Type 1. *Pediatr Neurol*. doi:10.1016/j.pediatrneurol.2019.06.007

Andreassi, C., Zimmermann, C., Mitter, R., Fusco, S., De Vita, S., Saiardi, A., & Riccio, A. (2010). An NGF-responsive element targets myo-inositol monophosphatase-1 mRNA to sympathetic neuron axons. *Nat Neurosci, 13*(3), 291-301. doi:10.1038/nn.2486

Aschrafi, A., Gioio, A. E., Dong, L., & Kaplan, B. B. (2017). Disruption of the Axonal Trafficking of Tyrosine Hydroxylase mRNA Impairs Catecholamine Biosynthesis in the Axons of Sympathetic Neurons. *eNeuro, 4*(3). doi:10.1523/ENEURO.0385-16.2017

Au - Murray, L., Au - Gillingwater, T. H., & Au - Kothary, R. (2014). Dissection of the Transversus Abdominis Muscle for Whole-mount Neuromuscular Junction Analysis. *JoVE*(83), e51162. doi:doi:10.3791/51162

Autilio, L. A., Appel, S. H., Pettis, P., & Gambetti, P. L. (1968). Biochemical studies of synapses in vitro. I. Protein synthesis. *Biochemistry, 7*(7), 2615-2622. doi:10.1021/bi00847a025

Bacrot, S., Doyard, M., Huber, C., Alibeu, O., Feldhahn, N., Lehalle, D., . . . Cormier-Daire, V. (2015). Mutations in SNRPB, Encoding Components of the Core Splicing Machinery, Cause Cerebro-Costo-Mandibular Syndrome. *Human Mutation, 36*(2), 187-190. doi:10.1002/humu.22729

Baleriola, J., & Hengst, U. (2015). Targeting axonal protein synthesis in neuroregeneration and degeneration. *Neurotherapeutics, 12*(1), 57-65. doi:10.1007/s13311-014-0308-8

Baleriola, J., Walker, C. A., Jean, Y. Y., Crary, J. F., Troy, C. M., Nagy, P. L., & Hengst, U. (2014). Axonally synthesized ATF4 transmits a neurodegenerative signal across brain regions. *Cell, 158*(5), 1159-1172. doi:10.1016/j.cell.2014.07.001

Batista, A. F., & Hengst, U. (2016). Intra-axonal protein synthesis in development and beyond. *Int J Dev Neurosci, 55*, 140-149. doi:10.1016/j.ijdevneu.2016.03.004

Battle, D. J., Lau, C. K., Wan, L., Deng, H., Lotti, F., & Dreyfuss, G. (2006). The Gemin5 protein of the SMN complex identifies snRNAs. *Mol Cell, 23*(2), 273-279.

Baumer, D., Lee, S., Nicholson, G., Davies, J. L., Parkinson, N. J., Murray, L. M., . . . Talbot, K. (2009). Alternative splicing events are a late feature of pathology in a mouse model of spinal muscular atrophy. *PLoS Genet, 5*(12), e1000773. doi:10.1371/journal.pgen.1000773

Ben-Yaakov, K., Dagan, S. Y., Segal-Ruder, Y., Shalem, O., Vuppalanchi, D., Willis, D. E., . . . Fainzilber, M. (2012). Axonal transcription factors signal retrogradely in lesioned peripheral nerve. *EMBO J, 31*(6), 1350-1363. doi:10.1038/emboj.2011.494

Bernabo, P., Tebaldi, T., Groen, E. J. N., Lane, F. M., Perenthaler, E., Mattedi, F., . . . Viero, G. (2017). In Vivo Translatome Profiling in Spinal Muscular Atrophy Reveals a Role for SMN Protein in Ribosome Biology. *Cell Rep, 21*(4), 953-965. doi:10.1016/j.celrep.2017.10.010

Bernabò, P., Tebaldi, T., Groen, E. J. N., Lane, F. M., Perenthaler, E., Mattedi, F., . . . Viero, G. (2017). In Vivo Translatome Profiling in Spinal Muscular Atrophy Reveals a Role for SMN Protein in Ribosome Biology. *Cell reports, 21*(4), 953-965. doi:https://doi.org/10.1016/j.celrep.2017.10.010

Bi, J., Tsai, N. P., Lu, H. Y., Loh, H. H., & Wei, L. N. (2007). Copb1-facilitated axonal transport and translation of kappa opioid-receptor mRNA. *Proc Natl Acad Sci U S A, 104*(34), 13810-13815. doi:10.1073/pnas.0703805104

Bigler, R. L., Kamande, J. W., Dumitru, R., Niedringhaus, M., & Taylor, A. M. (2017). Messenger RNAs localized to distal projections of human stem cell derived neurons. *Sci Rep, 7*(1), 611. doi:10.1038/s41598-017-00676-w

Bogeat-Triboulot, M. B., Brosche, M., Renaut, J., Jouve, L., Le Thiec, D., Fayyaz, P., . . . Dreyer, E. (2007). Gradual soil water depletion results in reversible changes of gene expression, protein profiles, ecophysiology, and growth performance in Populus euphratica, a poplar growing in arid regions. *Plant Physiol, 143*(2), 876-892. doi:10.1104/pp.106.088708

Boido, M., & Vercelli, A. (2016). Neuromuscular Junctions as Key Contributors and Therapeutic Targets in Spinal Muscular Atrophy. *Frontiers in Neuroanatomy, 10*, 6. doi:10.3389/fnana.2016.00006

Bowerman, M., Murray, L. M., Beauvais, A., Pinheiro, B., & Kothary, R. (2012). A critical smn threshold in mice dictates onset of an intermediate spinal muscular atrophy phenotype associated with a distinct neuromuscular junction pathology. *Neuromuscular Disorders, 22*(3), 263-276. doi:https://doi.org/10.1016/j.nmd.2011.09.007

Bowerman, M., Murray, L. M., Beauvais, A., Pinheiro, B., & Kothary, R. (2012). A critical smn threshold in mice dictates onset of an intermediate spinal muscular atrophy phenotype associated with a distinct neuromuscular junction pathology. *Neuromuscul Disord, 22*(3), 263-276. doi:10.1016/j.nmd.2011.09.007

Boyer, J. G., Murray, L. M., Scott, K., De Repentigny, Y., Renaud, J. M., & Kothary, R. (2013). Early onset muscle weakness and disruption of muscle proteins in mouse models of spinal muscular atrophy. *Skelet Muscle, 3*(1), 24. doi:10.1186/2044-5040-3-24

Brahms, H., Meheus, L., de Brabandere, V., Fischer, U., & Luhrmann, R. (2001). Symmetrical dimethylation of arginine residues in spliceosomal Sm protein B/B' and the Sm-like protein LSm4, and their interaction with the SMN protein. *Rna, 7*(11), 1531-1542.

Brangwynne, C. P., Eckmann, C. R., Courson, D. S., Rybarska, A., Hoege, C., Gharakhani, J., . . . Hyman, A. A. (2009). Germline P granules are liquid droplets that localize by controlled dissolution/condensation. *Science, 324*(5935), 1729-1732. doi:10.1126/science.1172046

Briese, M., Esmaeili, B., & Sattelle, D. B. (2005). Is spinal muscular atrophy the result of defects in motor neuron processes? *Bioessays, 27*(9), 946-957. doi:10.1002/bies.20283

Briese, M., Saal, L., Appenzeller, S., Moradi, M., Baluapuri, A., & Sendtner, M. (2016). Whole transcriptome profiling reveals the RNA content of motor axons. *Nucleic Acids Research, 44*(4), e33-e33. doi:10.1093/nar/gkv1027

Bronicki, L. M., & Jasmin, B. J. (2013). Emerging complexity of the HuD/ELAVl4 gene; implications for neuronal development, function, and dysfunction. *RNA, 19*(8), 1019-1037. doi:10.1261/rna.039164.113

Buchan, J. R. (2014). mRNP granules. Assembly, function, and connections with disease. *RNA Biol, 11*(8), 1019-1030. doi:10.4161/15476286.2014.972208

Burgart, A. M., Magnus, D., Tabor, H. K., Paquette, E. D., Frader, J., Glover, J. J., . . . Feudtner, C. (2017). Ethical Challenges Confronted When Providing Nusinersen Treatment for Spinal Muscular Atrophy. *JAMA Pediatr*. doi:10.1001/jamapediatrics.2017.4409

Burgess, R. W., Nguyen, Q. T., Son, Y. J., Lichtman, J. W., & Sanes, J. R. (1999). Alternatively spliced isoforms of nerve- and muscle-derived agrin: Their roles at the neuromuscular junction. *Neuron, 23*(1), 33-44. doi:Doi 10.1016/S0896-6273(00)80751-5

Burghes, A. H., & Beattie, C. E. (2009). Spinal muscular atrophy: why do low levels of survival motor neuron protein make motor neurons sick? *Nat Rev Neurosci, 10*(8), 597-609. doi:nrn2670 [pii]

10.1038/nrn2670
Burghes, A. H. M., & Beattie, C. E. (2009). Spinal Muscular Atrophy: Why do low levels of SMN make motor neurons sick? *Nature reviews. Neuroscience, 10*(8), 597-609. doi:10.1038/nrn2670
Cajigas, I. J., Tushev, G., Will, T. J., tom Dieck, S., Fuerst, N., & Schuman, E. M. (2012). The local transcriptome in the synaptic neuropil revealed by deep sequencing and high-resolution imaging. *Neuron, 74*(3), 453-466. doi:10.1016/j.neuron.2012.02.036
Calliari, A., Farias, J., Puppo, A., Canclini, L., Mercer, J. A., Munroe, D., . . . Sotelo-Silveira, J. R. (2014). Myosin Va associates with mRNA in ribonucleoprotein particles present in myelinated peripheral axons and in the central nervous system. *Dev Neurobiol, 74*(3), 382-396. doi:10.1002/dneu.22155
Campbell, L., Potter, A., Ignatius, J., Dubowitz, V., & Davies, K. (1997). Genomic variation and gene conversion in spinal muscular atrophy: implications for disease process and clinical phenotype. *Am J Hum Genet, 61*(1), 40-50. doi:10.1086/513886
Carra, S., Seguin, S. J., Lambert, H., & Landry, J. (2008). HspB8 Chaperone Activity toward Poly(Q)-containing Proteins Depends on Its Association with Bag3, a Stimulator of Macroautophagy. *Journal of Biological Chemistry, 283*(3), 1437-1444. doi:10.1074/jbc.M706304200
Carrel, T. L., McWhorter, M. L., Workman, E., Zhang, H. L., Wolstencroft, E. C., Lorson, C., . . . Beattie, C. E. (2006). Survival motor neuron function in motor axons is independent of functions required for small nuclear ribonucleoprotein biogenesis. *Journal of Neuroscience, 26*(43), 11014-11022. doi:10.1523/Jneurosci.1637-06.2006
Castello, A., Fischer, B., Eichelbaum, K., Horos, R., Beckmann, B. M., Strein, C., . . . Hentze, M. W. (2012). Insights into RNA biology from an atlas of mammalian mRNA-binding proteins. *Cell, 149*(6), 1393-1406. doi:10.1016/j.cell.2012.04.031
Castello, A., Fischer, B., Frese, C. K., Horos, R., Alleaume, A. M., Foehr, S., . . . Hentze, M. W. (2016). Comprehensive Identification of RNA-Binding Domains in Human Cells. *Mol Cell, 63*(4), 696-710. doi:10.1016/j.molcel.2016.06.029
Cauchi, R. J. (2010). SMN and Gemins: 'we are family' ... or are we?: insights into the partnership between Gemins and the spinal muscular atrophy disease protein SMN. *Bioessays, 32*(12), 1077-1089. doi:10.1002/bies.201000088
Ceolin, L., Bouquier, N., Vitre-Boubaker, J., Rialle, S., Severac, D., Valjent, E., . . . Puighermanal, E. (2017). Cell Type-Specific mRNA Dysregulation in Hippocampal CA1 Pyramidal Neurons of the Fragile X Syndrome Mouse Model. *Front Mol Neurosci, 10*, 340. doi:10.3389/fnmol.2017.00340
Cerveró, C., Blasco, A., Tarabal, O., Casanovas, A., Piedrafita, L., Navarro, X., . . . Calderó, J. (2018). Glial Activation and Central Synapse Loss, but Not Motoneuron Degeneration, Are Prevented by the Sigma-1 Receptor Agonist PRE-084 in the Smn2B/− Mouse Model of Spinal Muscular Atrophy. *Journal of Neuropathology & Experimental Neurology, 77*(7), 577-597. doi:10.1093/jnen/nly033
Chari, A., Golas, M. M., Klingenhäger, M., Neuenkirchen, N., Sander, B., Englbrecht, C., . . . Fischer, U. (2008). An Assembly Chaperone Collaborates with the SMN Complex to Generate Spliceosomal SnRNPs. *Cell, 135*(3), 497-509. doi:https://doi.org/10.1016/j.cell.2008.09.020
Charroux, B., Pellizzoni, L., Perkinson, R. A., Shevchenko, A., Mann, M., & Dreyfuss, G. (1999). Gemin3: A novel DEAD box protein that interacts with SMN, the spinal muscular atrophy gene product, and is a component of gems. *J Cell Biol, 147*(6), 1181-1194.
Chen, Y. Z., Bennett, C. L., Huynh, H. M., Blair, I. P., Puls, I., Irobi, J., . . . Chance, P. F. (2004). DNA/RNA helicase gene mutations in a form of juvenile amyotrophic lateral sclerosis (ALS4). *Am J Hum Genet, 74*(6), 1128-1135. doi:10.1086/421054
Chevalier-Larsen, E., & Holzbaur, E. L. (2006). Axonal transport and neurodegenerative disease. *Biochim Biophys Acta, 1762*(11-12), 1094-1108. doi:S0925-4439(06)00073-1 [pii]

