Utilizing Gene Therapy Methods to Probe the Genetic Requirements to Prevent Spinal Muscular Atrophy

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by

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DEDICATION

To my parents, Tim and Rebecca: thanks for getting me here.

To my fiancé, Simon: thanks for getting me through.

To many friends, family, and mentors along the way: simply, thanks.

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I would like to thank my mentor, Dr. Christian Lorson for inviting me into a world with which I quickly fell in love. Because of you I found a study that is truly worth my passion. Because of you Mizzou truly became a home.

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LIST OF ABBREVIATIONS

AAV	Adeno-Associated Virus
ALS	Amyotrophic Lateral Sclerosis
ARE	Antioxidant Response Element
ASO	Antisense Oligonucleotide
BBB	Blood Brain Barrier
BIPAP	Bilevel Positive Airway Pressure
C.e.Smn	*Caenorhabditis. elegans Smn*
CBs	Cajal (coiled) Bodies
CCR	Calpain Cleavage Region
CMAP	Compound Muscle Action Potential
CNS	Central Nervous System
D.mSmn	*Drosophila melanogaster Smn*
D.r.Smn	*Danio Rerio Smn*
FDA	Food and Drug Administration
FLSMN	Full-Length Survival Motor Neuron
IM	Intramuscular

ICV	Intracerebralventricular
iPSCs	Induced Pluripotent Stem Cells
ITR	Inverted Terminal Repeat
IV	Intravascular
mRNA	Messenger RNA
mRBPs	mRNA Binding Protein
MUNE	Motor Unit Number Estimates
NAIP	Neuronal Apoptosis Inhibiting Protein
NMJ	Neuromuscular Junction
PMRT1	Protein arginine N-methyltransferase 1
PND	Postnatal Day
scAAV	Self-Complementary Adeno-Associated Virus
ssAAV	Single-Stranded Adeno-Associated Virus
rAAV	Recombinant Adeno-Associated Virus
RNP	Ribonucleoprotein
ROS	Reactive Oxygen Species
EMA	European Medicines Agency

S.p.Smn	*Schizosaccharomyces pombe Smn*
SMA	Spinal Muscular Atrophy
SMN	Survival Motor Neuron
snRNP	Small Nuclear Ribonucleoprotein
TMS1	Trimethylguanosine Synthase 1
trs	Terminal Resolution Site
UNRIP	UNR-Interacting Protein
WT	Wild-type
X.l.Smn	*Xenopus laevis Smn*
ZBP1	Zipcode Binding Protein 1

RESEARCH ABSTRACT

Spinal Muscular Atrophy is clinically recognized as a progressive weakness within the trunk and proximal limbs that will lead to breathing failure and death within infants. As a neurodegenerative genetic disease, SMA is caused by loss of motor neurons, which in turn is caused by low levels of the Survival Motor Neuron (SMN) protein. The mechanism by which a ubiquitously expressed protein such as SMN is able to cause the specific death of motor neurons is highly debated and of great interest.

Work presented here focuses on understanding the biological requirements of SMN and its downstream effects on the neuromuscular junction. To this end we utilize viral based gene delivery as a powerful tool to assess the effects of genes of interest *in vivo*. Our findings contribute to the conversation regarding whether SMA is truly a "motor neuron" disease, suggesting that astrocytes play a meaningful role in staving off SMA. Further, we investigate the domains within SMN needed to maintain its function in a mammalian system. We take a novel and challenging approach to identify a minimal domain capable of maintaining function. Finally, we demonstrate the practical use of morophological analysis of the neuromuscular junction as a means to characterize SMA pathology.

Chapter One: Introduction

1: Spinal Muscular Atrophy

Spinal Muscular Atrophy (SMA) is a neurodegenerative disease originally classified in the 1890s by Werdnig and Hoffmann. These physicians recognized symptoms in infants that resembled the muscular dystrophy usually found in older boys. These patients grew progressively weaker and died very young. In 1954, Kugelberg and Welander characterized a different type of dystrophy that was previously mistaken for limb-girdle dystrophy. They noted wasting of the muscles with no bulbar or mental changes, a "relatively favorable course," and likened biopsy results to those caused by lower motor neuron lesion[1]. Since, these diseases have been identified as belonging to different types within the spectrum of a single disease: Spinal Muscular Atrophy.

1.1: Clinical Manifestation of SMA

Spinal Muscular Atrophy (SMA) manifests in loss of α-motor neurons in the lower ventral horn of the spinal cord, which results in symmetrical muscle wasting. While the loss of motor neurons leads to loss of muscle control and tendon responses, cognition and sensation remain unaffected[2]. Patients experience a progressive weakness in stereotyped proximal muscle groups. Distal muscles are less affected and the upper limbs are less severely affected than are the lower limbs. While different classifications of SMA patients are now recognized to have the same molecular cause, the extreme range of symptoms originally had them classified as different diseases. Clinical separation into their respective subtypes is now based on the age of diagnosis as well as developmental milestones achieved.

SMA is clinically stratified based upon the age of onset of symptoms and the physical milestones achieved, with Type 0 being the most severe resulting in death prenatally or very shortly after birth and Type IV SMA resulting in an adolescent/adult form of the disease that only manifests later in life[3]. These extremes within the clinical spectrum are rare; most living patients are classified as Types I, II or III, presenting with differing severity of classical neurodegenerative features.

Diagnosis, Ambulation, and Life Expectancy

The most severe cases of SMA, Type 0, are rarely seen and it has only recently been recognized as lethality occurs prenatally or shortly after birth. While diagnosis is sometimes postmortem, these patients have a history of decreased movement in utero[4].

Type I patients exhibit muscle weakness very early, are diagnosed within 6 months, and without respiratory assistance will succumb to SMA by age 2. Developmentally, a main diagnosis criterion is that they are never able to sit unassisted. Type I patients are unable to lift the head and have poor limb control. Accordingly, SMA is one disorder that results in infant hypotonia, colloquially referred to as "floppy baby syndrome." Type I SMA is now recognized to be the same disease originally described as Werdnig-Hoffmann disease. These patients represent the greatest percentage of new cases annually[5].

Type II patients were formerly diagnosed with Dubowitz syndrome. These patients are diagnosed between 6-18 months of age and achieve the milestone of sitting, but not walking. They may have decreased head and/or arm control. Primary weakness is in the upper arms and legs; however, patients may experience spasms in the distal limbs.

These patients may use motorized wheelchairs and require assistance for many daily tasks. Without intervention these patients will live into adolescence or young adulthood.

Previously known as Kugelberg-Welander syndrome, symptom onset in Type III SMA begins between 2-17 years old. The clinical definition states that patients achieve the ability to sit and stand, but never run. They have an abnormal gait, experience fatigue, and fall often. They may have assistive walkers or manual wheelchairs for occasional or permanent use. [2]. These patients are expected to have normal or near-normal lifespans.

Type IV SMA is adult onset, presenting over the age of 18. These patients are able to achieve walking, running, and all normal milestones. These patients experience atrophy that affects their strength and mobility and may become wheelchair bound. SMA does not usually affect the patient's lifespan.

Current options for SMA assistance

More recent natural history data indicates a pronounced change in the life spans for Type I and II patients[6, 7]. While no treatments are specifically approved for SMA, the development of consensus statement for standard-of-care for SMA highlights a number of supportive measures that clearly have impacted life span as well as quality of life for SMA patients[8].

Orthopedics

Orthopedic interventions are typically provided for Type II and Type III patients. These patients experience orthopedic problems that are exacerbated by immobility. Type II patients develop an extreme progressive scoliosis as well as pelvic obliquity which can put further strain on the chest cavity[9]. Structural corsets cannot ameliorate the progression of scoliosis, but can aid in general function specifically by increasing seated

stability, relieving the arms from this task[9]. Spinal fusion is an option in severely affected patients, however for very young patients who are still growing, stabilization via growing rods is preferred[10]. Type III patients may have similar orthopedic issues, but to a lesser degree. Leg braces and other standing assistive devices are important to aid in normal development as standing slows the progression of scoliosis, reduces joint contractures, and increases respiratory function. Proper orthopedic support can help improve quality of life for these patients[9].

Gastrointestinal

Complications of SMA affect many parts of the GI tract. Type I patients are prone to tongue fasciculation, dysphagia, and fatigue during feeding, causing failure to thrive[8, 11]. This also leaves Type I patients prone to aspiration pneumonia; it is advised to implement external feeding tubes before pneumonia becomes a recurring problem. Bowel problems are common in Type I and Type II patients including constipation and impaction due to poor abdominal strength as well as immobility[11]. While SMA patients are prone to undernutrition, they are simultaneously vulnerable to becoming overweight. Typical BMI measurements can be skewed due to abnormal muscle loss, masking the additional weight gained[9]. With these issues considered, a critical part of patient care is diet.

Respiratory

SMA patients have very weak intercostals muscles and severe patients depend more on their diaphragm to breath. This has several consequences. 1) The lungs and chest wall are underdeveloped, resulting in pectus excavatum and a bell-shaped chest. 2) Patients are unable to cough, leaving them prone to infection and choking, and unable to clear lung secretions. 3) Hypoventilation during sleep results in poor oxygenation[12]. Without assistance, breathing failure will eventually lead to premature death. Various techniques

are employed to keep the airways clear. Secretion mobilization, postural drainage and cough assist are carried out multiple times daily for Type I patients and when necessary for Type II and Type III patients[12]. Respiratory aid can be noninvasive bilevel positive airway pressure (BIPAP) or mechanical ventilation, or invasive via tracheotomy. Less severe patients experience primarily sleep apnea or postoperative breathing distress[12].

1.2: SMA as a Genetic Disease

Incidence of SMA

Despite being classified as a "rare disease", Spinal Muscular Atrophy (SMA) is a devastating and surprisingly common disorder that affects approximately 1 in 6000-10,000 live births, resulting in a progressive muscle wasting[13, 14]. The disease is transmitted in an autosomal recessive pattern and is found to have a carrier frequency of approximately 1:50. This frequency is thought to be roughly universal with some variation: 1:47 for European populations, 1:72 for African Americans[15]. The largest exception to this pan-ethnic trend is within the Hutterite community in the northern United States and Canada. This population is found to have an SMA carrier frequency of nearly 1 in 8 due to a founder effect and limited genetic diversity[16].

While the majority of SMA cases are inherited from carrier parents, up to 2% of cases arise due to *de novo* mutations or deletions[17]. Despite the complex nature of SMA genetics that will be described shortly, carrier screening is available with accuracy ~90%. Based on the high carrier frequency, availability of accurate testing, and severity of disease, preconception carrier screening has been recommended to enable planning parents to choose from the fullest range of reproductive options[15, 18].

Identification of the SMA locus

The causative gene for SMA was localized to Chromosome 5q11.2-q13.3 using classic linkage analysis[19, 20]. However, this region contains multiple genes as well as repetitive elements which cause it to be a dynamic genomic region. Large deletions in this region seemed to be responsible for most cases of SMA, however these deletions included more than one 5q gene as seen in Figure 1. A heated debate began as to which was the SMA causative gene or whether more than one gene was involved[21, 22]. The two strongest SMA candidate genes were *Survival Motor Neuron 1 (SMN1)* and *Neuronal Apoptosis Inhibitor Protein (NAIP)*. Initially each was found to be deleted in a high percentage of patients and present in healthy controls. However, after a large body of work, including identifying patients with intact *NAIP* and single nucleotide mutations in *SMN1*, it was concluded that the latter is solely responsible for the 5q-linked SMA phenotype.