10.1016/j.bbadis.2006.04.002
Cifuentes-Diaz, C., Nicole, S., Velasco, M. E., Borra-Cebrian, C., Panozzo, C., Frugier, T., . . . Melki, J. (2002). Neurofilament accumulation at the motor endplate and lack of axonal sprouting in a spinal muscular atrophy mouse model. *Hum Mol Genet, 11*(12), 1439-1447.
Colak, D., Ji, S. J., Porse, B. T., & Jaffrey, S. R. (2013). Regulation of axon guidance by compartmentalized nonsense-mediated mRNA decay. *Cell, 153*(6), 1252-1265. doi:10.1016/j.cell.2013.04.056
Cornejo, V. H., Luarte, A., & Couve, A. (2017). Global and local mechanisms sustain axonal proteostasis of transmembrane proteins. *Traffic, 18*(5), 255-266. doi:10.1111/tra.12472
Cosker, K. E., Pazyra-Murphy, M. F., Fenstermacher, S. J., & Segal, R. A. (2013). Target-derived neurotrophins coordinate transcription and transport of bclw to prevent axonal degeneration. *J Neurosci, 33*(12), 5195-5207. doi:10.1523/JNEUROSCI.3862-12.2013
Costa, C. J., & Willis, D. E. (2017). To the end of the line: Axonal mRNA transport and local translation in health and neurodegenerative disease. *Dev Neurobiol*. doi:10.1002/dneu.22555
Courtney, N. L., Mole, A. J., Thomson, A. K., & Murray, L. M. (2019). Reduced P53 levels ameliorate neuromuscular junction loss without affecting motor neuron pathology in a mouse model of spinal muscular atrophy. *Cell Death & Disease, 10*(7), 515. doi:10.1038/s41419-019-1727-6
Cox, L. J., Hengst, U., Gurskaya, N. G., Lukyanov, K. A., & Jaffrey, S. R. (2008). Intra-axonal translation and retrograde trafficking of CREB promotes neuronal survival. *Nat Cell Biol, 10*(2), 149-159. doi:10.1038/ncb1677
Coyne, A. N., Zaepfel, B. L., & Zarnescu, D. C. (2017). Failure to Deliver and Translate-New Insights into RNA Dysregulation in ALS. *Front Cell Neurosci, 11*, 243. doi:10.3389/fncel.2017.00243
Custer, S. K., & Androphy, E. J. (2014). Autophagy dysregulation in cell culture and animals models of spinal muscular atrophy. *Mol Cell Neurosci, 61*, 133-140. doi:10.1016/j.mcn.2014.06.006
Custer, S. K., Gilson, T. D., Li, H., Todd, A. G., Astroski, J. W., Lin, H., . . . Androphy, E. J. (2016). Altered mRNA Splicing in SMN-Depleted Motor Neuron-Like Cells. *PLOS ONE, 11*(10), e0163954. doi:10.1371/journal.pone.0163954
Day, M., Wang, Z., Ding, J., An, X., Ingham, C. A., Shering, A. F., . . . Surmeier, D. J. (2006). Selective elimination of glutamatergic synapses on striatopallidal neurons in Parkinson disease models. *Nat Neurosci, 9*(2), 251-259. doi:10.1038/nn1632
Deglincerti, A., Liu, Y., Colak, D., Hengst, U., Xu, G., & Jaffrey, S. R. (2015). Coupled local translation and degradation regulate growth cone collapse. *Nat Commun, 6*, 6888. doi:10.1038/ncomms7888
Deguise, M. O., Boyer, J. G., McFall, E. R., Yazdani, A., De Repentigny, Y., & Kothary, R. (2016). Differential induction of muscle atrophy pathways in two mouse models of spinal muscular atrophy. *Sci Rep, 6*, 28846. doi:10.1038/srep28846
DiDonato, C. J., Lorson, C. L., De Repentigny, Y., Simard, L., Chartrand, C., Androphy, E. J., & Kothary, R. (2001). Regulation of murine survival motor neuron (Smn) protein levels by modifying Smn exon 7 splicing. *Hum Mol Genet, 10*(23), 2727-2736.
Diers, A., Kaczinski, M., Grohmann, K., Hubner, C., & Stoltenburg-Didinger, G. (2005). The ultrastructure of peripheral nerve, motor end-plate and skeletal muscle in patients suffering from spinal muscular atrophy with respiratory distress type 1 (SMARD1). *Acta Neuropathol, 110*(3), 289-297. doi:10.1007/s00401-005-1056-y
Doktor, T. K., Hua, Y., Andersen, H. S., Broner, S., Liu, Y. H., Wieckowska, A., . . . Andresen, B. S. (2017). RNA-sequencing of a mouse-model of spinal muscular atrophy reveals tissue-wide changes in splicing of U12-dependent introns. *Nucleic Acids Res, 45*(1), 395-416. doi:10.1093/nar/gkw731
Dombert, B., Sivadasan, R., Simon, C. M., Jablonka, S., & Sendtner, M. (2014). Presynaptic localization of Smn and hnRNP R in axon terminals of embryonic and postnatal mouse motoneurons. *PLOS ONE, 9*(10), e110846. doi:10.1371/journal.pone.0110846

Donlin-Asp, P. G., Bassell, G. J., & Rossoll, W. (2016). A role for the survival of motor neuron protein in mRNP assembly and transport. *Curr Opin Neurobiol, 39*, 53-61. doi:10.1016/j.conb.2016.04.004

Donlin-Asp, P. G., Fallini, C., Campos, J., Chou, C. C., Merritt, M. E., Phan, H. C., . . . Rossoll, W. (2017). The Survival of Motor Neuron Protein Acts as a Molecular Chaperone for mRNP Assembly. *Cell Rep, 18*(7), 1660-1673. doi:10.1016/j.celrep.2017.01.059

Donlin-Asp, P. G., Fallini, C., Rouanet, J. P., Williams, K. R., Bassell, G. J., & Rossoll, W. (2014). SMN functions as a chaperone for mRNP complex assembly. *Molecular Biology of the Cell, 25*.

Donnelly, C. J., Park, M., Spillane, M., Yoo, S., Pacheco, A., Gomes, C., . . . Twiss, J. L. (2013). Axonally synthesized beta-actin and GAP-43 proteins support distinct modes of axonal growth. *J Neurosci, 33*(8), 3311-3322. doi:10.1523/JNEUROSCI.1722-12.2013

Donnelly, C. J., Willis, D. E., Xu, M., Tep, C., Jiang, C., Yoo, S., . . . Twiss, J. L. (2011). Limited availability of ZBP1 restricts axonal mRNA localization and nerve regeneration capacity. *EMBO J, 30*(22), 4665-4677. doi:10.1038/emboj.2011.347

Dotto, G. P. (2000). p21(WAF1/Cip1): more than a break to the cell cycle? *Biochim Biophys Acta, 1471*(1), M43-56. doi:10.1016/s0304-419x(00)00019-6

Edens, B. M., Ajroud-Driss, S., Ma, L., & Ma, Y.-C. (2015). Molecular mechanisms and animal models of spinal muscular atrophy. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 1852*(4), 685-692. doi:https://doi.org/10.1016/j.bbadis.2014.07.024

Edstrom, A., & Sjostrand, J. (1969). Protein synthesis in the isolated Mauthner nerve fibre of goldfish. *J Neurochem, 16*(1), 67-81. doi:10.1111/j.1471-4159.1969.tb10344.x

Elsaid, M. F., Chalhoub, N., Ben-Omran, T., Kumar, P., Kamel, H., Ibrahim, K., . . . Aleem, A. A. (2017). Mutation in noncoding RNA RNU12 causes early onset cerebellar ataxia. *Ann Neurol, 81*(1), 68-78. doi:10.1002/ana.24826

Eom, T., Antar, L. N., Singer, R. H., & Bassell, G. J. (2003). Localization of a beta-actin messenger ribonucleoprotein complex with zipcode-binding protein modulates the density of dendritic filopodia and filopodial synapses. *J Neurosci, 23*(32), 10433-10444.

Eshraghi, M., McFall, E., Gibeault, S., & Kothary, R. (2016). Effect of genetic background on the phenotype of the Smn2B/- mouse model of spinal muscular atrophy. *Hum Mol Genet, 25*(20), 4494-4506. doi:10.1093/hmg/ddw278

Fallini, C., Bassell, G. J., & Rossoll, W. (2010). High-efficiency transfection of cultured primary motor neurons to study protein localization, trafficking, and function. *Mol Neurodegener, 5*, 17. doi:10.1186/1750-1326-5-17

Fallini, C., Bassell, G. J., & Rossoll, W. (2012). Spinal muscular atrophy: the role of SMN in axonal mRNA regulation. *Brain Res, 1462*, 81-92. doi:10.1016/j.brainres.2012.01.044

Fallini, C., Donlin-Asp, P. G., Rouanet, J. P., Bassell, G. J., & Rossoll, W. (2016). Deficiency of the Survival of Motor Neuron Protein Impairs mRNA Localization and Local Translation in the Growth Cone of Motor Neurons. *J Neurosci, 36*(13), 3811-3820. doi:10.1523/JNEUROSCI.2396-15.2016

Fallini, C., Rouanet, J. P., Donlin-Asp, P. G., Guo, P., Zhang, H., Singer, R. H., . . . Bassell, G. J. (2014). Dynamics of survival of motor neuron (SMN) protein interaction with the mRNA-binding protein IMP1 facilitates its trafficking into motor neuron axons. *Dev Neurobiol, 74*(3), 319-332. doi:10.1002/dneu.22111

Fallini, C., Zhang, H., Su, Y., Silani, V., Singer, R. H., Rossoll, W., & Bassell, G. J. (2011). The survival of motor neuron (SMN) protein interacts with the mRNA-binding protein HuD and regulates localization of poly(A) mRNA in primary motor neuron axons. *J Neurosci, 31*(10), 3914-3925. doi:10.1523/JNEUROSCI.3631-10.2011

Farías, G. G., Guardia, C. M., De Pace, R., Britt, D. J., & Bonifacino, J. S. (2017). BORC/kinesin-1 ensemble drives polarized transport of lysosomes into the axon. *Proceedings of the National Academy of Sciences, 114*(14), E2955-E2964. doi:10.1073/pnas.1616363114

Farrar, M. A., Park, S. B., Vucic, S., Carey, K. A., Turner, B. J., Gillingwater, T. H., . . . Kiernan, M. C. (2017). Emerging therapies and challenges in spinal muscular atrophy. *Annals of Neurology, 81*(3), 355-368. doi:10.1002/ana.24864

Fischer, L. R., Culver, D. G., Tennant, P., Davis, A. A., Wang, M., Castellano-Sanchez, A., . . . Glass, J. D. (2004). Amyotrophic lateral sclerosis is a distal axonopathy: evidence in mice and man. *Exp Neurol, 185*(2), 232-240.

Fischer, U., Liu, Q., & Dreyfuss, G. (1997). The SMN-SIP1 complex has an essential role in spliceosomal snRNP biogenesis. *Cell, 90*(6), 1023-1029. doi:10.1016/s0092-8674(00)80368-2

Foust, K. D., Nurre, E., Montgomery, C. L., Hernandez, A., Chan, C. M., & Kaspar, B. K. (2009). Intravascular AAV9 preferentially targets neonatal-neurons and adult-astrocytes in CNS. *Nature biotechnology, 27*(1), 59-65. doi:10.1038/nbt.1515

Frey, D., Laux, T., Xu, L., Schneider, C., & Caroni, P. (2000). Shared and unique roles of CAP23 and GAP43 in actin regulation, neurite outgrowth, and anatomical plasticity. *J Cell Biol, 149*(7), 1443-1454.

Friesen, W. J., Massenet, S., Paushkin, S., Wyce, A., & Dreyfuss, G. (2001). SMN, the product of the spinal muscular atrophy gene, binds preferentially to dimethylarginine-containing protein targets. *Mol Cell, 7*(5), 1111-1117.

Friesen, W. J., Paushkin, S., Wyce, A., Massenet, S., Pesiridis, G. S., Van Duyne, G., . . . Dreyfuss, G. (2001). The methylosome, a 20S complex containing JBP1 and pICln, produces dimethylarginine-modified Sm proteins. *Mol Cell Biol, 21*(24), 8289-8300. doi:10.1128/mcb.21.24.8289-8300.2001

Fukaya, M., Hayashi, Y., & Watanabe, M. (2005). NR2 to NR3B subunit switchover of NMDA receptors in early postnatal motoneurons. *Eur J Neurosci, 21*(5), 1432-1436. doi:10.1111/j.1460-9568.2005.03957.x

Fuller, H. R., Mandefro, B., Shirran, S. L., Gross, A. R., Kaus, A. S., Botting, C. H., . . . Sareen, D. (2016). Spinal Muscular Atrophy Patient iPSC-Derived Motor Neurons Have Reduced Expression of Proteins Important in Neuronal Development. *Frontiers in Cellular Neuroscience, 9*. doi:ARTN 506

10.3389/fncel.2015.00506

Gabanella, F., Butchbach, M. E., Saieva, L., Carissimi, C., Burghes, A. H., & Pellizzoni, L. (2007). Ribonucleoprotein assembly defects correlate with spinal muscular atrophy severity and preferentially affect a subset of spliceosomal snRNPs. *PLoS ONE, 2*(9), e921. doi:10.1371/journal.pone.0000921

Gabanella, F., Butchbach, M. E. R., Saieva, L., Carissimi, C., Burghes, A. H. M., & Pellizzoni, L. (2007). Ribonucleoprotein Assembly Defects Correlate with Spinal Muscular Atrophy Severity and Preferentially Affect a Subset of Spliceosomal snRNPs. *PLOS ONE, 2*(9), e921. doi:10.1371/journal.pone.0000921

Gabanella, F., Pisani, C., Borreca, A., Farioli-Vecchioli, S., Ciotti, M. T., Ingegnere, T., . . . Di Certo, M. G. (2016). SMN affects membrane remodelling and anchoring of the protein synthesis machinery. *J Cell Sci, 129*(4), 804-816. doi:10.1242/jcs.176750

Gama-Carvalho, M., M, L. G.-V., F, R. P., Besse, F., Weis, J., Voigt, A., . . . De Las Rivas, J. (2017). Linking amyotrophic lateral sclerosis and spinal muscular atrophy through RNA-transcriptome homeostasis: a genomics perspective. *J Neurochem, 141*(1), 12-30. doi:10.1111/jnc.13945

Gamerdinger, M., Hajieva, P., Kaya, A. M., Wolfrum, U., Hartl, F. U., & Behl, C. (2009). Protein quality control during aging involves recruitment of the macroautophagy pathway by BAG3. *The EMBO Journal, 28*(7), 889-901. doi:10.1038/emboj.2009.29

Gamerdinger, M., Kaya, A. M., Wolfrum, U., Clement, A. M., & Behl, C. (2011). BAG3 mediates chaperone-based aggresome-targeting and selective autophagy of misfolded proteins. *EMBO reports, 12*(2), 149-156. doi:10.1038/embor.2010.203

Garcia, E. L., Wen, Y., Praveen, K., & Matera, A. G. (2016). Transcriptomic comparison of Drosophila snRNP biogenesis mutants reveals mutant-specific changes in pre-mRNA processing: implications for spinal muscular atrophy. *Rna, 22*(8), 1215-1227. doi:10.1261/rna.057208.116

Gavrilina, T. O., McGovern, V. L., Workman, E., Crawford, T. O., Gogliotti, R. G., DiDonato, C. J., . . . Burghes, A. H. M. (2008). Neuronal SMN expression corrects spinal muscular atrophy in severe SMA mice while muscle-specific SMN expression has no phenotypic effect. *Human Molecular Genetics, 17*(8), 1063-1075. doi:10.1093/hmg/ddm379

Glinka, M., Herrmann, T., Funk, N., Havlicek, S., Rossoll, W., Winkler, C., & Sendtner, M. (2010). The heterogeneous nuclear ribonucleoprotein-R is necessary for axonal beta-actin mRNA translocation in spinal motor neurons. *Hum Mol Genet, 19*(10), 1951-1966. doi:10.1093/hmg/ddq073

Gogliotti, R. G., Quinlan, K. A., Barlow, C. B., Heier, C. R., Heckman, C. J., & Didonato, C. J. (2012). Motor neuron rescue in spinal muscular atrophy mice demonstrates that sensory-motor defects are a consequence, not a cause, of motor neuron dysfunction. *J Neurosci, 32*(11), 3818-3829. doi:10.1523/jneurosci.5775-11.2012

Goldstein, L. S. (2001). Kinesin molecular motors: transport pathways, receptors, and human disease. *Proc Natl Acad Sci U S A, 98*(13), 6999-7003. doi:10.1073/pnas.111145298

Gomes, C., Lee, S. J., Gardiner, A. S., Smith, T., Sahoo, P. K., Patel, P., . . . Twiss, J. L. (2017). Axonal localization of neuritin/CPG15 mRNA is limited by competition for HuD binding. *J Cell Sci*. doi:10.1242/jcs.201244

Gonzalez, C., Canovas, J., Fresno, J., Couve, E., Court, F. A., & Couve, A. (2016). Axons provide the secretory machinery for trafficking of voltage-gated sodium channels in peripheral nerve. *Proc Natl Acad Sci U S A, 113*(7), 1823-1828. doi:10.1073/pnas.1514943113

Gopal, P. P., Nirschl, J. J., Klinman, E., & Holzbaur, E. L. (2017). Amyotrophic lateral sclerosis-linked mutations increase the viscosity of liquid-like TDP-43 RNP granules in neurons. *Proc Natl Acad Sci U S A, 114*(12), E2466-E2475. doi:10.1073/pnas.1614462114

Gracias, N. G., Shirkey-Son, N. J., & Hengst, U. (2014). Local translation of TC10 is required for membrane expansion during axon outgrowth. *Nat Commun, 5*, 3506. doi:10.1038/ncomms4506

Gribling-Burrer, A. S., Leichter, M., Wurth, L., Huttin, A., Schlotter, F., Troffer-Charlier, N., . . . Allmang, C. (2017). SECIS-binding protein 2 interacts with the SMN complex and the methylosome for selenoprotein mRNP assembly and translation. *Nucleic Acids Res, 45*(9), 5399-5413. doi:10.1093/nar/gkx031

Gross, A., Schoendube, J., Zimmermann, S., Steeb, M., Zengerle, R., & Koltay, P. (2015). Technologies for Single-Cell Isolation. *Int J Mol Sci, 16*(8), 16897-16919. doi:10.3390/ijms160816897

Gruss, O. J., Meduri, R., Schilling, M., & Fischer, U. (2017). UsnRNP biogenesis: mechanisms and regulation. *Chromosoma, 126*(5), 577-593. doi:10.1007/s00412-017-0637-6

Gumy, L. F., Yeo, G. S., Tung, Y. C., Zivraj, K. H., Willis, D., Coppola, G., . . . Fawcett, J. W. (2011). Transcriptome analysis of embryonic and adult sensory axons reveals changes in mRNA repertoire localization. *RNA, 17*(1), 85-98. doi:rna.2386111 [pii]

10.1261/rna.2386111

Gygi, S. P., Rochon, Y., Franza, B. R., & Aebersold, R. (1999). Correlation between protein and mRNA abundance in yeast. *Mol Cell Biol, 19*(3), 1720-1730. doi:10.1128/mcb.19.3.1720

Haidar, M., Asselbergh, B., Adriaenssens, E., De Winter, V., Timmermans, J.-P., Auer-Grumbach, M., . . . Timmerman, V. (2019). Neuropathy-causing mutations in HSPB1 impair autophagy by disturbing the formation of SQSTM1/p62 bodies. *Autophagy, 15*(6), 1051-1068. doi:10.1080/15548627.2019.1569930

Haimon, Z., Volaski, A., Orthgiess, J., Boura-Halfon, S., Varol, D., Shemer, A., . . . Jung, S. (2018). Re-evaluating microglia expression profiles using RiboTag and cell isolation strategies. *Nat Immunol, 19*(6), 636-644. doi:10.1038/s41590-018-0110-6

Hammond, S. M., Gogliotti, R. G., Rao, V., Beauvais, A., Kothary, R., & DiDonato, C. J. (2010). Mouse survival motor neuron alleles that mimic SMN2 splicing and are inducible rescue embryonic lethality early in development but not late. *PLoS One, 5*(12), e15887. doi:10.1371/journal.pone.0015887

Han, T. W., Kato, M., Xie, S., Wu, L. C., Mirzaei, H., Pei, J., . . . McKnight, S. L. (2012). Cell-free formation of RNA granules: bound RNAs identify features and components of cellular assemblies. *Cell, 149*(4), 768-779. doi:10.1016/j.cell.2012.04.016

Hao le, T., Duy, P. Q., Jontes, J. D., & Beattie, C. E. (2015). Motoneuron development influences dorsal root ganglia survival and Schwann cell development in a vertebrate model of spinal muscular atrophy. *Hum Mol Genet, 24*(2), 346-360. doi:10.1093/hmg/ddu447

Hao, L. T., Duy, P. Q., An, M., Talbot, J., Iyer, C. C., Wolman, M., & Beattie, C. E. (2017). HuD and the Survival Motor Neuron protein interact in motoneurons and are essential for motoneuron development, function and mRNA regulation. *J Neurosci*. doi:10.1523/JNEUROSCI.1528-17.2017

Hayes, M. J., Shao, D., Bailly, M., & Moss, S. E. (2006). Regulation of actin dynamics by annexin 2. *EMBO J, 25*(9), 1816-1826. doi:10.1038/sj.emboj.7601078

Hayhurst, M., Wagner, A. K., Cerletti, M., Wagers, A. J., & Rubin, L. L. (2012). A cell-autonomous defect in skeletal muscle satellite cells expressing low levels of survival of motor neuron protein. *Dev Biol, 368*(2), 323-334. doi:10.1016/j.ydbio.2012.05.037

He, G., Siddik, Z. H., Huang, Z., Wang, R., Koomen, J., Kobayashi, R., . . . Kuang, J. (2005). Induction of p21 by p53 following DNA damage inhibits both Cdk4 and Cdk2 activities. *Oncogene, 24*(18), 2929-2943. doi:10.1038/sj.onc.1208474

Hedlund, E., Karlsson, M., Osborn, T., Ludwig, W., & Isacson, O. (2010). Global gene expression profiling of somatic motor neuron populations with different vulnerability identify molecules and pathways of degeneration and protection. *Brain, 133*(8), 2313-2330. doi:10.1093/brain/awq167

Hengst, U., Cox, L. J., Macosko, E. Z., & Jaffrey, S. R. (2006). Functional and selective RNA interference in developing axons and growth cones. *J Neurosci, 26*(21), 5727-5732.

Hengst, U., Deglincerti, A., Kim, H. J., Jeon, N. L., & Jaffrey, S. R. (2009). Axonal elongation triggered by stimulus-induced local translation of a polarity complex protein. *Nat Cell Biol, 11*(8), 1024-1030. doi:10.1038/ncb1916

Hentze, M. W., Castello, A., Schwarzl, T., & Preiss, T. (2018). A brave new world of RNA-binding proteins. *Nat Rev Mol Cell Biol*. doi:10.1038/nrm.2017.130

Hirokawa, N., & Takemura, R. (2005). Molecular motors and mechanisms of directional transport in neurons. *Nat Rev Neurosci, 6*(3), 201-214. doi:10.1038/nrn1624

Holt, C. E., & Schuman, E. M. (2013). The central dogma decentralized: new perspectives on RNA function and local translation in neurons. *Neuron, 80*(3), 648-657. doi:10.1016/j.neuron.2013.10.036

Hoy, S. M. (2017). Nusinersen: First Global Approval. *Drugs, 77*(4), 473-479. doi:10.1007/s40265-017-0711-7

Hsieh-Li, H. M., Chang, J. G., Jong, Y. J., Wu, M. H., Wang, N. M., Tsai, C. H., & Li, H. (2000). A mouse model for spinal muscular atrophy. *Nat Genet, 24*(1), 66-70. doi:10.1038/71709

Hsu, W. L., Chung, H. W., Wu, C. Y., Wu, H. I., Lee, Y. T., Chen, E. C., . . . Chang, Y. C. (2015). Glutamate Stimulates Local Protein Synthesis in the Axons of Rat Cortical Neurons by Activating alpha-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid (AMPA) Receptors and Metabotropic Glutamate Receptors. *J Biol Chem, 290*(34), 20748-20760. doi:10.1074/jbc.M115.638023

Hua, Y., Sahashi, K., Hung, G., Rigo, F., Passini, M. A., Bennett, C. F., & Krainer, A. R. (2010). Antisense correction of SMN2 splicing in the CNS rescues necrosis in a type III SMA mouse model. *Genes Dev, 24*(15), 1634-1644. doi:10.1101/gad.1941310

Hua, Y., & Zhou, J. (2004). Survival motor neuron protein facilitates assembly of stress granules. *FEBS Lett, 572*(1-3), 69-74.

Hubers, L., Valderrama-Carvajal, H., Laframboise, J., Timbers, J., Sanchez, G., & Cote, J. (2011). HuD interacts with survival motor neuron protein and can rescue spinal muscular atrophy-like neuronal defects. *Hum Mol Genet, 20*(3), 553-579. doi:10.1093/hmg/ddq500

Hunter, G., Aghamaleky Sarvestany, A., Roche, S. L., Symes, R. C., & Gillingwater, T. H. (2014). SMN-dependent intrinsic defects in Schwann cells in mouse models of spinal muscular atrophy. *Hum Mol Genet, 23*(9), 2235-2250. doi:10.1093/hmg/ddt612

Huo, Q., Kayikci, M., Odermatt, P., Meyer, K., Michels, O., Saxena, S., . . . Schumperli, D. (2014). Splicing changes in SMA mouse motoneurons and SMN-depleted neuroblastoma cells: evidence for involvement of splicing regulatory proteins. *RNA Biol, 11*(11), 1430-1446. doi:10.1080/15476286.2014.996494

Huttelmaier, S., Zenklusen, D., Lederer, M., Dictenberg, J., Lorenz, M., Meng, X., . . . Singer, R. H. (2005). Spatial regulation of beta-actin translation by Src-dependent phosphorylation of ZBP1. *Nature, 438*(7067), 512-515.

Ito, Y., Kumada, S., Uchiyama, A., Saito, K., Osawa, M., Yagishita, A., . . . Hayashi, M. (2004). Thalamic lesions in a long-surviving child with spinal muscular atrophy type I: MRI and EEG findings. *Brain and Development, 26*(1), 53-56. doi:https://doi.org/10.1016/S0387-7604(03)00075-5

Iyer, C. C., McGovern, V. L., Murray, J. D., Gombash, S. E., Zaworski, P. G., Foust, K. D., . . . Burghes, A. H. (2015). Low levels of Survival Motor Neuron protein are sufficient for normal muscle function in the SMNDelta7 mouse model of SMA. *Hum Mol Genet, 24*(21), 6160-6173. doi:10.1093/hmg/ddv332

Jablonka, S., Bandilla, M., Wiese, S., Buhler, D., Wirth, B., Sendtner, M., & Fischer, U. (2001). Co-regulation of survival of motor neuron (SMN) protein and its interactor SIP1 during development and in spinal muscular atrophy. *Hum Mol Genet, 10*(5), 497-505.