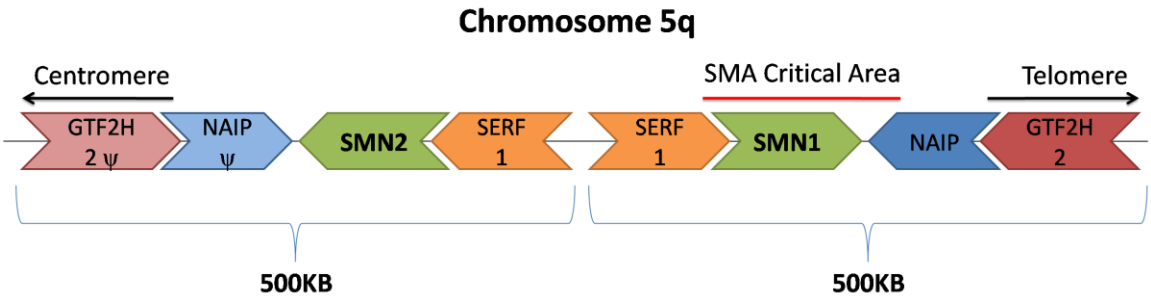


Figure 1: The SMA critical region is within a duplicated region on Chromosome 5q.

A 500kb region within Chromosome 5q was duplicated within humans and this region was linked with SMA. The presence of multiple genes within this duplication confounded the positive identification of the SMA causative gene. While Neuronal Apoptosis Inhibiting Protein (NAIP) was proposed to be responsible for SMA, ultimately loss or deletion of Survival Motor Neuron (SMN) 1 with preservation of *SMN2* was found to be necessary and sufficient to cause the SMA condition.

Non 5q Spinal Muscular Atrophies

While 95% of SMA cases are due to loss of *SMN1*, a number of patients presenting similar phenotypes test negative for 5q defects. Many of these diseases present similarly, because motor neurons are negatively affected, but their molecular etiologies are distinct. Until identification of the disease causing gene in each case, noting the pattern of inheritance helped distinguish these diseases from each other. For the purpose of contrasting these diseases, the classical SMA caused by *SMN1* mutation will be briefly referred to as 5qSMA

SMAX1—Spinal and Bulbar Muscular Atrophy (Kennedy Disease, OMIM #313200)

CAG (glutamine) repeat expansions in exon 1 of the Androgen Receptor genes cause SMAX1. The mutated protein acts in a dominant negative fashion, causing toxicity in nerves and muscles. Clinical symptoms include progressive limb weakness, bulbar involvement causing dysphagia and facial fasciculations. Disease onset is delayed, typically to the third to fifth decade of life and due to its X-linked inheritance SMAX1 primarily affects men[23].

SMAX2 X-Linked Infantile Spinal Musuclar Atrophy

The clinical manifestation of X-linked SMA is quite similar to 5qSMA Type I. SMAX2 presents with hypotonia, scoliosis, chest deformities, and infantile death due to respiratory failure. As indicated by the name, this disease is clinically distinct in its X-linked nature and predominantly affects males. This disease is caused by mutations in *UBA1* (previously *UBE1*)[24, 25]. Interestingly, it was recently suggested that *UBA1* could be linked to the motor axon defects found in SMN deficient zebrafish[26].

X–linked distal Spinal Muscular Atrophy Type 3

Certain missense mutations in the copper transport gene *ATP7A* can result in SMAX3[27]. This results in a loss-of-function which affects motor neurons, causing a progressive muscle atrophy phenotype[28]. This disease affects distal muscles such as the foot more prominently than other SMAs and follows an X-linked inheritance.

SMARD1—Spinal Muscular Atrophy with Respiratory Distress

Unlike 5qSMA, respiratory distress in SMARD1 occurs within the first year of life and precedes muscle atrophy. Atrophy can manifest as hand drop, fatty pad buildup This autosomal recessive disease is caused by mutations within the *IGHMBP2* gene. While a high level of clinical heterogeneity exists, prognosis is generally poor[29]. Pre-clinical results suggest that SMARD1 may be a viable candidate for gene replacement therapy[30].

Chromosome 5q

Genetic confirmation of the SMA causative gene was further complicated by a genomic duplication event early in hominid evolution. A 500 kb region on Chromosome 5q was duplicated and inverted, resulting in two very similar sets of genes[31]. Among these genes are a pseudogene *ψNAIP*, and a gene closely related to *SMN1* called *SMN2*. It was found that deletion or mutation of *SMN1*, but not *SMN2* results in Spinal Muscular Atrophy; up to 15% of healthy individuals have no *SMN2* gene[18]. While *SMN1* is known to be the SMA causative gene, *SMN2* acts as a critical disease modifier; more copies of *SMN2* result in milder forms of SMA, as will be discussed later.

The typical makeup of the SMN locus has one *SMN1* gene and one *SMN2* gene and can be annotated as *SMN1:SMN2* 1:1. However, because this region is prone to rearrangement, copy numbers of *SMN1* and *SMN2* can be variable on each Chromosome. Via intergenic meiotic recombination, variant forms of this locus are produced at a

frequency of 1 in 10,000[15]. This most typically occurs during paternal meiosis[18]. Approximately 5% of the normal population carries three copies of *SMN1* and *SMN2* copies have been found to range from 0-5[18]. The presence of 2 copies of *SMN1* on the same chromosome masks some carriers of SMA because the quantitative assays used to determine copy number do not distinguish between separate alleles. Evolution should select against alleles carrying no *SMN1* because homozygous deletions usually result in death before reproductive age, but the instability within Chromosome 5 keeps them around. It is thought that this high rate of *de novo* mutation is thought to be responsible for the relatively high SMA carrier frequency[18].

Several genetic scenarios can cause loss of functional *SMN1*, resulting in Spinal Muscular Atrophy. Most commonly *SMN1* is deleted on one of each parental chromosomes [18]. A number of patient mutations have also been identified which make the mutated protein from *SMN1* non-functional or unstable[32]. Approximately 5% of patients are found to be compound heterozygous at the SMN locus: one *SMN1* allele has a mutation while the other is deleted[18]. In cases of gene conversion, *SMN1* can be converted into *SMN2* through intra-or interchromosomal recombinations[15, 33]. This is the only scenario in which the copy number of *SMN2* is simultaneously altered and is thought to lead to less severe Types of SMA[21].

1.3: SMN1 and SMN2

While 99% identical at the nucleotide level, *SMN1* and *SMN2* are functionally different. Each gene is capable of producing a pre-mRNA containing 8 exons (1, 2a, 2b, 3-8) which is known as full length SMN (FLSMN). A stop codon present in exon 7 terminates this

protein product, thus creating a polypeptide of 294 amino acids. The resulting protein is 38kD and considered stable.

In contrast, *SMN2* harbors few single nucleotide differences from *SMN1* and one in particular causes it to produce a truncated protein[34]. This dynamic process is illustrated in Figure 2. In position 6 of exon 7, *SMN1* encodes a C, allowing for a complete exonic splicing enhancer site that recruits SR proteins, thus defining exon 7 for splicing inclusion. Alternatively, *SMN2* harbors a T in this position, which disrupts the exonic splicing enhancer site and further creates binding sites for SR and hnRNP proteins, serving as an exonic splicing silencer for exon 7[35]. Therefore, *SMN2* primarily produces a pre-mRNA that preferentially splices out exon 7, resulting in a truncated 282 amino acid protein termed SMNΔ7.

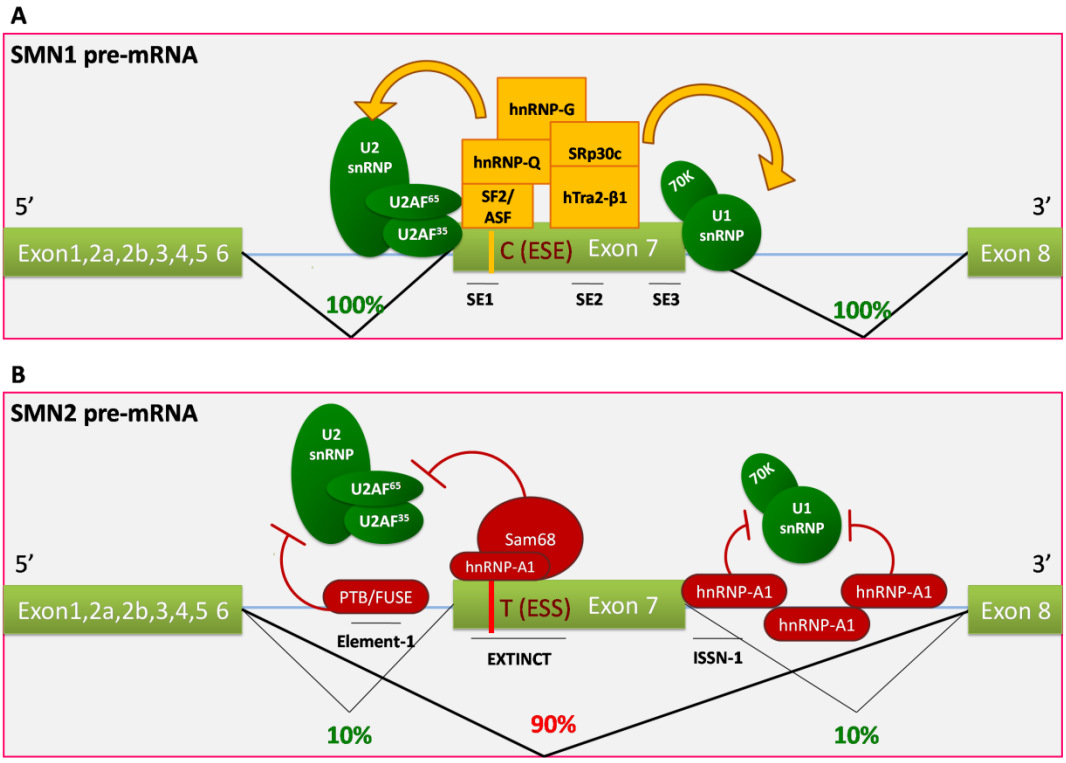


Figure 2: *SMN1* and *SMN2* display differing splicing patterns

Within exon 7 nucleotide 6, *SMN1* and *SMN2* harbor a single nucleotide difference. C present in *SMN1* forms an exonic splicing enhancer known as splicing enhancer 1 (SE1). This promotes aggregation of enhancer proteins resulting in the splicing inclusion of SMN exon 7 in 100% of transcripts. In *SMN2* this position carries a T, which alters SE1 into a exonic splicing silencer known as EXTINCT. The repressor proteins that bind EXTINCT further block enhancer proteins and cause the preferential skipping of exon 7 in 90% of transcripts.

Modified from Coady and Lorson, 2011[36]

Importantly, approximately 10% of *SMN2* transcripts include exon 7, in turn producing FLSMN protein while the other 90% of transcripts yield SMNΔ7. Additional copies of *SMN2* each provided additive levels of FLSMN and SMNΔ7. The product SMNΔ7 is not toxic, however it is unstable and therefore less functional[35]. The mechanism behind SMNΔ7 instability has recently been elucidated. Previously it was presumed that a sequence within exon 7 conferred stability on FLSMN and that the SMNΔ7 product was simply lacking that important stability region. However, exon 7 encodes the FLSMN stop codon, so the splicing of exon 6 to exon 8 SMNΔ7 products read an extra four codons into exon 8 before encountering a stop codon. The YG box found in exon 6 in combination with the extra four amino acids (EMLA) within exon 8 have been shown to act as a degradation signal. In these experiments, luciferase fused with YG and EMLA together had a similar half-life to luciferase tagged with SMNΔ7, an effect that was abolished upon mutation within the YG box[37].