Jablonka, S., Beck, M., Lechner, B. D., Mayer, C., & Sendtner, M. (2007). Defective Ca2+ channel clustering in axon terminals disturbs excitability in motoneurons in spinal muscular atrophy. *Journal of Cell Biology, 179*(1), 139-149. doi:10.1083/jcb.200703187

Jablonka, S., Karle, K., Sandner, B., Andreassi, C., von Au, K., & Sendtner, M. (2006). Distinct and overlapping alterations in motor and sensory neurons in a mouse model of spinal muscular atrophy. *Hum Mol Genet, 15*(3), 511-518. doi:10.1093/hmg/ddi467

Jablonka, S., Schrank, B., Kralewski, M., Rossoll, W., & Sendtner, M. (2000). Reduced survival motor neuron (Smn) gene dose in mice leads to motor neuron degeneration: an animal model for spinal muscular atrophy type III. *Hum Mol Genet, 9*(3), 341-346.

Jablonka, S., & Sendtner, M. (2017). Developmental regulation of SMN expression: pathophysiological implications and perspectives for therapy development in spinal muscular atrophy. *Gene Ther, 24*(9), 506-513. doi:10.1038/gt.2017.46

Jablonka, S., Wiese, S., & Sendtner, M. (2004). Axonal defects in mouse models of motoneuron disease. *J Neurobiol, 58*(2), 272-286. doi:10.1002/neu.10313

Jangi, M., Fleet, C., Cullen, P., Gupta, S. V., Mekhoubad, S., Chiao, E., . . . Staropoli, J. F. (2017). SMN deficiency in severe models of spinal muscular atrophy causes widespread intron retention and DNA damage. *Proc Natl Acad Sci U S A, 114*(12), E2347-E2356. doi:10.1073/pnas.1613181114

Jansen, R. P., Niessing, D., Baumann, S., & Feldbrugge, M. (2014). mRNA transport meets membrane traffic. *Trends Genet, 30*(9), 408-417. doi:10.1016/j.tig.2014.07.002

Javaherian, A., & Cline, H. T. (2005). Coordinated motor neuron axon growth and neuromuscular synaptogenesis are promoted by CPG15 in vivo. *Neuron, 45*(4), 505-512. doi:10.1016/j.neuron.2004.12.051

Ji, S.-J., & Jaffrey, S. R. (2014). Axonal transcription factors: Novel regulators of growth cone-to-nucleus signaling. *Dev Neurobiol, 74*(3), 245-258. doi:10.1002/dneu.22112

Jinn, S., Drolet, R. E., Cramer, P. E., Wong, A. H.-K., Toolan, D. M., Gretzula, C. A., . . . Stone, D. J. (2017). TMEM175 deficiency impairs lysosomal and mitochondrial function and increases α-synuclein aggregation. *Proceedings of the National Academy of Sciences, 114*(9), 2389-2394. doi:10.1073/pnas.1616332114

Jodelka, F. M., Ebert, A. D., Duelli, D. M., & Hastings, M. L. (2010). A feedback loop regulates splicing of the spinal muscular atrophy-modifying gene, SMN2. *Hum Mol Genet, 19*(24), 4906-4917. doi:10.1093/hmg/ddq425

Jung, J., & Jung, H. (2016). Methods to analyze cell type-specific gene expression profiles from heterogeneous cell populations. *Animal Cells and Systems, 20*(3), 113-117. doi:10.1080/19768354.2016.1191544

Kalous, A., Stake, J. I., Yisraeli, J. K., & Holt, C. E. (2014). RNA-binding protein Vg1RBP regulates terminal arbor formation but not long-range axon navigation in the developing visual system. *Dev Neurobiol, 74*(3), 303-318. doi:10.1002/dneu.22110

Kang, S. S., Ebbert, M. T. W., Baker, K. E., Cook, C., Wang, X., Sens, J. P., . . . Fryer, J. D. (2018). Microglial translational profiling reveals a convergent APOE pathway from aging, amyloid, and tau. *The Journal of Experimental Medicine, 215*(9), 2235-2245. doi:10.1084/jem.20180653

Kanning, K. C., Kaplan, A., & Henderson, C. E. (2010). Motor neuron diversity in development and disease. *Annu Rev Neurosci, 33*, 409-440. doi:10.1146/annurev.neuro.051508.135722

Kaplan, A., Spiller, K. J., Towne, C., Kanning, K. C., Choe, G. T., Geber, A., . . . Henderson, C. E. (2014). Neuronal matrix metalloproteinase-9 is a determinant of selective neurodegeneration. *Neuron, 81*(2), 333-348. doi:10.1016/j.neuron.2013.12.009

Kar, A. N., Lee, S. J., & Twiss, J. L. (2017). Expanding Axonal Transcriptome Brings New Functions for Axonally Synthesized Proteins in Health and Disease. *Neuroscientist*, 1073858417712668. doi:10.1177/1073858417712668

Kar, A. N., MacGibeny, M. A., Gervasi, N. M., Gioio, A. E., & Kaplan, B. B. (2013). Intra-axonal Synthesis of Eukaryotic Translation Initiation Factors Regulate Local Protein Synthesis and Axon Growth in Rat Sympathetic Neurons. *J Neurosci, 33*(17), 7165-7174. doi:10.1523/JNEUROSCI.2040-12.2013

Kar, A. N., Sun, C. Y., Reichard, K., Gervasi, N. M., Pickel, J., Nakazawa, K., . . . Kaplan, B. B. (2014). Dysregulation of the axonal trafficking of nuclear-encoded mitochondrial mRNA alters neuronal mitochondrial activity and mouse behavior. *Dev Neurobiol, 74*(3), 333-350. doi:10.1002/dneu.22141

Kariya, S., Park, G. H., Maeno-Hikichi, Y., Leykekhman, O., Lutz, C., Arkovitz, M. S., . . . Monani, U. R. (2008). Reduced SMN protein impairs maturation of the neuromuscular junctions in mouse models of spinal muscular atrophy. *Hum Mol Genet, 17*(16), 2552-2569. doi:ddn156 [pii]

10.1093/hmg/ddn156

Kato, M., Han, T. W., Xie, S., Shi, K., Du, X., Wu, L. C., . . . McKnight, S. L. (2012). Cell-free formation of RNA granules: low complexity sequence domains form dynamic fibers within hydrogels. *Cell, 149*(4), 753-767. doi:10.1016/j.cell.2012.04.017

Katz, Y., Wang, E. T., Airoldi, E. M., & Burge, C. B. (2010). Analysis and design of RNA sequencing experiments for identifying isoform regulation. *Nat Methods, 7*(12), 1009-1015. doi:10.1038/nmeth.1528

Kiebler, M. A., & Bassell, G. J. (2006). Neuronal RNA granules: movers and makers. *Neuron, 51*(6), 685-690. doi:10.1016/j.neuron.2006.08.021

Kim, J. K., Caine, C., Awano, T., Herbst, R., & Monani, U. R. (2017). Motor neuronal repletion of the NMJ organizer, Agrin, modulates the severity of the spinal muscular atrophy disease phenotype in model mice. *Hum Mol Genet, 26*(13), 2377-2385. doi:10.1093/hmg/ddx124

Kim, S. G., Ravi, G., Hoffmann, C., Jung, Y. J., Kim, M., Chen, A., & Jacobson, K. A. (2002). p53-Independent induction of Fas and apoptosis in leukemic cells by an adenosine derivative, Cl-IB-MECA. *Biochemical pharmacology, 63*(5), 871-880. doi:10.1016/s0006-2952(02)00839-0

Kislauskis, E. H., & Singer, R. H. (1992). Determinants of mRNA localization. *Curr Opin Cell Biol, 4*(6), 975-978.

Kline, R. A., Kaifer, K. A., Osman, E. Y., Carella, F., Tiberi, A., Ross, J., . . . Murray, L. M. (2017). Comparison of independent screens on differentially vulnerable motor neurons reveals alpha-synuclein as a common modifier in motor neuron diseases. *PLoS Genet, 13*(3), e1006680-e1006680. doi:10.1371/journal.pgen.1006680

Koenig, E. (1967). Synthetic mechanisms in the axon. IV. In vitro incorporation of [3H]precursors into axonal protein and RNA. *J Neurochem, 14*(4), 437-446. doi:10.1111/j.1471-4159.1967.tb09542.x

Koenig, E., Martin, R., Titmus, M., & Sotelo-Silveira, J. R. (2000). Cryptic peripheral ribosomal domains distributed intermittently along mammalian myelinated axons. *J Neurosci, 20*(22), 8390-8400.

Kolb, S. J., Battle, D. J., & Dreyfuss, G. (2007). Molecular Functions of the SMN Complex. *Journal of Child Neurology, 22*(8), 990-994. doi:10.1177/0883073807305666

Kolb, S. J., & Kissel, J. T. (2011). Spinal muscular atrophy: a timely review. *Arch Neurol, 68*(8), 979-984. doi:10.1001/archneurol.2011.74

Kong, L., Wang, X., Choe, D. W., Polley, M., Burnett, B. G., Bosch-Marce, M., . . . Sumner, C. J. (2009). Impaired synaptic vesicle release and immaturity of neuromuscular junctions in spinal muscular atrophy mice. *J Neurosci, 29*(3), 842-851. doi:10.1523/JNEUROSCI.4434-08.2009

Kulikov, R., Letienne, J., Kaur, M., Grossman, S. R., Arts, J., & Blattner, C. (2010). Mdm2 facilitates the association of p53 with the proteasome. *Proceedings of the National Academy of Sciences, 107*(22), 10038-10043. doi:10.1073/pnas.0911716107

Kye, M. J., Niederst, E. D., Wertz, M. H., Goncalves Ido, C., Akten, B., Dover, K. Z., . . . Sahin, M. (2014). SMN regulates axonal local translation via miR-183/mTOR pathway. *Hum Mol Genet, 23*(23), 6318-6331. doi:10.1093/hmg/ddu350

Lau, C. K., Bachorik, J. L., & Dreyfuss, G. (2009). Gemin5-snRNA interaction reveals an RNA binding function for WD repeat domains. *Nat Struct Mol Biol, 16*(5), 486-491. doi:10.1038/nsmb.1584

Le, T. T., Pham, L. T., Butchbach, M. E., Zhang, H. L., Monani, U. R., Coovert, D. D., . . . Burghes, A. H. (2005). SMNDelta7, the major product of the centromeric survival motor neuron (SMN2) gene, extends survival in mice with spinal muscular atrophy and associates with full-length SMN. *Hum Mol Genet, 14*(6), 845-857. doi:10.1093/hmg/ddi078

Lee, Y. I., Mikesh, M., Smith, I., Rimer, M., & Thompson, W. (2011). Muscles in a mouse model of spinal muscular atrophy show profound defects in neuromuscular development even in the absence of failure in neuromuscular transmission or loss of motor neurons. *Dev Biol, 356*(2), 432-444. doi:10.1016/j.ydbio.2011.05.667

Lefebvre, S., Burglen, L., Reboullet, S., Clermont, O., Burlet, P., Viollet, L., . . . et al. (1995). Identification and characterization of a spinal muscular atrophy-determining gene. *Cell, 80*(1), 155-165.

Leung, K. M., van Horck, F. P., Lin, A. C., Allison, R., Standart, N., & Holt, C. E. (2006). Asymmetrical beta-actin mRNA translation in growth cones mediates attractive turning to netrin-1. *Nat Neurosci, 9*(10), 1247-1256.

Li, D. K., Tisdale, S., Lotti, F., & Pellizzoni, L. (2014a). SMN control of RNP assembly: From post-transcriptional gene regulation to motor neuron disease. *Semin Cell Dev Biol*. doi:10.1016/j.semcdb.2014.04.026

Li, D. K., Tisdale, S., Lotti, F., & Pellizzoni, L. (2014b). SMN control of RNP assembly: from post-transcriptional gene regulation to motor neuron disease. *Semin Cell Dev Biol, 32*, 22-29. doi:10.1016/j.semcdb.2014.04.026

Li, H. X., Custer, S. K., Gilson, T., Hao, L. T., Beattie, C. E., & Androphy, E. J. (2015). alpha-COP binding to the survival motor neuron protein SMN is required for neuronal process outgrowth. *Human Molecular Genetics, 24*(25), 7295-7307. doi:10.1093/hmg/ddv428

Li, X., Yu, B., Sun, Q., Zhang, Y., Ren, M., Zhang, X., . . . Qiu, Z. (2018). Generation of a whole-brain atlas for the cholinergic system and mesoscopic projectome analysis of basal forebrain cholinergic neurons. *Proceedings of the National Academy of Sciences, 115*(2), 415-420. doi:10.1073/pnas.1703601115

Li, Y.-F., Zheng, Y., Vemireddy, L. R., Panda, S. K., Jose, S., Ranjan, A., . . . Sunkar, R. (2018). Comparative transcriptome and translatome analysis in contrasting rice genotypes reveals differential mRNA translation in salt-tolerant Pokkali under salt stress. *BMC Genomics, 19*(10), 935. doi:10.1186/s12864-018-5279-4

Lin, Y., Protter, D. S., Rosen, M. K., & Parker, R. (2015). Formation and Maturation of Phase-Separated Liquid Droplets by RNA-Binding Proteins. *Mol Cell, 60*(2), 208-219. doi:10.1016/j.molcel.2015.08.018

Ling, K. K. Y., Lin, M.-Y., Zingg, B., Feng, Z., & Ko, C.-P. (2010). Synaptic Defects in the Spinal and Neuromuscular Circuitry in a Mouse Model of Spinal Muscular Atrophy. *PLoS ONE, 5*(11), e15457. doi:10.1371/journal.pone.0015457

Liu, Q., & Dreyfuss, G. (1996). A novel nuclear structure containing the survival of motor neurons protein. *Embo J, 15*(14), 3555-3565.

Liu, Q., Fischer, U., Wang, F., & Dreyfuss, G. (1997). The spinal muscular atrophy disease gene product, SMN, and its associated protein SIP1 are in a complex with spliceosomal snRNP proteins. *Cell, 90*(6), 1013-1021.