1.4: SMN and SMA: Genotype-Phenotype Correlation

In SMA, patients have deleted or mutated copies of *SMN1* and must rely on protein provided by *SMN2*. Due to the fact that it produces low levels of full length SMN, the presence of *SMN2* acts as a critical disease modifier in SMA; patients with a higher *SMN2* copy number produce proportionately more full length protein and accordingly experience more mild symptoms as illustrated in Figure 3[38]. Individuals have been identified that are genetically classified as "SMA" as they lack a functional *SMN1* gene, yet the presence of 5 or more copies of *SMN2* can fully compensate for the absence of *SMN1* [38, 39]. In other species, complete depletion of SMN protein is lethal early in development. This seems to be true in humans also, as patients with 0-1 copies of SMN are rarely identified. For this reason, Type 0 patients have only recently been recognized

on the SMA spectrum, presumably because most of these infants are miscarried pre-term. While other genes may have an effect on SMA pathology and have been proposed as SMA modifier genes, currently *SMN2* is the only accepted modifier of SMA and therefore is an attractive therapeutic target.

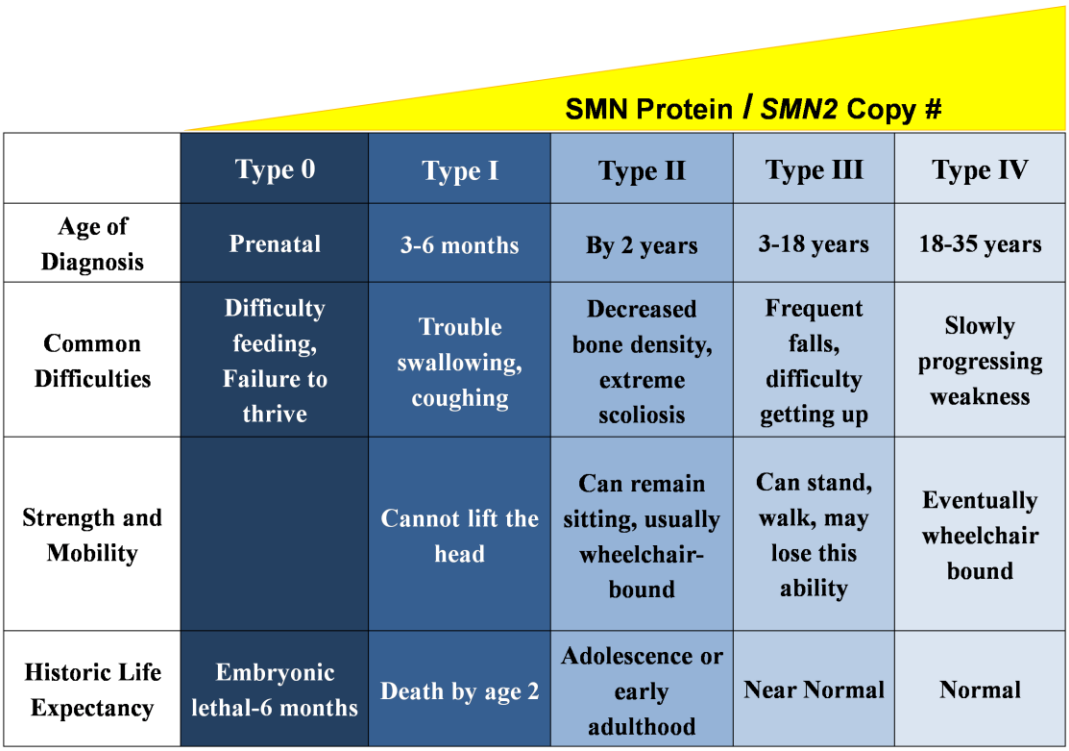


Figure 3: Severity of SMA patient symptoms correlate with *SMN2* copy number.

SMA patients are diagnosed based on clinical observations regarding age of onset and death as well as developmental milestones that are achieved. Type 0 patients are the most severe while Type IV demonstrate the mildest forms of SMA symptoms. Patients with increasing copy numbers of *SMN2* produce higher levels of stable SMN protein and accordingly exhibit more mild symptoms.

SMN protein is ubiquitously expressed and can be found in the cytoplasm and nucleus. Within the nucleus, SMN can be visualized in puncta known as Gemini of coiled bodies, or gems, appropriately named as they closely associate with coiled bodies[40]. Also referred to as Cajal bodies, coiled bodies (CBs) are DNA-free nonmembrane-bound subnuclear aggregates of RNA and protein. CBs are marked by the presence of Coilin and are seen in cells that are dividing as well as those that are metabolically active, such as neurons. These aggregates could serve as sites of maturation and higher order complexing for the small nuclear ribonucleoproteins (snRNPs) that carry out splicing. Under certain conditions, gems and CBs closely associate, and SMN can physically interact with Coilin, however under alternate conditions, gems and CBs occupy distinct spaces. Knockdown of SMN is known to disrupt the formation of Gems and CBs alike, whereas depletion of Coilin disrupts formation of CBs and promotes the formation of gems[41, 42]. While their exact function is unknown, practically, gems are used as an indirect measure of SMN abundance[43].

1.5: SMN Molecular Function

Several functions for SMN protein have been established, however the contribution of these functions to SMA pathology is contested. The canonical function of SMN is as a scaffold protein aiding in the assembly and transport of small nuclear ribonucleoproteins (snRNPs). Further, SMN has functions that seem to be motor neuron specific. SMN can bind to mRNA binding proteins, and through these proteins is thought to transport key mRNAs from the neuronal cell body to the dendrite. Additionally, SMN's profilin-interacting domain is thought to help maintain neuronal cytoskeleton integrity. Each of these features will be discussed at length.

In order to examine the function of SMN, it is important to have an understanding of its interactions. While the SMN structure itself will be discussed at length in chapter 2, presently it will suffice to mention key regions within SMN and provide a basic map of the interacting partners currently understood as seen in Figure 4. As demonstrated, SMN has many binding partners and several distinct domains. Protein-protein interactions are important not only for function, but for SMN stability[44]. Between FLSMN and SMNΔ7 proteins, these regions are mostly conserved. However, as the normal translational stop codon is lost when exon 7 is excluded, four amino acids from exon 8 are included in the SMNΔ7 protein before reaching another stop codon. This protein becomes rapidly degraded. Protein levels of SMNΔ7 drop precipitously within 1-3 hours, whereas FLSMN remains steady within this time[35]. Detailed comparison of the turnover rate demonstrates that SMNΔ7 has a half-life of 2.15 hours compared to that of FLSMN which is 4.35 hours [44]. Both products are targeted for degradation via the proteasome[44]. The half-life of SMNΔ7 is further shortened in the absence of FLSMN[44]. Whereas the structure of the protein is largely unchanged, the shortened half-life renders SMNΔ7 unable to perform the standard functions of SMN.

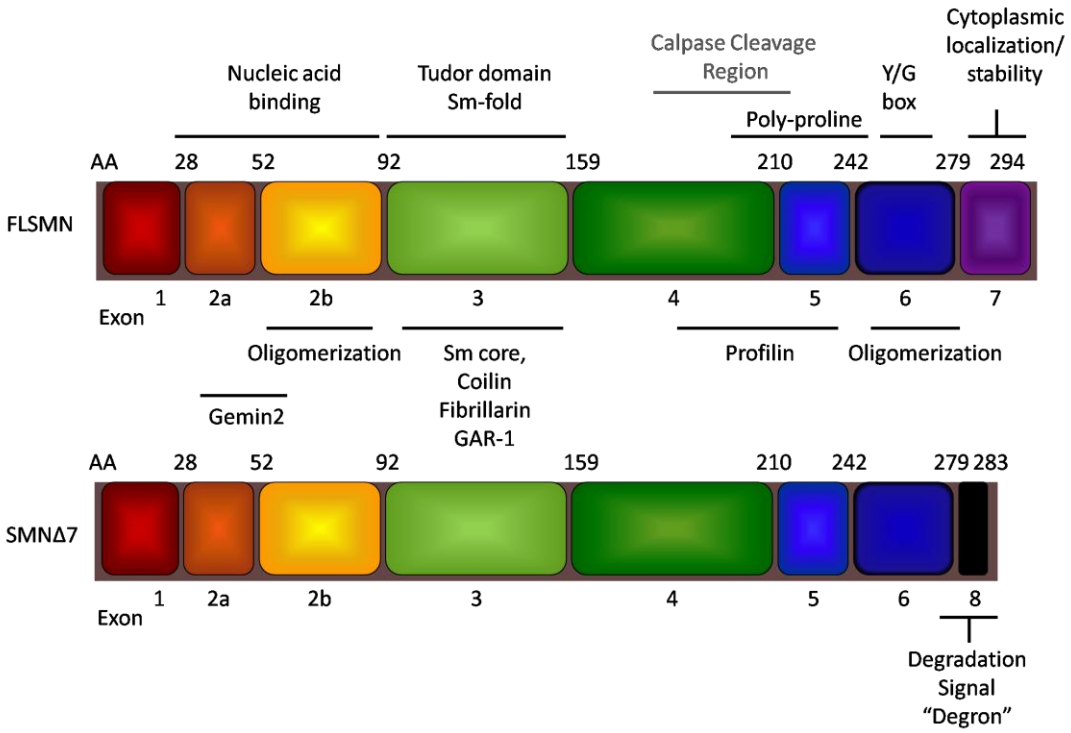


Figure 4: Schematic of full length SMN and the truncated SMNΔ7.

FLSMN protein map is divided into colored exon regions labeled with exon number (below) and amino acid exon definition (above). Functional regions within SMN that have been characterized by their sequence characteristics and/or binding partners are defined by black bars and labeled. Schematic of SMNΔ7 has identical properties throughout exons 1-6 which were not duplicated. At the C terminus within SMNΔ7, exon 7 is removed and the open reading frame continues into exon 8. The fusion of exon 6 and 8 creates a novel motif that acts as a degradation signal known as the "degron."

Figure modified from Coady and Lorson, 2011[36]

SMN in snRNP Biogenesis

SMN function in RNP biogenesis has gradually grown to include a number of important snRNPs involved in RNA processing. The most characterized role of SMN in this regard is its involvement with snRNPs that carry out the process of splicing. This process is modeled in Figure 5.

Eukaryotic cells have acquired an elaborate system of pre-mRNA splicing that is essential to their genetic diversity and is one mechanism of maintaining proper spatio-temporal regulation of transcripts. This is accomplished through a series of catalytic events each carried out by unique members of the spliceosome known as small nucleic ribonucleoproteins, or snRNPs. The protein component of snRNPs are 7 unique, but closely related Smith antigen proteins (Sm proteins) called B/B', D1, D2, D3, E, F, and G. These are small proteins that assume a β-barrel shape and need to form a heptameric ring in order to function. The RNA component is a small nucleic RNA sequence (snRNA) that recognizes conserved splicing signals within pre-mRNA. The uridine-rich snRNAs of interest are U1, U2, U4, U5 and U6. These will direct each assembled snRNP to the proper location.

The journey of snRNAs must begin in the nucleus with polymerase II dependent transcription. The transcript is then modified with 7-methylguanosine (m^7G) cap and modification that results in an extra stem-loop structure in the 3' end. A cap-binding complex (CBC) made up of CBP80 and CBP20 binds to the m^7G cap. Next, the phosphorylated adaptor of RNA export (PHAX) is recruited and recognized by the export receptor Exportin1 (Xpo1) bound to RanGTP, thus forming a functional nuclear export complex. Once the complex has entered the cytoplasm, PHAX becomes

dephosphorylated upon GTP hydrolysis Ran and Xpo1 are dissociated from the complex, leaving dephosphorylated PHAX, CBC, and the pre-snRNA intact.