Liu, S., & Trapnell, C. (2016). Single-cell transcriptome sequencing: recent advances and remaining challenges [version 1; peer review: 2 approved]. *F1000Research, 5*(182). doi:10.12688/f1000research.7223.1

Lorson, C. L., Hahnen, E., Androphy, E. J., & Wirth, B. (1999). A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy. *Proc Natl Acad Sci U S A, 96*(11), 6307-6311. doi:10.1073/pnas.96.11.6307

Lotti, F., Imlach, W. L., Saieva, L., Beck, E. S., Hao le, T., Li, D. K., . . . Pellizzoni, L. (2012). An SMN-dependent U12 splicing event essential for motor circuit function. *Cell, 151*(2), 440-454. doi:10.1016/j.cell.2012.09.012

Luarte, A., Cornejo, V. H., Bertin, F., Gallardo, J., & Couve, A. (2017). The axonal endoplasmic reticulum: One organelle-many functions in development, maintenance, and plasticity. *Dev Neurobiol*. doi:10.1002/dneu.22560

Lunn, M. R., & Wang, C. H. (2008). Spinal muscular atrophy. *Lancet, 371*(9630), 2120-2133. doi:10.1016/s0140-6736(08)60921-6

Ma, Y., Dostie, J., Dreyfuss, G., & Van Duyne, G. D. (2005). The Gemin6-Gemin7 Heterodimer from the Survival of Motor Neurons Complex Has an Sm Protein-like Structure. *Structure, 13*(6), 883-892. doi:https://doi.org/10.1016/j.str.2005.03.014

Maday, S., Twelvetrees, A. E., Moughamian, A. J., & Holzbaur, E. L. (2014). Axonal transport: cargo-specific mechanisms of motility and regulation. *Neuron, 84*(2), 292-309. doi:10.1016/j.neuron.2014.10.019

Martin, J. E., Nguyen, T. T., Grunseich, C., Nofziger, J. H., Lee, P. R., Fields, D., . . . Foran, E. (2017). Decreased Motor Neuron Support by SMA Astrocytes due to Diminished MCP1 Secretion. *J Neurosci, 37*(21), 5309-5318. doi:10.1523/jneurosci.3472-16.2017

Martin, K. C., & Ephrussi, A. (2009). mRNA localization: gene expression in the spatial dimension. *Cell, 136*(4), 719-730. doi:10.1016/j.cell.2009.01.044

Martinez-Hernandez, R., Bernal, S., Also-Rallo, E., Alias, L., Barcelo, M. J., Hereu, M., . . . Tizzano, E. F. (2013). Synaptic defects in type I spinal muscular atrophy in human development. *J Pathol, 229*(1), 49-61. doi:10.1002/path.4080

Martinez-Hernandez, R., Soler-Botija, C., Also, E., Alias, L., Caselles, L., Gich, I., . . . Tizzano, E. F. (2009). The developmental pattern of myotubes in spinal muscular atrophy indicates prenatal delay of muscle maturation. *J Neuropathol Exp Neurol, 68*(5), 474-481. doi:10.1097/NEN.0b013e3181a10ea1

Marzluff, W. F., Wagner, E. J., & Duronio, R. J. (2008). Metabolism and regulation of canonical histone mRNAs: life without a poly(A) tail. *Nat Rev Genet, 9*(11), 843-854. doi:10.1038/nrg2438

McAndrew, P. E., Parsons, D. W., Simard, L. R., Rochette, C., Ray, P. N., Mendell, J. R., . . . Burghes, A. H. (1997). Identification of proximal spinal muscular atrophy carriers and patients by analysis of SMNT and SMNC gene copy number. *Am J Hum Genet, 60*(6), 1411-1422. doi:10.1086/515465

McCarty, D. M. (2008). Self-complementary AAV vectors; advances and applications. *Mol Ther, 16*(10), 1648-1656. doi:10.1038/mt.2008.171

McCarty, D. M., Monahan, P. E., & Samulski, R. J. (2001). Self-complementary recombinant adeno-associated virus (scAAV) vectors promote efficient transduction independently of DNA synthesis. *Gene Ther, 8*(16), 1248-1254. doi:10.1038/sj.gt.3301514

McGivern, J. V., Patitucci, T. N., Nord, J. A., Barabas, M.-E. A., Stucky, C. L., & Ebert, A. D. (2013). Spinal muscular atrophy astrocytes exhibit abnormal calcium regulation and reduced growth factor production. *Glia, 61*(9), 1418-1428. doi:10.1002/glia.22522

McGovern, V. L., Iyer, C. C., Arnold, W. D., Gombash, S. E., Zaworski, P. G., Blatnik, A. J., 3rd, . . . Burghes, A. H. (2015). SMN expression is required in motor neurons to rescue electrophysiological deficits in the SMNDelta7 mouse model of SMA. *Hum Mol Genet, 24*(19), 5524-5541. doi:10.1093/hmg/ddv283

McGovern, V. L., Iyer, C. C., Arnold, W. D., Gombash, S. E., Zaworski, P. G., Blatnik, A. J., . . . Burghes, A. H. M. (2015). SMN expression is required in motor neurons to rescue electrophysiological deficits in the SMNΔ7 mouse model of SMA. *Human Molecular Genetics, 24*(19), 5524-5541. doi:10.1093/hmg/ddv283

McKinley, J. W., Shi, Z., Kawikova, I., Hur, M., Bamford, I. J., Sudarsana Devi, S. P., . . . Bamford, N. S. (2019). Dopamine Deficiency Reduces Striatal Cholinergic Interneuron Function in Models of Parkinson's Disease. *Neuron, 103*(6), 1056-1072.e1056. doi:10.1016/j.neuron.2019.06.013

McWhorter, M. L., Boon, K. L., Horan, E. S., Burghes, A. H., & Beattie, C. E. (2008). The SMN binding protein Gemin2 is not involved in motor axon outgrowth. *Dev Neurobiol, 68*(2), 182-194. doi:10.1002/dneu.20582

McWhorter, M. L., Monani, U. R., Burghes, A. H., & Beattie, C. E. (2003). Knockdown of the survival motor neuron (Smn) protein in zebrafish causes defects in motor axon outgrowth and pathfinding. *J Cell Biol, 162*(5), 919-931.

Meister, G., Buhler, D., Pillai, R., Lottspeich, F., & Fischer, U. (2001). A multiprotein complex mediates the ATP-dependent assembly of spliceosomal U snRNPs. *Nat Cell Biol, 3*(11), 945-949.

Meister, G., Eggert, C., Bühler, D., Brahms, H., Kambach, C., & Fischer, U. (2001). Methylation of Sm proteins by a complex containing PRMT5 and the putative U snRNP assembly factor pICln. *Current Biology, 11*(24), 1990-1994. doi:https://doi.org/10.1016/S0960-9822(01)00592-9

Meister, G., Eggert, C., & Fischer, U. (2002). SMN-mediated assembly of RNPs: a complex story. *Trends in Cell Biology, 12*(10), 472-478. doi:https://doi.org/10.1016/S0962-8924(02)02371-1

Mentis, G. Z., Blivis, D., Liu, W., Drobac, E., Crowder, M. E., Kong, L., . . . O'Donovan, M. J. (2011). Early functional impairment of sensory-motor connectivity in a mouse model of spinal muscular atrophy. *Neuron, 69*(3), 453-467. doi:10.1016/j.neuron.2010.12.032

Merianda, T., & Twiss, J. (2013). Peripheral nerve axons contain machinery for co-translational secretion of axonally-generated proteins. *Neurosci Bull, 29*(4), 493-500. doi:10.1007/s12264-013-1360-9

Merianda, T. T., Gomes, C., Yoo, S., Vuppalanchi, D., & Twiss, J. L. (2013). Axonal localization of neuritin/CPG15 mRNA in neuronal populations through distinct 5' and 3' UTR elements. *J Neurosci, 33*(34), 13735-13742. doi:10.1523/JNEUROSCI.0962-13.2013

Merianda, T. T., Lin, A. C., Lam, J. S., Vuppalanchi, D., Willis, D. E., Karin, N., . . . Twiss, J. L. (2009). A functional equivalent of endoplasmic reticulum and Golgi in axons for secretion of locally synthesized proteins. *Mol Cell Neurosci, 40*(2), 128-142. doi:10.1016/j.mcn.2008.09.008

Miller, N., Shi, H., Zelikovich, A. S., & Ma, Y.-C. (2016). Motor neuron mitochondrial dysfunction in spinal muscular atrophy. *Hum Mol Genet, 25*(16), 3395-3406. doi:10.1093/hmg/ddw262

Miller, N., Shi, H., Zelikovich, A. S., & Ma, Y. C. (2016). Motor neuron mitochondrial dysfunction in spinal muscular atrophy. *Hum Mol Genet, 25*(16), 3395-3406. doi:10.1093/hmg/ddw262

Minces, V., Pinto, L., Dan, Y., & Chiba, A. A. (2017). Cholinergic shaping of neural correlations. *Proceedings of the National Academy of Sciences, 114*(22), 5725-5730. doi:10.1073/pnas.1621493114

Minis, A., Dahary, D., Manor, O., Leshkowitz, D., Pilpel, Y., & Yaron, A. (2014). Subcellular transcriptomics-dissection of the mRNA composition in the axonal compartment of sensory neurons. *Dev Neurobiol, 74*(3), 365-381. doi:10.1002/dneu.22140

Mitrea, D. M., & Kriwacki, R. W. (2016). Phase separation in biology; functional organization of a higher order. *Cell Commun Signal, 14*, 1. doi:10.1186/s12964-015-0125-7

Molliex, A., Temirov, J., Lee, J., Coughlin, M., Kanagaraj, A. P., Kim, H. J., . . . Taylor, J. P. (2015). Phase separation by low complexity domains promotes stress granule assembly and drives pathological fibrillization. *Cell, 163*(1), 123-133. doi:10.1016/j.cell.2015.09.015

Monani, U. R., Lorson, C. L., Parsons, D. W., Prior, T. W., Androphy, E. J., Burghes, A. H. M., & McPherson, J. D. (1999). A Single Nucleotide Difference That Alters Splicing Patterns Distinguishes the SMA Gene SMN1 From the Copy Gene SMN2. *Hum Mol Genet, 8*(7), 1177-1183. doi:10.1093/hmg/8.7.1177

Monani, U. R., Sendtner, M., Coovert, D. D., Parsons, D. W., Andreassi, C., Le, T. T., . . . Burghes, A. H. (2000). The human centromeric survival motor neuron gene (SMN2) rescues embryonic lethality in Smn(-/-) mice and results in a mouse with spinal muscular atrophy. *Hum Mol Genet, 9*(3), 333-339. doi:10.1093/hmg/9.3.333

Moore, M. J., & Proudfoot, N. J. (2009). Pre-mRNA processing reaches back to transcription and ahead to translation. *Cell, 136*(4), 688-700. doi:10.1016/j.cell.2009.02.001

Moradi, M., Sivadasan, R., Saal, L., Luningschror, P., Dombert, B., Rathod, R. J., . . . Sendtner, M. (2017). Differential roles of alpha-, beta-, and gamma-actin in axon growth and collateral branch formation in motoneurons. *J Cell Biol, 216*(3), 793-814. doi:10.1083/jcb.201604117

Moreira, M. C., Klur, S., Watanabe, M., Nemeth, A. H., Le Ber, I., Moniz, J. C., . . . Koenig, M. (2004). Senataxin, the ortholog of a yeast RNA helicase, is mutant in ataxia-ocular apraxia 2. *Nat Genet, 36*(3), 225-227. doi:10.1038/ng1303

Morgan, I. G., & Austin, L. (1969). Energy metabolism and synaptosomal protein synthesis. *Life Sci, 8*(2), 79-84. doi:10.1016/0024-3205(69)90120-9

Mourelatos, Z., Abel, L., Yong, J., Kataoka, N., & Dreyfuss, G. (2001). SMN interacts with a novel family of hnRNP and spliceosomal proteins. *EMBO J, 20*(19), 5443-5452. doi:10.1093/emboj/20.19.5443

Murashov, A. K., Chintalgattu, V., Islamov, R. R., Lever, T. E., Pak, E. S., Sierpinski, P. L., . . . Van Scott, M. R. (2007). RNAi pathway is functional in peripheral nerve axons. *The FASEB Journal, 21*(3), 656-670. doi:10.1096/fj.06-6155com

Murata, Y., Yasaka, T., Takano, M., & Ishihara, K. (2016). Neuronal and glial expression of inward rectifier potassium channel subunits Kir2.x in rat dorsal root ganglion and spinal cord. *Neurosci Lett, 617*, 59-65. doi:10.1016/j.neulet.2016.02.007

Murray, L. M., Beauvais, A., Gibeault, S., Courtney, N. L., & Kothary, R. (2015). Transcriptional profiling of differentially vulnerable motor neurons at pre-symptomatic stage in the Smn(2b/-) mouse model of spinal muscular atrophy. *Acta Neuropathologica Communications, 3*, 55. doi:10.1186/s40478-015-0231-1

Mutsaers, C. A., Lamont, D. J., Hunter, G., Wishart, T. M., & Gillingwater, T. H. (2013). Label-free proteomics identifies Calreticulin and GRP75/Mortalin as peripherally accessible protein biomarkers for spinal muscular atrophy. *Genome Medicine, 5*. doi:ARTN 95

10.1186/gm498

Nag, S., Qin, J., Srivenugopal, K. S., Wang, M., & Zhang, R. (2013). The MDM2-p53 pathway revisited. *Journal of biomedical research, 27*(4), 254-271. doi:10.7555/JBR.27.20130030

Naryshkin, N. A., Weetall, M., Dakka, A., Narasimhan, J., Zhao, X., Feng, Z., . . . Metzger, F. (2014). Motor neuron disease. SMN2 splicing modifiers improve motor function and longevity in mice with spinal muscular atrophy. *Science, 345*(6197), 688-693. doi:10.1126/science.1250127