Meanwhile within the cytoplasm the Sm proteins must form a heptameric ring. To do this, the first critical interaction is with chloride conductance regulatory protein (pICln), which helps prevent premature binding with incorrect RNA sequences. This protein is part of a larger complex colloquially known as the methylosome, or protein arginine methyltransferase 5 (PRMT5). Interaction between PMRT5 and PMRT7 results in dimethylation of the Sm proteins which increases their affinity for binding with SMN. Several Gemin proteins are vital in the process of binding the snRNPs to the complex. pICln1 pre-forms Sm proteins into higher order structures B/D3 and F/E/D to prevent premature RNA binding[45]. These two complexes seem to bind to the SMN complex in a pentameric arc before the last two proteins complete the heptameric ring. Until the proper snRNA is ready to bind, this pentameric structure is kept in a narrower, more compact formation than it will be as a completed ring. This compact shape contorts the Sm proteins' RNA binding domains and accordingly prevents binding with alternative RNAs[46]. This intermediate maintains its narrow structure and is stabilized by Gemin 2 which contacts Sm proteins D1 and D2 with its C-terminal domain, and further reaches F, E and D with its N-terminal domain. The far end of the N-terminal domain wraps around into the center of the Sm pocket and makes contact with highly conserved residues within the Sm proteins that are responsible for binding the highly conserved Us within its snRNA partners. Thus it seems that Gemin 2 provides an additional mechanism of preventing spurious RNA binding. Gemins 6/7 share no amino acid homology with Sm proteins, yet heterodimerize to form a quaternary structure that closely resembles that of

Sm proteins and the SMN Tudor domain. This five-fold β-sheet motif allows for binding of Sm proteins and it is thought that this complex could be involved in acting as a placeholder, interacting with the other Sm proteins after their hypermethylation and before B/D3 are ready to join and close the Sm core ring[36, 45]. The Gemin6/7 heterodimer is connected to the SMN complex through Gemin 8[47]. UNRIP has a WD repeat region that could aid in the process moving Sm B/D3 into their place and complete the heptameric ring[45]. Unlike the other Gemins, UNRIP is found only in cytosolic fractions. While it is not essential for snRNP assembly, it seems to function in SMN localization as depletion of UNRIP leads to stronger nuclear SMN signal[45, 48].

The Sm core must finally bind snRNA and complete the snRNP. Toward this end, Gemin 5 is able to specifically identify RNAs that have the Sm binding sequence through its WD repeats. Gemin 2 brings in this snRNP as it binds to the complex. Gemin 3 is known to have DEAD-box RNA helicase activity and ATP-dependent 5'-3' unwinding functions. It is thought that this could act as a chaperone for RNA or RNP complexes, likely with Gemin 4 acting intimately as a cofactor[45]. The snRNP must be further processed by hypermethylation of the m^7G cap to a 2,2,7-trimethylguanosine (TMG) cap which is catalyzed by trimethylguanosine synthase 1(TGS1) and nucleotide trimming of the snRNA 3' end[45, 46]. At this point, the TMG cap acts as a nuclear localization signal and is recognized by Surportin-1. The complex binds to the nuclear import receptor Importin-β. Gemin 5 and UNRIP (unr interacting protein) dissociate either prior to nuclear import, or are exported immediately afterward. The import proteins also dissociate and the snRNPs accumulate in Cajal Bodies whereas SMN localizes to gems. The snRNPs will undergo further steps for maturation including addition of other

proteins, chemical modifications and coupling into complexes such as the U4/U6-U5 tri-snRNP[45].

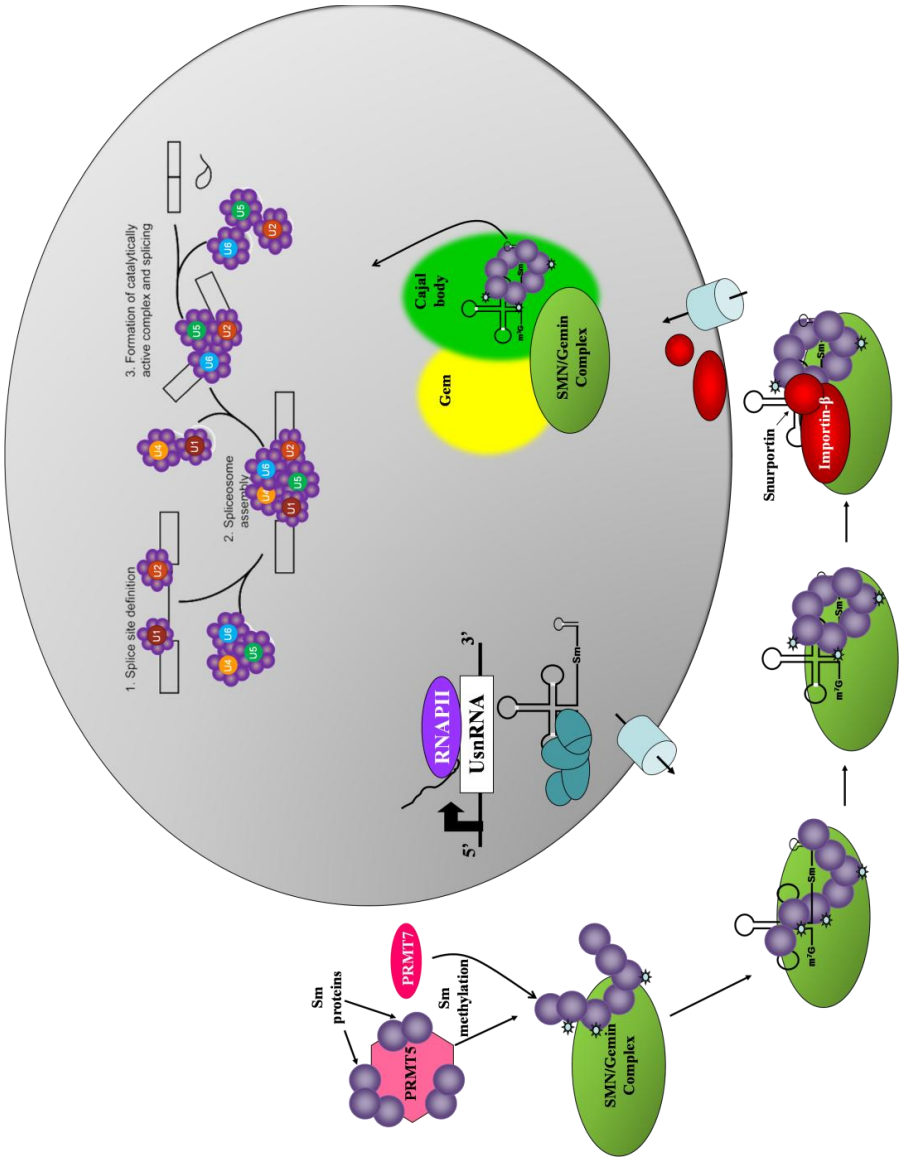


Figure 5: SMN acts as a scaffold for snRNP biogenesis.

U snRNAs are transcribed by DNA polymerase II and shuttled out of the cytoplasm. Meanwhile, Sm proteins have been methylated by PMRT1 to increase affinity for the SMN complex. In combination with Gemins 2-8 and UNRIP, SMN secures the Sm proteins in an incomplete ring formation at which point the U snRNA can bind. Once the U snRNA is bound, the final Sm proteins complete the heptameric ring. This complex is transported into the nucleus by Importin B and Snurportin. Once inside the nucleus, these

proteins dissociate. The snRNA and the SMN complex dissociate but aggregate nearby each other in Cajal Bodies or Gems, respectively. The completed snRNP will used to catalyze pre-mRNA splicing.

Figure modified from Coady and Lorson, 2011[36]

SMN's essential role in building the working parts of the spliceosome is thought to be the mechanism behind early cell death seen in SMN depleted organisms as well as the reason behind such high levels of conservation. Given that *S. pombe* as well as *Arabadopsis thaliana* and *Oryza sativa* all contain orthologs of SMN and Gemin 2, it is thought that these two proteins may have composed the earliest version of the SMN complex and that more interacting partners were added as splicing mechanisms became more complex[49].

Through similar mechanisms SMN also aids in assembly of the U7 snRNP which is in turn responsible for 3' modification of histone RNAs. Different Sm proteins play a role in this process: Sm-like proteins LSm10 and LSm11 replace SmD1 and SmD2, these substitutes are important as they allow for stabilization and cofactor recruitment of the snRNP at the histone 3' tail. Consistent with its role in this process, depleting SMN leads to accumulation of U7 snRNA and aberrant histone RNA 3' modification[46].

SMN's involvement with the formation of other classes of snRNPs is less understood. One such class is the LSm class snRNPs U6 and U6atac. Production of these snRNPs is carried out in the nucleus and involves a mostly LSm core[45]. Toward this, LSm 2-8 bind the uridine-rich regions of U6 and U6atac to facilitate the formation of the U4/U6 complex that is involved in splicing as well as snRNP recycling[46]. SMN may additionally be involved in assembly of the LSm1-7 complex which is responsible for mRNA turnover via binding to the 3' region of its targets and recruiting decapping proteins to aid in target 5'-3' degradation[46].

The means by which one or more of these snRNP assembly processes could contribute to the dramatic effect within neurons is highly hypothesized but poorly established. Presumably aberration of such essential housekeeping functions would result in global dysfunction within SMA patient tissues, however some evidence suggests that motor neurons may rely more heavily on high levels of SMN. It is known that snRNPs are not all equally affected by SMN depletion and that relative snRNP depletion is dependent on cell type.

One candidate explanation for the selective sensitivity of motor neurons states that these cells are actually more sensitive to disruption of the minor spliceosome. Most pre-mRNAs are spliced using the major spliceosome consisting of U1, U2, U4/U6, U5 snRNPs. Alternatively, the minor spliceosome utilizes U7, U11, U12, U4atac/U6atac, and U5 snRNPs. These snRNPs recognize alternative splicing signals and are responsible for splicing ~600-700 genes which represent approximately 1% of all introns within the transcriptome. Several of the U snRNP, and particularly U11 are disturbed in severe SMA mice. This effect was most profound in neural tissue compared to other organs such as kidney[47]. Further, in vertebrate knockdown models of SMA, providing purified snRNPs alleviates model-specific axonal defects[50, 51]. The few minor spliceosome-processed introns are not evenly distributed across the genome; instead, a subset of genes contains multiple of these introns. A cluster of voltage-gated ion channel genes was identified as having the non-canonical splicing sites defining their introns. One such gene, *Stasimon*, was identified as aberrantly spliced in a low SMN context. This evolutionarily conserved transmembrane protein is responsible for normal synaptic transmission within motor neurons[52]. It is compelling that *Stasimon* knockout in

Drosophila causes neural defects reminiscent of SMN depletion models in *Drosophila* and zebrafish. Unsurprisingly, *Stasimon* expression was insufficient to rescue phenotypes associated with SMN knockdown, implying that *Stasimon* could be partly, but not wholly responsible for the SMA phenotype. This provides an avenue for future work as well as establishes a proof-of-concept that snRNP biogenesis and the downstream splicing events associated with it could contribute to motor neuron loss.

This effect could further extend to major spliceosome-containing genes. Recently *neurexin* and *UBA1*were found to be linked to the motor axon defects found in zebrafish[26, 46]. These leads will require more investigation. It is of interest to note that mutation in the *UBA1* gene is responsible for the non-5qSMA known as SMAX2 mentioned previously.

In the scenario that snRNP biogenesis is responsible for SMA, a series of thresholds can be imagined: 1) As SMN protein levels decrease, snRNPs U11 and U12 get depleted more rapidly than other snRNPs and this process affects their synapse-relevant genes. 2) Upon further SMN depletion, snRNPs involved in the major spliceosome are compromised and cause more widespread alterations including cardiac defects. 3) Finally, complete loss of SMN disrupts even the most basic splicing functions and results in very early embryonic termination.

Several arguments counter the hypothesis of snRNP dysregulation resulting in neuronal defects. First is that low levels of SMN have a dramatic effect on snRNP biogenesis, but have little effect on the overall baseline levels of snRNPs. Next it is important to note that many of the snRNP decreases seen *in viv*o were in end-stage SMA.

Earlier timepoints did not demonstrate strong decreases. This calls into question the causative effect snRNPs could have on MN atrophy and suggests that they could be a secondary effect of SMA pathogenesis. Results in *Drosophila* show that while snRNP biogenesis is defective in Smn knockout flies, minor splicing pathways seemed not to be disturbed even though knockout flies die in an early developmental stage[53, 54].

SMN's role in Axons

Several lines of evidence led researchers to believe SMN may have a neuronal-specific function aside from its role in snRNP biogenesis. Early searches for SMN within the spinal cord found the protein associated with polyribosomes in dendrites[55]. SMN also localizes in neural growth cones and is found in axons[56]. Through the course of CNS maturation, SMN transitions from being primarily localized within the nucleus to cytoplasmic and then to axonal localization[57]. SMN is even shown to move rapidly in bi-directional and cytoskeletal-dependent movements consistent with motor protein-dependent fast axonal trafficking[58 2003, 59, 60].

Loss-of-function studies have also been informative toward studying SMN in axons. SMN depletion in MNs led to accumulation of neurofilament at the cell body as well as at the motor plate[61]. Exon 7 was required for the fast trafficking of SMN, implying that SMNΔ7 would be deficient in this process[58]. SMN depleted cells have also demonstrated shortened axons in absence of any viability or dendritic phenotypes[62].

Upon investigating the axonal roles of SMN, evidence suggested that SMN has a role in localizing mRNAs and mRNA binding proteins (mRBPs) to distal ends of the neuron. Within axons, SMN has been shown to interact with a number of RNA-binding proteins to regulate transport and/or stability of key mRNAs.

β-actin is a key component of the cytoskeleton and is involved in the dynamic protrusion of the growth cone and synaptic formation. It has been shown that within SMN depleted cells β-actin mRNA and protein accumulate within at growth cones and distal axons, implying that their normal dynamic state is disrupted[62]. Interestingly, restoration of β-actin is able to restore morphological axonal defects *in vitro* and promote motor neuron survival *in vivo*[63, 64]. Several mRBPs link β-actin to SMN including ZBP which binds to the β-actin mRNA via the zip codes present its 3' UTR, HuD which stabilizes mRNAs through AU-rich elements (AREs) in their 3' UTR and hnRNP-R which was shown to bind to the 3'UTR of β-actin mRNAs through an interaction that required the presence of Smn[65 62]. Knockdown of hnRNP-R in cultured mouse motor neurons lead to morphological defects within the growth cone similar to that observed in SMA motor neurons[62, 66]. While many lines of evidence suggest that β-actin regulation is of importance in SMA, it has been shown that deletion of the β-actin locus in motor neurons does not have a deleterious effects on motor neuron outgrowth or NMJ morphology[67]. Thus it seems that low levels of distal β-actin mRNA cannot solely account for motor neuron deficits.

If β-actin is not sufficient to cause synaptic defects, others have hypothesized more general defects in the localization of polyadenylated mRNAs to distal ribosomes. An unbiased screen for dysregulated mRNAs in a decreased SMN context reveals a number of altered transcript levels for proteins involved in many functions, some of which relate to actin dynamics[68]. This is consistent with findings that other SMN interacting partners, HuD and ZPB1, are decreased within the axon[59, 69]. Both mRBPs affect local translation of several transcripts.

SMN also has a role in regulating levels of candidate plasticity-related gene 15 (cpg15)/neuritin and the stability of cytoskeleton-associated growth-associated protein GAP43. CPG15 is a small GPI-anchored protein known to be involved in neuronal outgrowth, neuromuscular synaptogenesis, axon branching, and axon regeneration. *Cpg15* is stabilized by HuD binding to the ARE within its 3' UTR. *Cpg15* mRNA, HuD, and SMN all colocalize within motor axons[70]. Levels of *cpg15* are reduced upon SMN depletion and its overexpression yields improvement of axonal defects within a zebrafish model of SMA[70]. HuD and ZBP have also been shown to interact with GAP43. This protein is known to be a positive regulator of axonal outgrowth as well as growth cone stability. GAP43 mRNA is decreased at the growth cone of SMA mouse model neurons *ex vivo.* Upon overexpression of ZBP and HuD, growth cones expressed higher levels of GAP43 and also demonstrated improved axonal morphology in this SMN-deficient environment[71].

SMN function is also linked with cytoskeletal integrity and actin dynamics. Stathmin, a microtubule-destabilizing protein was found to be upregulated upon SMN depletion and was seen responsible for poor structure within the microtubule network as well as reduced microtubule densities in distal axons *in vivo*. Upon knockdown in cells and in mice, microtubule defects were reduced and axon growth was ameliorated, respectively[72]. Other SMN binding partners are considered important for recycling and tethering of synaptic vesicles, all processes that involve the actin cytoskeleton. SMN is able to modulate actin polymerization and bind small actin-binding proteins profilin I and II in motor neurons[73]. In consequence, SMN deficient cells undergo an inappropriate activation of the RhoA actin-remodeling pathway due to SMN interaction with profilin

II[74]. Interestingly, another gene of interest that has been proposed as a naturally-occurring genetic modifier of SMA acts in actin bundling. Plastin 3 was identified in siblings whose genotypes at Chromosome 5q match, but clinically display differing levels of SMA serverity such that they are classified as different Types. Overexpression of Plastin 3 has beneficial effects on axons in an SMA context including cultured motor neurons from the severe mouse model and zebrafish embryos after knockdown of SMN. Plastin 3 and profilin have been linked at a genetic level but the consequences for SMA are unclear. By crossing the intermediate *Smn*$^{2B/-}$ mouse with a plastin 3 deficient mouse, researchers demonstrated that knocking down profilin could increase plastin 3 levels, however the SMA phenotype remained unchanged[75].

Some studies suggest that members of the canonical SMN complex join SMN in its cross-axon trafficking. Early studies reported that Gemin 2 did not interact with SMN in axons [56], however, utilizing sophisticated high resolution imaging techniques, it was later found that up to 48% of axonal SMN granules contain Gemin2 and Gemin3[76]. Double label immunofluorescence studies have further identified other Gemin proteins and UNRIP involved with SMN in axons[59, 77, 78]. Superficially this seems like evidence further supporting SMN's role in snRNP biogenesis, however these results could reflect alternative functions that still require one or more partners within the SMN complex. Indeed, SMN complex members have been shown to be involved with other types of transcriptional regulation such as translational repression[79-81] as well as cap- and IRES-depedent translation initiation steps[82-84]. UNRIP has also been implicated in the assembly of mRNA transport complexes[85].

Great contention has come in establishing whether one or both SMN's function in snRNP biosynthesis or mRPB transport are responsible for the symptoms of SMA. Each option has a theoretical basis by which it could affect motor neurons more dramatically than other cell types.

2: Tools to Study Spinal Muscular Atrophy

The field of SMA research is currently in an advanced state where many underlying causes of the disease are understood and where approved treatments seem to be on the horizon. While much important work is still performed in cell culture, understanding the fundamentals of SMA has allowed research to branch into areas of preclinical application and studying the disease within complex model organisms. As such, the tools of the trade are highly tailored to fit the exact questions remaining within SMA. Utilizing viral vectors in SMA mouse models to both study and treat SMA has become of high interest. The details of these methods will be discussed here.

2.1 Animal Models of SMA

Animal models within SMA have been utilized for two overlapping purposes: to study the pathology of SMA, and to test therapeutic strategies for SMA. Because humans are the only species that carries a copy of *SMN2*, all animal models for SMA must be genetically designed. A wide range of organisms have been utilized to this end including vertebrate and invertebrate models.

Schizosaccharomyces pombe

Yeast models provide a simple context to examine basic cell biology as well as conduct high throughput genetic screens. The two commonly utilized lab yeast are *Saccharomyces cerevisia* (budding yeast) and *Schizosaccharomyces pombe* (fission yeast). While both are yeast, these organisms are highly diverged; *S. pombe* is as closely

related to humans as it is to *S. cerevisia*[86]. A homolog to human SMN was identified in *S. pombe* in 2000, however no equivalent was found in *S. cerevisia*. However, *S. cerevisia* does harbor an apparent homolog of Gemin 2. It is thought that *S. cerevisia* lost its SMN homolog after splitting from the common ancestor shared with *S. pombe*[49].

Also known as Yab8, this 156 amino acid protein was called *S.p.Smn*. Similarity between *S.p.Smn* and human SMN is reported to be only 24%, mostly restricted to *S.p.Smn*'s N- and C-terminal domains[87]. Interestingly, *S.p.Smn* lacks any sequence homologous to the Tudor domain. This is unexpected because the Tudor domain is highly conserved in all other known SMN sequences and is considered central to SMN's ability to act as scaffolding for assembly of snRNPs.

Presence of SMN could be expected in fission yeast because in many ways the *S. pombe* resembles mammalian cells more closely than those from *S. cerevisia.* Approximately 40% of transcripts from *S. pombe* include one or more introns. In contrast, *S. cerevisia* has only a few hundred intron-containing genes, most of which contain only a single intron[88]. Other species of fission yeast also contain introns, and each of these species contain either an SMN homolog, or a paralog known as Spf30[89]. *S. pombe* also utilizes splicing signals that are more similar to mammals[90] This implies that yeast and humans could utilize SMN for similar splicing-related functions.

Yeast SMN seems to have partially overlapping function with human SMN. As in humans, knockout of endogenous SMN is lethal in yeast[87, 90]. Exogenous *S.p.Smn* expression in the context of endogenous *S.p.Smn* knockout is sufficient to rescue this

lethality. Further, *S.p.Smn* preferentially forms large order self-oligomers, as human SMN does[87].

Surprisingly, overexpression of *S.p.Smn* in a WT yeast strain was shown to increase growth rate, implying that *S.p.Smn* levels may be a growth limiting factor for these yeast. However, upon deletion of the N- or C-terminal domain or mutations mimicking human patient mutations, overexpression yielded a dominant-negative effect. The growth rate of each overexpressed strain slowed significantly compared to vector alone[90]. The dominant-negative effect of these mutant proteins could be suppressed upon coexpression with mouse Smn. This implies that the mouse Smn is able to interact with WT and/or mutant *S.p.Smn*.

Fission yeast seem to have a primitive version of the SMN complex consisting of homologs of SMN and Gemin 2[49]. *S.p.Smn* can bind to Yip1p, a yeast homolog of the human Gemin 2 (formerly called SIP1). When the N-terminus of *S.p.Smn* is deleted, binding with Yip1p is severely decreased, very similar to interactions between SMN and Gemin 2[91]. It is unknown whether *S.p.Smn* is able to function in a similar capacity to human SMN. Under some *in vitro* circumstances *S.p.Smn* was shown to bind Sm B, D2 and D3[87]. However, a different group has shown that *S.p.Smn* cannot bind to either human or yeast Sm proteins *in vitro*[91].