Nash, L. A., Burns, J. K., Chardon, J. W., Kothary, R., & Parks, R. J. (2016). Spinal Muscular Atrophy: More than a Disease of Motor Neurons? *Current Molecular Medicine, 16*(9), 779-792. doi:http://dx.doi.org/10.2174/1566524016666161128113338

Ning, K., Drepper, C., Valori, C. F., Ahsan, M., Wyles, M., Higginbottom, A., . . . Sendtner, M. (2010). PTEN depletion rescues axonal growth defect and improves survival in SMN-deficient motor neurons. *Hum Mol Genet, 19*(16), 3159-3168. doi:10.1093/hmg/ddq226

Noma, K., Goncharov, A., Ellisman, M. H., & Jin, Y. (2017). Microtubule-dependent ribosome localization in C. elegans neurons. *Elife, 6*. doi:10.7554/eLife.26376

Oprişoreanu, A.-M., Smith, H. L., Arya, S., Webster, R., Zhong, Z., Wehner, D., . . . Becker, C. G. (2019). Interaction of Axonal Chondrolectin with Collagen XIXa1 Is Necessary for Precise Neuromuscular Junction Formation. *Cell reports, 29*(5), 1082-1098.e1010. doi:https://doi.org/10.1016/j.celrep.2019.09.033

Otter, S., Grimmler, M., Neuenkirchen, N., Chari, A., Sickmann, A., & Fischer, U. (2007). A Comprehensive Interaction Map of the Human Survival of Motor Neuron (SMN) Complex. *Journal of Biological Chemistry, 282*(8), 5825-5833. doi:10.1074/jbc.M608528200

Paez-Colasante, X., Seaberg, B., Martinez, T. L., Kong, L., Sumner, C. J., & Rimer, M. (2013). Improvement of Neuromuscular Synaptic Phenotypes without Enhanced Survival and Motor Function in Severe Spinal Muscular Atrophy Mice Selectively Rescued in Motor Neurons. *PLOS ONE, 8*(9), e75866. doi:10.1371/journal.pone.0075866

Pagliardini, S., Giavazzi, A., Setola, V., Lizier, C., Di Luca, M., DeBiasi, S., & Battaglia, G. (2000). Subcellular localization and axonal transport of the survival motor neuron (SMN) protein in the developing rat spinal cord. *Hum Mol Genet, 9*(1), 47-56.

Park, G. H., Maeno-Hikichi, Y., Awano, T., Landmesser, L. T., & Monani, U. R. (2010). Reduced survival of motor neuron (SMN) protein in motor neuronal progenitors functions cell autonomously to cause spinal muscular atrophy in model mice expressing the human centromeric (SMN2) gene. *J Neurosci, 30*(36), 12005-12019. doi:10.1523/jneurosci.2208-10.2010

Patel, A., Lee, H. O., Jawerth, L., Maharana, S., Jahnel, M., Hein, M. Y., . . . Alberti, S. (2015). A Liquid-to-Solid Phase Transition of the ALS Protein FUS Accelerated by Disease Mutation. *Cell, 162*(5), 1066-1077. doi:10.1016/j.cell.2015.07.047

Patel, A. A., & Steitz, J. A. (2003). Splicing double: insights from the second spliceosome. *Nat Rev Mol Cell Biol, 4*(12), 960-970. doi:10.1038/nrm1259

Paushkin, S., Gubitz, A. K., Massenet, S., & Dreyfuss, G. (2002). The SMN complex, an assemblyosome of ribonucleoproteins. *Curr Opin Cell Biol, 14*(3), 305-312.

Pearn, J. (1978). Incidence, prevalence, and gene frequency studies of chronic childhood spinal muscular atrophy. *J Med Genet, 15*(6), 409-413.

Pease-Raissi, S. E., Pazyra-Murphy, M. F., Li, Y., Wachter, F., Fukuda, Y., Fenstermacher, S. J., . . . Segal, R. A. (2017). Paclitaxel Reduces Axonal Bclw to Initiate IP3R1-Dependent Axon Degeneration. *Neuron, 96*(2), 373-386 e376. doi:10.1016/j.neuron.2017.09.034

Pellizzoni, L. (2007). Chaperoning ribonucleoprotein biogenesis in health and disease. *EMBO Reports, 8*(4), 340-345. doi:10.1038/sj.embor.7400941

Pellizzoni, L., Charroux, B., Rappsilber, J., Mann, M., & Dreyfuss, G. (2001). A functional interaction between the survival motor neuron complex and RNA polymerase II. *J Cell Biol, 152*(1), 75-85.

Pellizzoni, L., Yong, J., & Dreyfuss, G. (2002). Essential role for the SMN complex in the specificity of snRNP assembly. *Science, 298*(5599), 1775-1779.

Peter, C. J., Evans, M., Thayanithy, V., Taniguchi-Ishigaki, N., Bach, I., Kolpak, A., . . . Androphy, E. J. (2011). The COPI vesicle complex binds and moves with survival motor neuron within axons. *Human Molecular Genetics, 20*(9), 1701-1711. doi:10.1093/hmg/ddr046

Piazzon, N., Rage, F., Schlotter, F., Moine, H., Branlant, C., & Massenet, S. (2008). In vitro and in cellulo evidences for association of the survival of motor neuron complex with the fragile X mental retardation protein. *J Biol Chem, 283*(9), 5598-5610. doi:10.1074/jbc.M707304200

Pigino, G., Morfini, G., Pelsman, A., Mattson, M. P., Brady, S. T., & Busciglio, J. (2003). Alzheimer's presenilin 1 mutations impair kinesin-based axonal transport. *J Neurosci, 23*(11), 4499-4508.

Piper, M., Lee, A. C., van Horck, F. P., McNeilly, H., Lu, T. B., Harris, W. A., & Holt, C. E. (2015). Differential requirement of F-actin and microtubule cytoskeleton in cue-induced local protein synthesis in axonal growth cones. *Neural Dev, 10*, 3. doi:10.1186/s13064-015-0031-0

Piras, A., Schiaffino, L., Boido, M., Valsecchi, V., Guglielmotto, M., De Amicis, E., . . . Vercelli, A. (2017). Inhibition of autophagy delays motoneuron degeneration and extends lifespan in a mouse model of spinal muscular atrophy. *Cell Death & Disease, 8*(12), 3223. doi:10.1038/s41419-017-0086-4

Prasad, V. (2018). Nusinersen for Spinal Muscular Atrophy: Are We Paying Too Much for Too Little? *JAMA Pediatrics, 172*(2), 123-124. doi:10.1001/jamapediatrics.2017.4360

Praveen, K., Wen, Y., & Matera, A. G. (2012). A Drosophila model of spinal muscular atrophy uncouples snRNP biogenesis functions of survival motor neuron from locomotion and viability defects. *Cell Rep, 1*(6), 624-631. doi:10.1016/j.celrep.2012.05.014

Prior, T. W., Snyder, P. J., Rink, B. D., Pearl, D. K., Pyatt, R. E., Mihal, D. C., . . . Garner, S. (2010). Newborn and carrier screening for spinal muscular atrophy. *American Journal of Medical Genetics Part A, 152A*(7), 1608-1616. doi:10.1002/ajmg.a.33474

Prior, T. W., Snyder, P. J., Rink, B. D., Pearl, D. K., Pyatt, R. E., Mihal, D. C., . . . Garner, S. (2010). Newborn and carrier screening for spinal muscular atrophy. *Am J Med Genet A, 152A*(7), 1608-1616. doi:10.1002/ajmg.a.33474

Rage, F., Boulisfane, N., Rihan, K., Neel, H., Gostan, T., Bertrand, E., . . . Soret, J. (2013). Genome-wide identification of mRNAs associated with the protein SMN whose depletion decreases their axonal localization. *Rna, 19*(12), 1755-1766. doi:10.1261/rna.040204.113

Rajendra, T. K., Gonsalvez, G. B., Walker, M. P., Shpargel, K. B., Salz, H. K., & Matera, A. G. (2007). A Drosophila melanogaster model of spinal muscular atrophy reveals a function for SMN in striated muscle. *The Journal of Cell Biology, 176*(6), 831-841. doi:10.1083/jcb.200610053

Ramaswami, M., Taylor, J. P., & Parker, R. (2013). Altered ribostasis: RNA-protein granules in degenerative disorders. *Cell, 154*(4), 727-736. doi:10.1016/j.cell.2013.07.038

Rathod, R., Havlicek, S., Frank, N., Blum, R., & Sendtner, M. (2012). Laminin induced local axonal translation of beta-actin mRNA is impaired in SMN-deficient motoneurons. *Histochem Cell Biol, 138*(5), 737-748. doi:10.1007/s00418-012-0989-1

Rezaul, K., Gupta, D., Semenova, I., Ikeda, K., Kraikivski, P., Yu, J., . . . Rodionov, V. (2016). Engineered Tug-of-War Between Kinesin and Dynein Controls Direction of Microtubule Based Transport In Vivo. *Traffic, 17*(5), 475-486. doi:10.1111/tra.12385

Riccio, A., Pierchala, B. A., Ciarallo, C. L., & Ginty, D. D. (1997). An NGF-TrkA-mediated retrograde signal to transcription factor CREB in sympathetic neurons. *Science, 277*(5329), 1097-1100.

Rigo, F., Chun, S. J., Norris, D. A., Hung, G., Lee, S., Matson, J., . . . Bennett, C. F. (2014). Pharmacology of a central nervous system delivered 2'-O-methoxyethyl-modified survival of motor neuron splicing oligonucleotide in mice and nonhuman primates. *J Pharmacol Exp Ther, 350*(1), 46-55. doi:10.1124/jpet.113.212407

Rindt, H., Feng, Z., Mazzasette, C., Glascock, J. J., Valdivia, D., Pyles, N., . . . Lorson, C. L. (2015). Astrocytes influence the severity of spinal muscular atrophy. *Hum Mol Genet, 24*(14), 4094-4102. doi:10.1093/hmg/ddv148

Ripolone, M., Ronchi, D., Violano, R., Vallejo, D., Fagiolari, G., Barca, E., . . . Moggio, M. (2015). Impaired Muscle Mitochondrial Biogenesis and Myogenesis in Spinal Muscular AtrophyBiogenesis and Myogenesis in Spinal Muscular AtrophyBiogenesis and Myogenesis in Spinal Muscular Atrophy. *JAMA Neurology, 72*(6), 666-675. doi:10.1001/jamaneurol.2015.0178

Rossner, M. J., Hirrlinger, J., Wichert, S. P., Boehm, C., Newrzella, D., Hiemisch, H., . . . Nave, K.-A. (2006). Global Transcriptome Analysis of Genetically Identified Neurons in the Adult Cortex. *The Journal of Neuroscience, 26*(39), 9956-9966. doi:10.1523/jneurosci.0468-06.2006

Rossoll, W., & Bassell, G. J. (2009a). Spinal Muscular Atrophy and a Model for Survival of Motor Neuron Protein Function in Axonal Ribonucleoprotein Complexes. *Results Probl Cell Differ*. doi:10.1007/400_2009_4

Rossoll, W., & Bassell, G. J. (2009b). Spinal muscular atrophy and a model for survival of motor neuron protein function in axonal ribonucleoprotein complexes. *Results Probl Cell Differ, 48*, 289-326. doi:10.1007/400_2009_4

Rossoll, W., Jablonka, S., Andreassi, C., Kroning, A. K., Karle, K., Monani, U. R., & Sendtner, M. (2003). Smn, the spinal muscular atrophy-determining gene product, modulates axon growth and localization of beta-actin mRNA in growth cones of motoneurons. *J Cell Biol, 163*(4), 801-812. doi:10.1083/jcb.200304128

Rossoll, W., Kroning, A. K., Ohndorf, U. M., Steegborn, C., Jablonka, S., & Sendtner, M. (2002). Specific interaction of Smn, the spinal muscular atrophy determining gene product, with hnRNP-R and gry-rbp/hnRNP-Q: a role for Smn in RNA processing in motor axons? *Hum Mol Genet, 11*(1), 93-105.