While use of *S. pombe* for SMA research has not been widespread, it has proven useful in studying SMN in cell biology. In yeast it was found that levels of *S.p.Smn* affects splicing and formation of U1, U2, U4, and U5 snRNPs, while leaving U6 and U3

unperturbed. Downstream effects included improper mRNA splicing that was sometimes linked to an increased polypyrimidine tract[92].

Caenorhabditis elegans

Due to its simplicity, the nematode worm is perhaps the most thoroughly characterized multicellular organism. The ultimate fate of each early cell has been identified. The number of motor neurons is known to be less than one hundred.; therefore it becomes an attractive model in which to study a disorder of motor neurons.

The *C. elegans* Smn gene was characterized in 1996 and named *C.e.Smn*. This 5 exon gene yields a 207 amino acid homolog of human SMN. Unlike *S.p.Smn*, *C.e.Smn* has a Tudor domain in addition to overall conservation of the N-terminus and YG box. The N-terminus has been shown to bind its homolog of Gemin 2, similar to the human case. Its SMN complex consists of SMN, Gemins 2, 3, 6, 7 and 8[45]. The protein is expressed in various tissues including the nervous system and body wall muscle.

Disrupting *C.e.Smn* carried the expected deleterious effects. Knockdown using RNAi resulted in larval lethality, however genetic knockout of the *C.e.Smn* region allowed the worms to live for several days off of maternal transcripts. Homozygous knockout animals are identical to their unaffected counterparts until maternal Smn decreases. They become comparatively shorter, slow moving, sterile, and most die before adulthood[93] Genetic aberration can be partially rescued by neuronal, but not muscle, expression of *C.e.Smn*[94]. Further, exogenous overexpression of *C.e.Smn* can lead to motor defects as well as reduced fitness[95].

Drosophila melanogaster

The characterization of an SMN homolog in *Drosophila melanogaster* in 1999 introduced a new high-throughput system with which to study SMN function and genetic interactions[96]. Termed *D.m.Smn*, this is a single-exon gene[97] present on Chromosome3 and given the genetic annotation 73A7-9[96]. Maternal *D.m.Smn* is highly expressed in embryos and maintained through early larval stages. Expression of *D.m.Smn* is high throughout embryogenesis, however levels drop during later development[97].

Several unique mutant lines demonstrate that loss of *D.m.Smn* is lethal at a late larval stage and that these animals have defects at the NMJ[98]. The embryos seem to survive to this stage due to maternal *D.m.Smn* expressed in the egg. Tissue-specific mutants demonstrate that *D.m.Smn* is required in muscles as well as neurons[99]. Interestingly, human SMN has a dominant negative effect when overexpressed in flies. A similar effect occurs when the N- or C-terminus of *D.m.Smn* is deleted and the truncated *D.m.Smn* is expressed.

The canonical SMN function in assisting snRNP biogenesis appears to be conserved in flies as *D.m.Smn* knockdown *in vitro* yields fewer intact Sm cores[97]. In humans this process is carried out by SMN as well as the other protein members of the SMN complex. *Drosophila* Smn complex contains homologs of SMN as well as Gemins 2, 3 and 5.

Overall the 226 amino acid *D.m.Smn* protein shares 25% identity with human SMN. This conservation is especially strong in certain regions of *D.m.Smn* including the centrally located Gemin2 binding and Tudor domains as well as a highly conserved C terminal YXXG box.

Interestingly, when modeled in *Drosophila*, patient mutations within the tudor domain have variable effects. Mutations that are associated with Type I (W92S) and III (G95R and Y130C) patients still maintain the ability to rescue a *D.m.Smn* deficient fly[100]. One mutation associated with Type III SMA (Y130C) rescued only to an intermediate phenotype described as pupal lethal[100]. Upon biochemical analysis, these mutants demonstrated no appreciable difference in binding to SmD1 indicating that the mutation would not disrupt SMN's function in snRNP biogenesis[100].

Disruption of *D.m.Smn* can be demonstrated by modeling a human mutation in the Gemin 2 binding region. To this respect, the *D.mSmn* mutant D20V displays an intermediate SMA phenotype[100]. This is consistent with the human situation in which D44V is associated with milder Type III SMA.

The *D.m.Smn* YG box is well characterized. It is through this YXXG region that *D.m.Smn* is able to self-oligomerize as well as bind human SMN. Whereas introduction of human SMN ameliorates the SMA phenotype in other SMN knockout models including mice[101] and zebrafish[102], human SMN has a dominant negative effect in flies[95]. This effect was not seen with introduction of *D.m.Smn in trans*, however truncated *D.m.Smn* sequences containing only the C terminus was necessary and sufficient to cause this effect[95]. Mutations in this region have also been shown to be deleterious. By screening for recessive lethality in EMS mutagenized male lines, *D.m.Smn* missense mutations S201F and G202 have been shown to disrupt *D.m.Smn* function and used to create transgenic fly lines smn^{B} smn^{73Ao} and, respectively [97, 99]. While these lines have been crossed with isogenized fly lines to ensure no other homozygous lethal mutations are present in the genetic background, flies homozygous for each of these mutations die

within late larval stages[99]. When these two mutations are expressed in conjunction with each other, the phenotype is only slightly more mild[99]. Further, upon expression of homologs of SMA patient mutations, Praveen *et al.* found expression of *D.m.Smn* YG mutations M194R, Y203C and G206S to have a dominant negative effect such that displayed a lower frequency of eclosion than the $SMN^{X7/X7}$ flies[100]. Through *in vitro* binding assays as well as *in silico* modeling, *D.m.Smn* containing the most severe Y203C mutation was shown to be deficient in self-oligomerization yet still able to bind native *D.m.Smn* [53, 100]. In this way it is hypothesized that mutation Y203C forms nonfunctional complexes selectively with *D.m.Smn* thus limiting the functional *D.m.Smn* pool[100].

Xenopus

Two different species of frog have been examined in an SMA context—*X. laevis* and *X. tropicalis.* Each SMN sequence has been characterized and was found to be expressed ubiquitously and continuously throughout development[50]. *Xenopus* Smn sequences share 52-52% identity with human SMN. This identity is particularly high in the Tudor domain (67%) and other domains can be recognized in *Xenopus* SMN such as the N-terminus, polyproline region, and YG box [50, 103].

X. laevis was studied at very early levels of development, while *X. tropicalis* was followed into the tadpole stage. Each study utilized morpholino-based RNAi to deplete SMN levels *in vivo*. Injection of RNAi against Smn or Gemin2 at the two cell blastula stage resulted in protein depletion at the gastrula stage. Approximately 0.2μM of morpholinos targeting their respective mRNAs were injected. This had a very severe effect; SMN depletion caused ~50-80% of the embryos exhibited developmental arrest and the embryos did not finish gastrulation. Embryos that completed gastrulation failed to

close the blastoporus while the control injected embryos were successfully forming the neural tube. A more significant effect was true upon Gemin 2 depletion: 95% of embryos failed to complete gastrulation. Interestingly, these effects were ameliorated upon exogenous delivery of a completed snRNP cocktail. This striking effect in *X. laevis* embryos highlights the essential housekeeping function SMN and Gemin2 play within the cell[50].

In *X. tropicalis*, morpholinos were delivered at a dose of approximately 8pM, resulting in significantly less dramatic effects. SMN protein depletion did not occur until approximately 3 days post injection, at the tadpole stage. Morphants exhibited a short body axis phenotype at the tail bud stage at which point there were no defects in muscle patterning observed. At the tadpole stage, a much more pronounced curved body phenotype was common and this was attributed to poor muscle mass according to histological analysis. Because early muscle patterning was unaffected, the gross phenotype was hypothesized to be due to innervations defects. Indeed, researchers found defects in axonal pathfinding, outgrowth and branching. These abnormal axons seemed to make functional synapses once they reached their unnatural destination. The rate of all phenotypes was reduced upon co-injection with mRNA from *X. laevis* Smn as this transcript contains no sites for the *X. tropicalis* targeting morpholinos[104].

Xenopus egg extracts or cDNA libraries have also been used for important *in vitro* work. SMN binding interactions have been mapped in this system. This includes interactions between SMN and fibrillin[103], snRNPs [105], and snRNAs[106].

Danio rerio

While smaller organisms such as *Drosophila* and *C. elegans* are helpful for high throughput screening, many tests should be performed in more complex environments. For this purpose, the zebrafish has been an excellent vertebrate resource. Due to its small size, quick generation time, and abundance of embryos, zebrafish can be utilized for drug screening as well as targeted therapeutics. Further, one of the unique strengths of this model is the ability to observe neuronal populations *in vivo* without terminating the animal. The *D.r.Smn* sequence was characterized in 1996. The protein is 281 amino acids in length and shares 52% identity and 75% similarity with human SMN. Regions of identity can be found spanning the entire protein, including the N-terminus, Tudor domain, poly-proline region, and C-terminus[107]. Consistent with other SMN homologs, *D.r.Smn* is ubiquitously expressed[51].

This first zebrafish model for SMA was created using morpholinos to knock down SMN levels. Morpholino technology is particularly well-suited to egg-laying animal models and SMA research specifically. Eggs are collected and microinjected at the one cell stage to allow ensure the treatment is included in every cell thereafter. While each cell may contain variable amounts of morpholino, causing variation in phenotype, the large numbers of eggs produced in a short amount of time allows for a large number of replicates and therefore robust statistical analysis to tease apart these differences. Toward SMA research, morpholino knockdown more closely resembles the human condition than complete SMN knockout. This further allows for dose-dependent SMN knockdown in which one could mimic the differing severities of Type I, II, and III patients.

Zebrafish have a very stereotyped neuromuscular system. Within each spinal cord hemisegment, three motor neurons will develop within 24 hours post fertilization (hpf). Subsequently, secondary motor neuron bodies are born, extend axons, and innervate the trunk muscle segments that have already been formed. By 72hpf this process has completed and the fish is at the end of its embryonic stage.

The first SMA model in zebrafish utilized a translation-blocking morpholino to knock down *Smn*. A high dose of morpholino caused high levels of embryonic lethality before 24hpf. A lower dose still yielded high mortality (55%), and the remaining embryos were amenable to studying their developing neuromuscular phenotypes. At 24hpf, morphant fish had no fewer motor neuron bodies than control fish. However, these motor neurons and the later developing secondary motor neurons exhibited axonal defects such as shortened axons and branching defects. These neurons did reach their intended muscles, however they often didn't reach to the furthest regions within the muscle. Defects were seen in the neurons reaching the pectoral fin, homologous to the human hind limb, and the facial musculature, which is seen to be affected n severe patients. Several classes of sensory neurons investigated were not affected. These neurons were able to establish synapses on their respective muscles, however those synapses were not maintained[51]. Co-injection of the morpholino with human SMN mRNA significantly ameliorated these phenotypes, however this did not extend to alternative versions of SMN including SMNΔ7, or SMN with patient mutations A111G, Y272C and G279V. A small motif within exon 7 (QNQKE) has been shown to be important for axonal transport of SMN. When researchers co-injected morpholino with SMNΔ7-VDQNQKE, the rescue effect was restored, dependent on the first Q in the

sequence. Random amino acids added to the end did not restore rescue effect. In attempt to delineate whether these effects were due to SMN's contribution to splicing, researchers determined the ability of each construct to bind to appropriate cofactors. A construct's ability to rescue the SMA phenotype could not predict its ability to oligomerize with SMN or bind to Sm proteins. These results were inconsistent with the hypothesis that aberrant splicing could cause the axonal defects seen in zebrafish. Further, depletion of Gemin2 had highly deleterious effects on zebrafish, but axonal defects were attributed to defects in the animal's overall body structure[76].

In order to establish a zebrafish model with homogeneous phenotype and without the requirement of injecting morpholino, transgenic lines were created. These three lines were all based on point mutations: two nonsense mutations and a missense mutation homologous to a human patient mutation. Smn protein levels were low, in line with unstable protein production. The fish lived into the second week of development from contributions of maternal Smn. However, in their shortened life they were smaller and demonstrated synaptic defects, namely a decrease in the synaptic vesicle protein SV2. Upon motor neuron-specific addition of human *SMN2*, this defect was ameliorated, however overall survival was only modestly improved. This model has helped establish that SMN may have a direct or indirect role in synaptic integrity and also that SMN expression in motor neurons cannot compensate for overall defects[108].

Mus musculus

Non-mammalian models have been ideal for high throughput screening, carrying out both forward and reverse genetics, and identifying relevant pathways in SMA. However rodent models have been irreplaceable for early testing of therapeutics and have helped

researchers understand the basics of SMA biology. Indeed, therapeutic benefits demonstrated in mice have helped many strategies advance to clinical trials.

The first SMN knockout mouse revealed that complete loss of *M.m.Smn* is lethal before implantation into the uterine wall. At the time this was helpful in solidifying the case that SMN, not NAIP, was related to SMA disease pathology[109]. This further highlighted the importance of SMN in every cell, but could not represent a true model of SMA because it yielded a loss, rather than depletion of SMN. To more closely match the human genetic situation, the entire *SMN2* gene was introduced into a *M.m.Smn* knockout model. Animals that had 1-2 copies of *SMN2* integrated could be divided into two groups: most of the animals were in the early lethal group that dies within 6 hours of birth, and approximately 25% of the animals survived 6 days after birth[110]. Bridging past embryonic lethality was a great accomplishment and allowed for pathogenic analysis throughout a very rapid decline. However, only a limited number of clinically meaningful observations and even fewer therapeutic interventions would be possible within such a short timeframe. Within the same experiment, a different founder was identified which carried 8 copies of *SMN2*. Knockout pups from this line were quite healthy and lived an excess of 18 months[110, 111]. Toward the same end, another group chose to integrate a larger section around *SMN2*. Their construct included neighboring regions within human Chromosome 5q13 and resulted in a spectrum of severities within the knockout mice. The group characterized their "Type 1" mice, which died by day 10, "Type 2" died within 2-4 weeks, and "Type 3" were long lived with only a blunted tail to discriminate them from healthy mice[112]. It was seen that addition of two copies of *SMN2* rescues embryonic lethality, whereas 8 copies provides complete rescue from the SMA phenotype.

Therefore, it was hypothesized that a stepwise addition of *SMN2* copies would provide an increasingly mild phenotype, as is suggested from the human spectrum of SMA severities. In order to minimize variability with different target mutations and transgenes, researchers simply replaced the *M.m.Smn* locus with 0, 1, 2, 3, 4, 5, 6, or 8 copies of *SMN2*[113]. This series did not result in the gradual phenotype changes expected but rather in discreet phenotypic sets. 0-2 copies of *M.m.Smn* resulted in embryonic lethality, which may have been predicted, but 3 or more copies resulted in surprisingly healthy animals. Only moderate behavioral and physiological phenotypes could be observed in these animals despite only restoring SMN levels to 30-40% of wildtype levels. While this failed to meet the original objective of building a usable mouse series, some valuable points can be inferred from this data. The most intuitive lesson is that titrating levels of SMN can be difficult using transgenic means. This should be considered when making other transgenic models and also gives credence to the importance of vector-based and pharmacologically induced models for SMA. Toward therapeutics this evidence suggests that even a modest increase in SMN can yield profound results *in vivo*. This further supports therapies designed to enhance FLSMN yield from *SMN2*.

Animal models that have a transgenic copy of *SMN2* are ideal for translational work because they allow researchers to test an abundance of *SMN2* targeted therapeutics. Accordingly, the most widely used mouse model was built upon the previously discussed *SMN2*-based model. The "delta 7" model carries 2 copies of *SMN2* and many copies of the *SMNΔ7* cDNA. This model was originally developed as a means to demonstrate that the SMNΔ7 protein product is not toxic, simply insufficient[114]. While the model certainly proved this point, these animals also exhibited a very consistent SMA-like phenotype.

These animals were born asymptomatic, develop symptoms after 5-6 days, and experience a sharp decline in muscle strength before dying at 10-14 days. This disease course has allowed researchers to study and treat mice at presymptomatic, early, and late stages of severe SMA. Further, the very reproducible survival curve has been useful in teasing apart relatively subtle differences due to treatment.

While the SMNΔ7 model has been highly utilized, the field also recognized the need for a model that represents the more mild Types of SMA. This of course would be valuable toward studying Type II and Type III patients, but it would also open up options for testing more mild or non-SMN based therapeutics. Many groups are interested in repurposing drugs or developing compounds that enhance motor neuron viability, regardless of function. These drugs may be too weak to rescue a severe mouse model, but could have meaningful effects on patients who have more copies of *SMN2* to enhance or are experiencing a slower decline in motor neuron populations. Therefore, several key animals have been developed to serve as an "intermediate" model of SMA.

The SMN^{RT} model was developed building upon the SMNΔ7 model. Previous work using aminoglycosides to fornce a translational readthrough event demonstrated that the presence of an additional few amino acids onto the SMNΔ7 C-terminus could confer greater stability onto the protein. Accordingly, this amino acid sequence was cloned into the SMNΔ7 transgenic cassette and derived in the FVB/BL6 animal strain, exactly as the SMNΔ7 model. One founder line resulted in SMA mice that lived an average of 32 days and displayed "intermediate" SMA phenotypes—clearly affected compared to wildtype, but less severe than SMNΔ7 mice. Thus the "readthrough" or SMN^{RT} mouse represents an *SMN2*-based intermediate model of SMA.

Another strategy to mimic a murine SMA condition resulted in a widely used intermediate model. Within SMN exon 7 of both human and mouse SMN lie three enhancer binding regions, numbered SE1, SE2 and SE3[35]. Within enhancer region 2 lies segments A, B, and C. A potent splicing enhancer, Tra2β, binds at this site and helps mark exon 7 for splicing inclusion. In 2010 researchers made a mouse model that carries a point mutation in this position[115]. The mutation simulated the C-T effect in human SMN and a proportion of the mouse transcripts now excluded exon 7. They named the allele *SMN2*^{B} and found that the homozygous condition, *Smn*$^{2B/2B}$maintained enough SMN and was a healthy mouse. However, when crossed with a *M.mSmn*$^{+/-}$ strain, 50% of the litter that inherits the *Smn*$^{2B/-}$ alleles acquired a distinct atrophy and died at approximately 25 days. Toward the end stage, mice are much smaller than their unaffected littermates and exhibit pronounced kyphosis and tremors. While this model carries no *SMN2* and cannot be used for testing a number of SMA therapeutics, it still has been widely utilized for examining SMA pathology and testing gene replacement therapies as well as proposed genetic modifier and non-SMN targets.

Interestingly, these and other SMA models are kept on mixed genetic backgrounds. This is not the ideal situation because it increases the number of genetic variable confounding the genotype/phenotype relationship. For example, the SMNΔ7 mouse is on a mixed FVB/BL6 background and lives approximately 14 days. When bred toward a congenic FVB or BL6 background, the knockout animals are much more severe. The term “hybrid vigor” has been adopted to describe this effect in SMA mouse lines. Still, to achieve a clean genetic background researchers are working toward congenic backgrounds for the *Smn*$^{2B/2B}$ line.

A number of other murine models have been developed for SMA. Some investigators have included mutated SMN variants including patient mutations A2G and A111G into a *M.m.Smn* null model. While these can have beneficial effects in the mice that express low amounts of FLSMN from *SMN2, n*either construct was able to rescue embryonic lethality in the way that *SMN2* can[116, 117].

Several mouse lines have also been created to study conditional expression of SMN. These have been used to test the requirements of SMN in specific tissue types as well as developmental stages. The first inducible model featured CRE/*loxP* technology that allowed exon 7 to be excised upon tissue specific expression of CRE recombinase, resulting in complete SMN ablation within the cell[61, 118-121]. It is therefore not surprising that these models developed phenotypes, as SMN knockout invariably leads to apoptosis. More recently, these experiments have been conducted in mouse lines that already express *SMN2*, creating a more SMA-like environment[122-125].

Research utilizing mouse models has contributed significantly to the understanding of SMA pathogenesis and treatment. Indeed, several therapeutics have been advanced to clinical trial with efficacy data based largely on results in mice. Even upon development of larger animal models, this will continue to be a valuable model in SMA.

Sus scroffa

For translational research, it is imperative to test therapeutics in a model that mimics the human condition as closely as possible. For this reason, pig models are highly valuable. The internal anatomy of a pig is quite similar to a human and they share a high level of similarity at the genetic level as well. Longer-lived models such as the pig are also well

suited to studying the slow progression of SMA, long term effects and toxicology of treatments, and underlying physiological issues that may arise if the initial treatment of motor neurons is achieved but other tissues outgrow initial therapeutic dosages.

The porcine Smn is highly similar to human SMN. While a few amino acids are different between the two sequences, still they share 90% identity and 93% similarity. Porcine Smn runs at approximately 38kd, similar to SMN and has all of the major binding regions identified within human SMN. The protein is expressed throughout all tissues examined and is found within the cytoplasm and in nuclear gems. Further, the porcine cellular environment is amenable to splicing of human *SMN1* and *SMN2*, which is important for integrating genes that can be targeted by splicing therapeutics. Every indicator suggests that porcine SMN should act similarly to human SMN and faithfully mimic SMA[126, 127].

While an *SMN2*-based pig model is currently being developed, a transient model has been used to knock Smn levels down to SMA-like levels. Using a scAAV9 vector to deliver shRNA against porcine Smn, researchers demonstrated that this effect will faithfully mimic human SMA. These pigs exhibited overt proximal muscle weakness, loss of motor neurons and decreased functionality as measured by compound muscle action potential (CMAP) CMAP and motor unit number estimates (MUNE)[128].

2.2 Other models of SMA

Patient fibroblast cells

For years, cell cultures from patient biopsies served as the only true model of SMA because they are lacking *SMN1* and have one or more functional copies of *SMN2*. While skin cells are not considered to be affected in SMA, this ex-vivo system allowed

researchers to study the inner workings of an SMN-deficient cell as well as test the effects of therapeutics on SMN expression. Common readouts from fibroblast lines include the percentage of SMN transcripts that include exon 7 as well as RNA and protein levels. Levels of SMN can be easily assayed by counting the number of gems that fluoresce with anti-SMN antibodies.