Rotem, N., Magen, I., Ionescu, A., Gershoni-Emek, N., Altman, T., Costa, C. J., . . . Perlson, E. (2017). ALS Along the Axons - Expression of Coding and Noncoding RNA Differs in Axons of ALS models. *Sci Rep, 7*, 44500. doi:10.1038/srep44500

Rozani, I., Tsapara, G., Witts, E. C., Deaville, S. J., Miles, G. B., & Zagoraiou, L. (2019). Pitx2 cholinergic interneurons are the source of C bouton synapses on brainstem motor neurons. *Sci Rep, 9*(1), 4936. doi:10.1038/s41598-019-39996-4

Rudnik-Schoneborn, S., Arning, L., Epplen, J. T., & Zerres, K. (2012). SETX gene mutation in a family diagnosed autosomal dominant proximal spinal muscular atrophy. *Neuromuscul Disord, 22*(3), 258-262. doi:10.1016/j.nmd.2011.09.006

Russell, K. A., Ng, R., Faulkner, J. A., Claflin, D. R., & Mendias, C. L. (2015). Mouse forepaw lumbrical muscles are resistant to age-related declines in force production. *Exp Gerontol, 65*, 42-45. doi:10.1016/j.exger.2015.03.003

Saal, L., Briese, M., Kneitz, S., Glinka, M., & Sendtner, M. (2014). Subcellular transcriptome alterations in a cell culture model of spinal muscular atrophy point to widespread defects in axonal growth and presynaptic differentiation. *Rna, 20*(11), 1789-1802. doi:10.1261/rna.047373.114

Sakakibara, T., Nemoto, Y., Nukiwa, T., & Takeshima, H. (2004). Identification and characterization of a novel Rho GTPase activating protein implicated in receptor-mediated endocytosis. *FEBS Lett, 566*(1-3), 294-300. doi:10.1016/j.febslet.2004.03.101

Salerno, V. P., Calliari, A., Provance, D. W., Jr., Sotelo-Silveira, J. R., Sotelo, J. R., & Mercer, J. A. (2008). Myosin-Va mediates RNA distribution in primary fibroblasts from multiple organs. *Cell Motil Cytoskeleton, 65*(5), 422-433. doi:10.1002/cm.20272

Salvi, J. S., & Mekhail, K. (2015). R-loops highlight the nucleus in ALS. *Nucleus, 6*(1), 23-29. doi:10.1080/19491034.2015.1004952

Sanchez, G., Dury, A. Y., Murray, L. M., Biondi, O., Tadesse, H., El Fatimy, R., . . . Cote, J. (2013). A novel function for the survival motoneuron protein as a translational regulator. *Hum Mol Genet, 22*(4), 668-684. doi:10.1093/hmg/dds474

Sanz, E., Yang, L., Su, T., Morris, D. R., McKnight, G. S., & Amieux, P. S. (2009). Cell-type-specific isolation of ribosome-associated mRNA from complex tissues. *Proc Natl Acad Sci U S A, 106*(33), 13939-13944. doi:10.1073/pnas.0907143106

Sanz, E., Yang, L., Su, T., Morris, D. R., McKnight, G. S., & Amieux, P. S. (2009). Cell-type-specific isolation of ribosome-associated mRNA from complex tissues. *Proceedings of the National Academy of Sciences, 106*(33), 13939-13944. doi:10.1073/pnas.0907143106

Sarvestany, A. A., Hunter, G., Tavendale, A., Lamont, D. J., Hurtado, M. L., Graham, L. C., . . . Gillingwater, T. H. (2014). Label-Free Quantitative Proteomic Profiling Identifies Disruption of Ubiquitin Homeostasis As a Key Driver of Schwann Cell Defects in Spinal Muscular Atrophy. *Journal of Proteome Research, 13*(11), 4546-4557. doi:10.1021/pr500492j

Sasaki, Y., Welshhans, K., Wen, Z., Yao, J., Xu, M., Goshima, Y., . . . Bassell, G. J. (2010). Phosphorylation of zipcode binding protein 1 is required for brain-derived neurotrophic factor signaling of local beta-actin synthesis and growth cone turning. *J Neurosci, 30*(28), 9349-9358. doi:30/28/9349 [pii]

10.1523/JNEUROSCI.0499-10.2010

Sauterer, R. A., Feeney, R. J., & Zieve, G. W. (1988). Cytoplasmic assembly of snRNP particles from stored proteins and newly transcribed snRNA's in L929 mouse fibroblasts. *Exp Cell Res, 176*(2), 344-359.

Schrank, B., Gotz, R., Gunnersen, J. M., Ure, J. M., Toyka, K. V., Smith, A. G., & Sendtner, M. (1997). Inactivation of the survival motor neuron gene, a candidate gene for human spinal muscular atrophy, leads to massive cell death in early mouse embryos. *Proc Natl Acad Sci U S A, 94*(18), 9920-9925.

See, K., Yadav, P., Giegerich, M., Cheong, P. S., Graf, M., Vyas, H., . . . Winkler, C. (2014). SMN deficiency alters Nrxn2 expression and splicing in zebrafish and mouse models of spinal muscular atrophy. *Hum Mol Genet, 23*(7), 1754-1770. doi:10.1093/hmg/ddt567

Shababi, M., Lorson, C. L., & Rudnik-Schoneborn, S. S. (2014). Spinal muscular atrophy: a motor neuron disorder or a multi-organ disease? *J Anat, 224*(1), 15-28. doi:10.1111/joa.12083

Shalek, A. K., Satija, R., Adiconis, X., Gertner, R. S., Gaublomme, J. T., Raychowdhury, R., . . . Regev, A. (2013). Single-cell transcriptomics reveals bimodality in expression and splicing in immune cells. *Nature, 498*(7453), 236-240. doi:10.1038/nature12172

Shankar, G. M., Li, S., Mehta, T. H., Garcia-Munoz, A., Shepardson, N. E., Smith, I., . . . Selkoe, D. J. (2008). Amyloid-beta protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory. *Nat Med, 14*(8), 837-842. doi:10.1038/nm1782

Sharma, A., Lambrechts, A., Hao le, T., Le, T. T., Sewry, C. A., Ampe, C., . . . Morris, G. E. (2005). A role for complexes of survival of motor neurons (SMN) protein with gemins and profilin in neurite-like cytoplasmic extensions of cultured nerve cells. *Exp Cell Res, 309*(1), 185-197. doi:10.1016/j.yexcr.2005.05.014

Shigeoka, T., Jung, H., Jung, J., Turner-Bridger, B., Ohk, J., Lin, Julie Q., . . . Holt, Christine E. (2016). Dynamic Axonal Translation in Developing and Mature Visual Circuits. *Cell, 166*(1), 181-192. doi:10.1016/j.cell.2016.05.029

Shigeoka, T., Jung, H., Jung, J., Turner-Bridger, B., Ohk, J., Lin, J. Q., . . . Holt, C. E. (2016). Dynamic Axonal Translation in Developing and Mature Visual Circuits. *Cell, 166*(1), 181-192. doi:10.1016/j.cell.2016.05.029

Shigeoka, T., Jung, J., Holt, C. E., & Jung, H. (2018). Axon-TRAP-RiboTag: Affinity Purification of Translated mRNAs from Neuronal Axons in Mouse In Vivo. *Methods Mol Biol, 1649*, 85-94. doi:10.1007/978-1-4939-7213-5_5

Shishikura, K., Hara, M., Sasaki, Y., & Misugi, K. (1983). A neuropathologic study of Werdnig-Hoffmann disease with special reference to the thalamus and posterior roots. *Acta Neuropathol, 60*(1-2), 99-106. doi:10.1007/BF00685353

Shukla, S., & Parker, R. (2016). Hypo- and Hyper-Assembly Diseases of RNA–Protein Complexes. *Trends in Molecular Medicine, 22*(7), 615-628. doi:https://doi.org/10.1016/j.molmed.2016.05.005

Simon, C. M., Dai, Y., Van Alstyne, M., Koutsioumpa, C., Pagiazitis, J. G., Chalif, J. I., . . . Mentis, G. Z. (2017). Converging Mechanisms of p53 Activation Drive Motor Neuron Degeneration in Spinal Muscular Atrophy. *Cell Rep, 21*(13), 3767-3780. doi:10.1016/j.celrep.2017.12.003

Singh, G., Pratt, G., Yeo, G. W., & Moore, M. J. (2015). The Clothes Make the mRNA: Past and Present Trends in mRNP Fashion. *Annu Rev Biochem, 84*, 325-354. doi:10.1146/annurev-biochem-080111-092106

Singh, N. N., Howell, M. D., Androphy, E. J., & Singh, R. N. (2017). How the discovery of ISS-N1 led to the first medical therapy for spinal muscular atrophy. *Gene Ther, 24*(9), 520-526. doi:10.1038/gt.2017.34

Singh, R. K., & Cooper, T. A. (2012). Pre-mRNA splicing in disease and therapeutics. *Trends Mol Med, 18*(8), 472-482. doi:10.1016/j.molmed.2012.06.006

Sison, S. L., Patitucci, T. N., Seminary, E. R., Villalon, E., Lorson, C. L., & Ebert, A. D. (2017). Astrocyte-produced miR-146a as a mediator of motor neuron loss in spinal muscular atrophy. *Hum Mol Genet, 26*(17), 3409-3420. doi:10.1093/hmg/ddx230

Sleigh, J. N., Barreiro-Iglesias, A., Oliver, P. L., Biba, A., Becker, T., Davies, K. E., . . . Talbot, K. (2014). Chondrolectin affects cell survival and neuronal outgrowth in in vitro and in vivo models of spinal muscular atrophy. *Hum Mol Genet, 23*(4), 855-869. doi:10.1093/hmg/ddt477

Smircich, P., Eastman, G., Bispo, S., Duhagon, M. A., Guerra-Slompo, E. P., Garat, B., . . . Sotelo-Silveira, J. R. (2015). Ribosome profiling reveals translation control as a key mechanism generating differential gene expression in Trypanosoma cruzi. *BMC Genomics, 16*(1), 443. doi:10.1186/s12864-015-1563-8

Smith, D. H. (2009). Stretch growth of integrated axon tracts: extremes and exploitations. *Prog Neurobiol, 89*(3), 231-239. doi:10.1016/j.pneurobio.2009.07.006

Sotelo-Silveira, J., Crispino, M., Puppo, A., Sotelo, J. R., & Koenig, E. (2008). Myelinated axons contain beta-actin mRNA and ZBP-1 in periaxoplasmic ribosomal plaques and depend on cyclic AMP and F-actin integrity for in vitro translation. *J Neurochem, 104*(2), 545-557. doi:JNC4999 [pii]

10.1111/j.1471-4159.2007.04999.x

Sotelo-Silveira, J. R., Calliari, A., Cardenas, M., Koenig, E., & Sotelo, J. R. (2004). Myosin Va and kinesin II motor proteins are concentrated in ribosomal domains (periaxoplasmic ribosomal plaques) of myelinated axons. *J Neurobiol, 60*(2), 187-196. doi:10.1002/neu.20015

Spiller, K. J., Khan, T., Dominique, M. A., Restrepo, C. R., Cotton-Samuel, D., Levitan, M., . . . Lee, V. M. Y. (2019). Reduction of matrix metalloproteinase 9 (MMP-9) protects motor neurons from TDP-43-triggered death in rNLS8 mice. *Neurobiol Dis, 124*, 133-140. doi:https://doi.org/10.1016/j.nbd.2018.11.013

Steward, O., & Levy, W. B. (1982). Preferential localization of polyribosomes under the base of dendritic spines in granule cells of the dentate gyrus. *J Neurosci, 2*(3), 284-291.

Strasswimmer, J., Lorson, C. L., Breiding, D. E., Chen, J. J., Le, T., Burghes, A. H., & Androphy, E. J. (1999). Identification of survival motor neuron as a transcriptional activator-binding protein. *Hum Mol Genet, 8*(7), 1219-1226.

Sugarman, E. A., Nagan, N., Zhu, H., Akmaev, V. R., Zhou, Z., Rohlfs, E. M., . . . Allitto, B. A. (2012). Pan-ethnic carrier screening and prenatal diagnosis for spinal muscular atrophy: clinical laboratory analysis of >72,400 specimens. *Eur J Hum Genet, 20*(1), 27-32. doi:10.1038/ejhg.2011.134

Sugino, K., Hempel, C. M., Miller, M. N., Hattox, A. M., Shapiro, P., Wu, C., . . . Nelson, S. B. (2006). Molecular taxonomy of major neuronal classes in the adult mouse forebrain. *Nat Neurosci, 9*(1), 99-107. doi:10.1038/nn1618

Suraweera, A., Lim, Y., Woods, R., Birrell, G. W., Nasim, T., Becherel, O. J., & Lavin, M. F. (2009). Functional role for senataxin, defective in ataxia oculomotor apraxia type 2, in transcriptional regulation. *Hum Mol Genet, 18*(18), 3384-3396. doi:10.1093/hmg/ddp278

Szymusiak, R. (1995). Magnocellular nuclei of the basal forebrain: substrates of sleep and arousal regulation. *Sleep, 18*(6), 478-500. doi:10.1093/sleep/18.6.478

Tadesse, H., Deschenes-Furry, J., Boisvenue, S., & Cote, J. (2008). KH-type splicing regulatory protein interacts with survival motor neuron protein and is misregulated in spinal muscular atrophy. *Hum Mol Genet, 17*(4), 506-524. doi:10.1093/hmg/ddm327

Taliaferro, J. M., Vidaki, M., Oliveira, R., Olson, S., Zhan, L., Saxena, T., . . . Burge, C. B. (2016). Distal Alternative Last Exons Localize mRNAs to Neural Projections. *Mol Cell, 61*(6), 821-833. doi:10.1016/j.molcel.2016.01.020

Taylor, A. M., Berchtold, N. C., Perreau, V. M., Tu, C. H., Li Jeon, N., & Cotman, C. W. (2009). Axonal mRNA in uninjured and regenerating cortical mammalian axons. *J Neurosci, 29*(15), 4697-4707. doi:29/15/4697 [pii]

10.1523/JNEUROSCI.6130-08.2009

Taylor, A. M., Wu, J., Tai, H. C., & Schuman, E. M. (2013). Axonal translation of beta-catenin regulates synaptic vesicle dynamics. *J Neurosci, 33*(13), 5584-5589. doi:10.1523/JNEUROSCI.2944-12.2013

Tcherkezian, J., Brittis, P. A., Thomas, F., Roux, P. P., & Flanagan, J. G. (2010). Transmembrane receptor DCC associates with protein synthesis machinery and regulates translation. *Cell, 141*(4), 632-644. doi:10.1016/j.cell.2010.04.008

Tisdale, S., Lotti, F., Saieva, L., Van Meerbeke, J. P., Crawford, T. O., Sumner, C. J., . . . Pellizzoni, L. (2013). SMN is essential for the biogenesis of U7 snRNP and 3'-end formation of histone mRNAs. *Cell reports, 5*(5), 10.1016/j.celrep.2013.1011.1012. doi:10.1016/j.celrep.2013.11.012

Todd, A. G., Lin, H., Ebert, A. D., Liu, Y. L., & Androphy, E. J. (2013). COPI transport complexes bind to specific RNAs in neuronal cells. *Human Molecular Genetics, 22*(4), 729-736. doi:10.1093/hmg/dds480

Töpert, C., Döring, F., Wischmeyer, E., Karschin, C., Brockhaus, J., Ballanyi, K., . . . Karschin, A. (1998). Kir2.4: A Novel K⁺ Inward Rectifier Channel Associated with Motoneurons of Cranial Nerve Nuclei. *The Journal of Neuroscience, 18*(11), 4096-4105. doi:10.1523/jneurosci.18-11-04096.1998

Tsai, M. S., Chiu, Y. T., Wang, S. H., Hsieh-Li, H. M., & Li, H. (2006). Abolishing Trp53-dependent apoptosis does not benefit spinal muscular atrophy model mice. *European Journal of Human Genetics, 14*(3), 372-375. doi:10.1038/sj.ejhg.5201556

Turunen, J. J., Niemelä, E. H., Verma, B., & Frilander, M. J. (2013). The significant other: splicing by the minor spliceosome. *Wiley Interdisciplinary Reviews. RNA, 4*(1), 61-76. doi:10.1002/wrna.1141

Twiss, J. L., Kalinski, A. L., Sachdeva, R., & Houle, J. D. (2016). Intra-axonal protein synthesis - a new target for neural repair? *Neural Regen Res, 11*(9), 1365-1367. doi:10.4103/1673-5374.191193

Van Alstyne, M., Simon, C. M., Sardi, S. P., Shihabuddin, L. S., Mentis, G. Z., & Pellizzoni, L. (2018). Dysregulation of Mdm2 and Mdm4 alternative splicing underlies motor neuron death in spinal muscular atrophy. *Genes & Development*. doi:10.1101/gad.316059.118

Verhaart, I. E. C., Robertson, A., Wilson, I. J., Aartsma-Rus, A., Cameron, S., Jones, C. C., . . . Lochmuller, H. (2017). Prevalence, incidence and carrier frequency of 5q-linked spinal muscular atrophy - a literature review. *Orphanet J Rare Dis, 12*(1), 124. doi:10.1186/s13023-017-0671-8

Verma, P., Chierzi, S., Codd, A. M., Campbell, D. S., Meyer, R. L., Holt, C. E., & Fawcett, J. W. (2005). Axonal protein synthesis and degradation are necessary for efficient growth cone regeneration. *J Neurosci, 25*(2), 331-342.

Vindry, C., Marnef, A., Broomhead, H., Twyffels, L., Ozgur, S., Stoecklin, G., . . . Standart, N. (2017). Dual RNA Processing Roles of Pat1b via Cytoplasmic Lsm1-7 and Nuclear Lsm2-8 Complexes. *Cell Rep, 20*(5), 1187-1200. doi:10.1016/j.celrep.2017.06.091

Wan, L., Battle, D. J., Yong, J., Gubitz, A. K., Kolb, S. J., Wang, J., & Dreyfuss, G. (2005). The survival of motor neurons protein determines the capacity for snRNP assembly: biochemical deficiency in spinal muscular atrophy. *Mol Cell Biol, 25*(13), 5543-5551. doi:10.1128/MCB.25.13.5543-5551.2005

Wang, E. T., Taliaferro, J. M., Lee, J. A., Sudhakaran, I. P., Rossoll, W., Gross, C., . . . Bassell, G. J. (2016). Dysregulation of mRNA Localization and Translation in Genetic Disease. *J Neurosci, 36*(45), 11418-11426. doi:10.1523/JNEUROSCI.2352-16.2016

Wedel, M. (2012). *A Monument of Inefficiency: The Presumed Course of the Recurrent Laryngeal Nerve in Sauropod Dinosaurs* (Vol. 57).

Wei, Z. B., Yuan, Y. F., Jaouen, F., Ma, M. S., Hao, C. J., Zhang, Z., . . . Li, W. (2016). SLC35D3 increases autophagic activity in midbrain dopaminergic neurons by enhancing BECN1-ATG14-PIK3C3 complex formation. *Autophagy, 12*(7), 1168-1179. doi:10.1080/15548627.2016.1179402

Welshhans, K., & Bassell, G. J. (2011). Netrin-1-Induced Local {beta}-Actin Synthesis and Growth Cone Guidance Requires Zipcode Binding Protein 1. *J Neurosci, 31*(27), 9800-9813. doi:31/27/9800 [pii]

10.1523/JNEUROSCI.0166-11.2011

Willis, D. E., van Niekerk, E. A., Sasaki, Y., Mesngon, M., Merianda, T. T., Williams, G. G., . . . Twiss, J. L. (2007). Extracellular stimuli specifically regulate localized levels of individual neuronal mRNAs. *J Cell Biol, 178*(6), 965-980. doi:10.1083/jcb.200703209

Willis, D. E., van Niekerk, E. A., Sasaki, Y., Mesngon, M., Merianda, T. T., Williams, G. G., . . . Twiss, J. L. (2007). Extracellular stimuli specifically regulate localized levels of individual neuronal mRNAs. *J Cell Biol, 178*(6), 965-980.

Winkler, C., Eggert, C., Gradl, D., Meister, G., Giegerich, M., Wedlich, D., . . . Fischer, U. (2005). Reduced U snRNP assembly causes motor axon degeneration in an animal model for spinal muscular atrophy. *Genes & Development, 19*(19), 2320-2330. doi:10.1101/gad.342005

Wirth, B., Barkats, M., Martinat, C., Sendtner, M., & Gillingwater, T. H. (2015). Moving towards treatments for spinal muscular atrophy: hopes and limits. *Expert Opin Emerg Drugs, 20*(3), 353-356. doi:10.1517/14728214.2015.1041375

Wishart, T. M., Huang, J. P.-W., Murray, L. M., Lamont, D. J., Mutsaers, C. A., Ross, J., . . . Gillingwater, T. H. (2010). SMN deficiency disrupts brain development in a mouse model of severe spinal muscular atrophy. *Hum Mol Genet, 19*(21), 4216-4228. doi:10.1093/hmg/ddq340

Wishart, T. M., Mutsaers, C. A., Riessland, M., Reimer, M. M., Hunter, G., Hannam, M. L., . . . Gillingwater, T. H. (2014). Dysregulation of ubiquitin homeostasis and beta-catenin signaling promote spinal muscular atrophy. *J Clin Invest, 124*(4), 1821-1834. doi:10.1172/JCI71318

Wong, H. H., Lin, J. Q., Strohl, F., Roque, C. G., Cioni, J. M., Cagnetta, R., . . . Holt, C. E. (2017). RNA Docking and Local Translation Regulate Site-Specific Axon Remodeling In Vivo. *Neuron, 95*(4), 852-868.e858. doi:10.1016/j.neuron.2017.07.016

Wu, D., & Hersh, L. B. (1994). Choline acetyltransferase: celebrating its fiftieth year. *J Neurochem, 62*(5), 1653-1663. doi:10.1046/j.1471-4159.1994.62051653.x

Wu, K. Y., Hengst, U., Cox, L. J., Macosko, E. Z., Jeromin, A., Urquhart, E. R., & Jaffrey, S. R. (2005). Local translation of RhoA regulates growth cone collapse. *Nature, 436*(7053), 1020-1024.

Wurth, L., Gribling-Burrer, A. S., Verheggen, C., Leichter, M., Takeuchi, A., Baudrey, S., . . . Allmang, C. (2014). Hypermethylated-capped selenoprotein mRNAs in mammals. *Nucleic Acids Res, 42*(13), 8663-8677. doi:10.1093/nar/gku580

Xing, L., & Bassell, G. J. (2013). mRNA localization: an orchestration of assembly, traffic and synthesis. *Traffic, 14*(1), 2-14. doi:10.1111/tra.12004

Xu, C. C., Denton, K. R., Wang, Z. B., Zhang, X., & Li, X. J. (2016). Abnormal mitochondrial transport and morphology as early pathological changes in human models of spinal muscular atrophy. *Dis Model Mech, 9*(1), 39-49. doi:10.1242/dmm.021766

Xu, M., Chung, S., Zhang, S., Zhong, P., Ma, C., Chang, W. C., . . . Dan, Y. (2015). Basal forebrain circuit for sleep-wake control. *Nat Neurosci, 18*(11), 1641-1647. doi:10.1038/nn.4143

Yamazaki, T., Chen, S., Yu, Y., Yan, B., Haertlein, T. C., Carrasco, M. A., . . . Reed, R. (2012). FUS-SMN protein interactions link the motor neuron diseases ALS and SMA. *Cell Rep, 2*(4), 799-806. doi:10.1016/j.celrep.2012.08.025

Yan, D., Wu, Z., Chisholm, A. D., & Jin, Y. (2009). The DLK-1 kinase promotes mRNA stability and local translation in C. elegans synapses and axon regeneration. *Cell, 138*(5), 1005-1018. doi:10.1016/j.cell.2009.06.023

Yao, F., Yu, F., Gong, L., Taube, D., Rao, D. D., & MacKenzie, R. G. (2005). Microarray analysis of fluoro-gold labeled rat dopamine neurons harvested by laser capture microdissection. *J Neurosci Methods, 143*(2), 95-106. doi:10.1016/j.jneumeth.2004.09.023

Yao, J., Sasaki, Y., Wen, Z., Bassell, G. J., & Zheng, J. Q. (2006). An essential role for beta-actin mRNA localization and translation in Ca2+-dependent growth cone guidance. *Nat Neurosci, 9*(10), 1265-1273. doi:10.1038/nn1773

Ymlahi-Ouazzani, Q., O, J. B., Paillard, E., Ballagny, C., Chesneau, A., Jadaud, A., . . . Pollet, N. (2010). Reduced levels of survival motor neuron protein leads to aberrant motoneuron growth in a Xenopus model of muscular atrophy. *Neurogenetics, 11*(1), 27-40. doi:10.1007/s10048-009-0200-6

Yoo, S., Kim, H. H., Kim, P., Donnelly, C. J., Kalinski, A. L., Vuppalanchi, D., . . . Twiss, J. L. (2013). A HuD-ZBP1 ribonucleoprotein complex localizes GAP-43 mRNA into axons through its 3' untranslated region AU-rich regulatory element. *J Neurochem, 126*(6), 792-804. doi:10.1111/jnc.12266

Young, P. J., Day, P. M., Zhou, J., Androphy, E. J., Morris, G. E., & Lorson, C. L. (2002). A direct interaction between the survival motor neuron protein and p53 and its relationship to spinal muscular atrophy. *J Biol Chem, 277*(4), 2852-2859. doi:10.1074/jbc.M108769200

Younts, T. J., Monday, H. R., Dudok, B., Klein, M. E., Jordan, B. A., Katona, I., & Castillo, P. E. (2016). Presynaptic Protein Synthesis Is Required for Long-Term Plasticity of GABA Release. *Neuron, 92*(2), 479-492. doi:10.1016/j.neuron.2016.09.040

Yu, J., Chen, M., Huang, H., Zhu, J., Song, H., Zhu, J., . . . Ji, S. J. (2017). Dynamic m6A modification regulates local translation of mRNA in axons. *Nucleic Acids Res*. doi:10.1093/nar/gkx1182

Zerres, K., Wirth, B., & Rudnik-Schöneborn, S. (1997). Spinal muscular atrophy—clinical and genetic correlations. *Neuromuscular Disorders, 7*(3), 202-207. doi:https://doi.org/10.1016/S0960-8966(97)00459-8

Zhang, H., Xing, L., Rossoll, W., Wichterle, H., Singer, R. H., & Bassell, G. J. (2006). Multiprotein complexes of the survival of motor neuron protein SMN with Gemins traffic to neuronal processes and growth cones of motor neurons. *J Neurosci, 26*(33), 8622-8632. doi:10.1523/JNEUROSCI.3967-05.2006

Zhang, H. L., Pan, F., Hong, D., Shenoy, S. M., Singer, R. H., & Bassell, G. J. (2003). Active transport of the survival motor neuron protein and the role of exon-7 in cytoplasmic localization. *J Neurosci, 23*(16), 6627-6637.

Zhang, Z., Lotti, F., Dittmar, K., Younis, I., Wan, L., Kasim, M., & Dreyfuss, G. (2008). SMN deficiency causes tissue-specific perturbations in the repertoire of snRNAs and widespread defects in splicing. *Cell, 133*(4), 585-600. doi:S0092-8674(08)00460-1 [pii]

10.1016/j.cell.2008.03.031

Zhang, Z., Pinto, A. M., Wan, L., Wang, W., Berg, M. G., Oliva, I., . . . Dreyfuss, G. (2013). Dysregulation of synaptogenesis genes antecedes motor neuron pathology in spinal muscular atrophy. *Proc Natl Acad Sci U S A, 110*(48), 19348-19353. doi:10.1073/pnas.1319280110

Zhao, D. Y., Gish, G., Braunschweig, U., Li, Y., Ni, Z., Schmitges, F. W., . . . Greenblatt, J. F. (2016). SMN and symmetric arginine dimethylation of RNA polymerase II C-terminal domain control termination. *Nature, 529*(7584), 48-53. doi:10.1038/nature16469

Zhong, Z., Ohnmacht, J., Reimer, M. M., Bach, I., Becker, T., & Becker, C. G. (2012). Chondrolectin Mediates Growth Cone Interactions of Motor Axons with an Intermediate Target. *The Journal of Neuroscience, 32*(13), 4426-4439. doi:10.1523/jneurosci.5179-11.2012

Zhou, C., Feng, Z., & Ko, C. P. (2016). Defects in Motoneuron-Astrocyte Interactions in Spinal Muscular Atrophy. *J Neurosci, 36*(8), 2543-2553. doi:10.1523/JNEUROSCI.3534-15.2016

Zhou, S., & Zhou, J. (2014). Neuritin, a neurotrophic factor in nervous system physiology. *Curr Med Chem, 21*(10), 1212-1219.

Zivraj, K. H., Tung, Y. C., Piper, M., Gumy, L., Fawcett, J. W., Yeo, G. S., & Holt, C. E. (2010). Subcellular profiling reveals distinct and developmentally regulated repertoire of growth cone mRNAs. *J Neurosci, 30*(46), 15464-15478. doi:30/46/15464 [pii]

10.1523/JNEUROSCI.1800-10.2010

Zou, J., Barahmand-pour, F., Blackburn, M. L., Matsui, Y., Chansky, H. A., & Yang, L. (2004). Survival motor neuron (SMN) protein interacts with transcription corepressor mSin3A. *J Biol Chem, 279*(15), 14922-14928. doi:10.1074/jbc.M309218200