Induced Pluripotent stem cells (iPSCs)

Programming of embryonic stem cells was introduced as recently as 1998. For the first time scientists were able to envision replacing missing or damaged cells within a patient by coaxing these stem cells down the correct developmental pathway. While this technology shows great promise, it also comes with ethical concerns regarding the use of fertilized human eggs. Fortunately, several of the most exciting features of embryonic stem cells are preserved in their successor: induced pluripotent stem cells (iPSCs). These new stem cells are adult cells, typically skin fibroblasts, that are reprogrammed by transducing them with the genes normally only found in true stem cells. Once they are undifferentiated, these cells are just like embryonic stem cells in that they can form teratomas consisting of endoderm, mesoderm and ectoderm. Using relatively simple cell culturing techniques, they can then be directed down developmental pathways toward various cell types including neurons that exhibit mature features such as neurotransmitters, transporters, and evoked action potentials. iPSCs have cells have additional advantages over embryonic stem cells due to the fact that they are derived from adult tissue: 1) generating iPSCs avoids ethical conundrums associated with embryonic work, 2) stem cells can be derived from patients with any known or unknown genetic background, and 3) toward therapeutic use, cells can be generated that genetically match the host and therefore are less likely to be rejected upon implantation[129].

As the only model that naturally has *SMN2*, SMA patient fibroblasts have long been useful in studying the disease, and they entered a new realm of technology once they were reprogrammed into iPSCs. Sets of iPSCs were made from commercially available patient fibroblasts taken from a 3 year old Type I SMA patient and his mother. Each line was able to form teratomas as expected and could then be lineage restricted while the SMA line still maintained its natural low levels of SMN expression compared to the WT line. After 4-8 weeks into differentiation, these cells exhibited motor neuron-like morphology and appropriate cell markers. In early stages of differentiation, there was no difference between the number or morphology of SMA iPS motor neurons compared to control. However, a few weeks later the SMA-derived cells underwent a selective reduction in number and size. This suggested that disease-relevant cells have no defect in motor neuron development, but rather a secondary degeneration is responsible for motor neuron defects in SMA[130, 131]. Patient-derived iPSCs have since become an established system for studying and treating SMA motor neurons with few other confounding factors[132-135].

2.3 Viral Gene Delivery

Characterization of Adeno-Associated Virus

Adeno-Associated Virus (AAV) is a small DNA virus that belongs to the family *Parvoviridae* and genus *Dependoparvovirus*[136]. All members of *Parvoviridae* are small (20-30nm), single stranded DNA viruses with an icosahedral capsid that infect vertebrate and invertebrate hosts. Each virus has a genome with limited coding capacity (~5 kb) flanked by palindromes known as inverted terminal repeats (ITRs) which serve as an origin for viral replication and encode information required for viral packaging. Due to their small genome, parvoviruses utilize complex alternative RNA processing tools to

produce multiple unique transcripts. This has allowed parvoviruses to serve as models of eukaryotic gene expression and as such they have provided insight into eukaryotic molecular mechanisms including transcription, pre-mRNA alternative splicing, and protein translation[137].

Unlike most members of *Parvoviridae,* dependoparvoviruses do not encode all the genes required for their own replication and are dependent upon co-infection by "helper" viruses. Dependoparvoviruses naturally encode only three open reading frames—one encoding replication proteins, one encoding capid proteins, and one known as the assembly-activating protein. Viral proteins VP1, VP2 and VP3 aggregate into a capsid comprised of 60 subunits with T=1 symmetry. This shell has narrow pores at its 5-fold axes that will allow newly replicated viral genomes to enter to complete the packaged virus. Viral replication is self-primed by the ITR and carried out by cellular polymerases. Viral proteins Rep78 and Rep68 provide ATP-dependent helicase activity followed by site-specific endonuclease activity at the terminal resolution site (trs). This allows for the separation of parent and daughter genomes; without nicking, replication continues to make one continuous strand of DNA. Completed genomes are unwound via the helicase action of Rep40 and Rep52 and one strand will be packaged into each capsid shell. Unlike autonomous parvoviruses, Dependoparvoviruses have homotelomeric ITRs. For this reason, there is no a 50% probability to package the plus or minus strand in the virion.

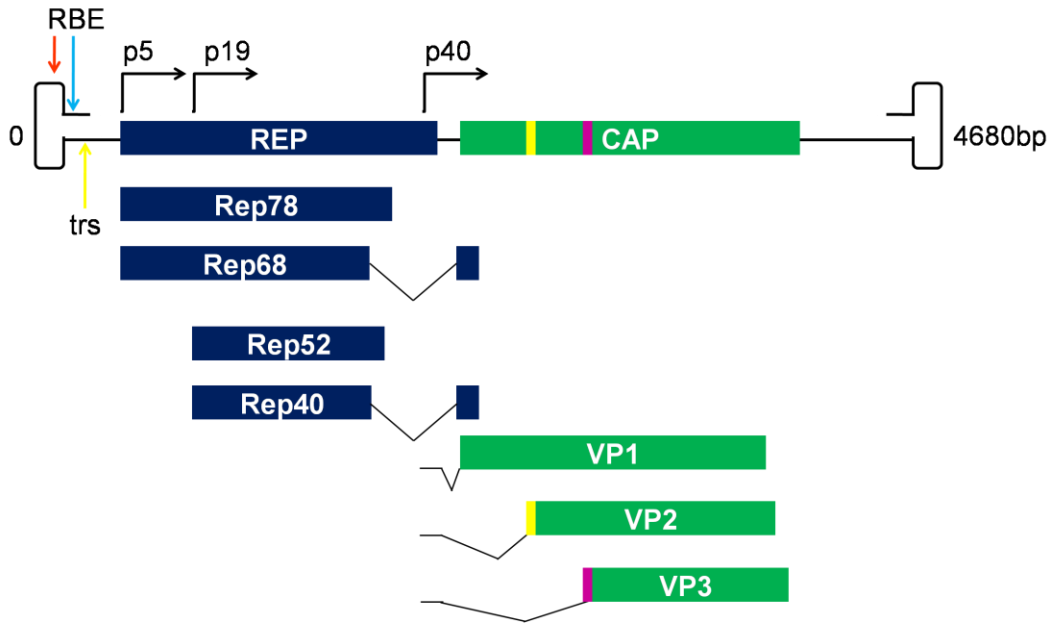


Figure 6: The AAV genome consists of two ITRs flanking rep and cap genes.

Rep genes utilize two promoters located at p5 and p19 and two splicing events to create four unique nonstructural proteins Rep78,68,52,40) named after their apparent molecular weight. One promoter at p40 drives Cap proteins VP1,2 and 3. Rep binding element (RBE) sites are shown near the 3' end of the genome. RBE indicated by the aqua arrow binds Rep68 and Rep78 while RBE indicated by the orange arrow stabilizes this binding. These proteins utilize a specific endonuclease catalytic domain in order to introduce a nick at the terminal resolution site (trs). Cap proteins are driven by a single promoter at p40. VP1 is the largest structural protein, while VP2 and VP3 utilize downstream noncanonical (yellow) and canonical (pink) start codons.

AAV Life Cycle

The most comprehensive understanding of AAV biology, including entry strategies, comes from studying AAV2. Previous studies have shown that AAVs attach to the cell surface using proteogylycan conjugates (AAV2 binds heparan sulfate proteoglycan) or O- or N-linked sugars (AAV9 binds N-linked galactose). It has been further suggested that protein receptors including fibroblast growth factor receptor-1 (FGFR1) and hepatocyte growth factor receptor (MET) act as receptors and dictate entry into the cytoplasm. However, recent data suggests that the contribution of FGFR1 and MET is modest compared to a type I transmembrane protein the authors term the "universal" AAV receptor (AAVR). This was seen to be necessary for a number of AAV serotypes including AAV9 [138]. It is established that bound AAV can be internalized in about 30-60 minutes; however current knowledge regarding the path by which AAV undergoes internalization is wrought with discrepancies. Some results suggest clatherin-dependent endocytosis, some suggest macropinocytosis, while other results challenge both of these hypotheses. These data could be confounded by the presence or absence of adenovirus in the cell culture process.

AAV intracellular transport is known to be a slow process by which AAV2 travels through endosomes, possibly through the endoplasmic reticulum (ER), and is ultimately expelled into the cytoplasm. Throughout this process, increased acidification in combination with proteolysis by endosomal enzymes induces complex conformational changes in AAV2's capsid; the N-terminal of VP1 becomes exposed and is thought to aid in AAV escape from the Golgi, Golgi-ER intermediate compartment, or (ER). This

conformational change is not conserved among all AAV serotypes and could explain differences in uncoating kinetics among different serotypes.

A body of evidence suggests that viral particles with modified capsids remain intact and travel into the nucleus. Along with allowing for endosomal escape, conformational change of the N-terminal region of VP1 exposes basic clusters thought to act as a non-classical nuclear localization signal. The full virion is imported through the nuclear pore complex and transported to the nucleolus and later into the nucleoplasm. It is here that the virus uncoats using mechanisms that appear to be cell- and serotype-specific. Like autonomous parvoviruses, it is likely that the viral genome escapes into the nucleus without complete disassembly of the viral capsid.

In the absence of helper virus infection, native AAV will enter a latent phase. This is accomplished by site-specific genome integration into a region known as AAVS1, located at Chromosome19q13.3-qter. The AAV genome integrates without directional preference and in a head-to-tail manner. This orientation was an early indicator that AAV forms a circular intermediate prior to integration[139]. Within the viral sequence, integration requires RBEs in the ITRs and Rep promoter region to act as *cis*-effectors and the largest Rep proteins to act in *trans*. AAVS1 contains the same tetranucleotide repeat that is found in the RBE of the left hand AAV ITR and is nicked at a region similar to the *trs.* Thus it seems that Rep is able to tether the viral genome and nick the AAVS1 site[140]. Successful integration allows AAV to remain dormant until active helper virus infection as well as ensuring viral DNA is replicated with cell division.

AAVS1 remains in an open chromatin conformation, presumably aiding in the integration process. This region is known to be a DNAse sensitive region and also

contains a genomic insulator that prevents heterochromatin from spreading over AAVS1 [141]. Along with making RBEs available for Rep binding and nicking, its openness to transcription factors also makes site-specific integration a tempting target for therapeutic vectors.

Latent AAV will reactivate in a cell that has experienced otherwise toxic conditions such as UV radiation, exposure to some carcinogens, or infection of a helper virus. Larger viruses such as adenovirus, herpes virus, and human papilloma virus serve as helper viruses as they provide additional factors that allow for successful AAV replication[142]. Upon activation, AAV will cause lysing the cell before these harmful viruses can replicate and spread, therefore protecting its host from potentially worse consequences[143, 144]. Further, Rep68 and Rep78 function to inhibit helper virus replication. Thus, while other parvoviruses have been associated with disease development such as the human virus B19[145], AAV has not been associated with pathogenesis in humans or animals. In contrast, AAV infection has been proposed to have a protective role in the host.

AAV as a Vector for Gene Transfer

Development of rAAV

As a non-pathogenic, replication deficient virus, early researchers could not anticipate a medical relevance for AAV research, presumably studying it out of scientific curiosity. AAV was initially discovered as a contaminant within viral preparations of Adenovirus[146, 147]. However, AAV would later become a powerful therapeutic tool. This transition occurred roughly fifteen years after the basic characterization of AAV when advancements in molecular biology allowed for the rapid exchange of genetic material

[148-150]. The process of gutting AAV is facilitated by the fact that AAV is a structurally simple virus. In the generation of recombinant vectors, the entire viral coding elements are removed, leaving only the terminal repeats. In addition to generating additional cloning space, this also reduces the possibility that viral gene products will be expressed within the vector recipient. While a variety of transgenes can be incorporated into the viral backbone, the total packaging capacity of the single-stranded recombinant AAV (rAAV) vector is only ~4.6-4.8 kb[151]. The simple nature of AAV also makes it customizable; modifying a few key residues in the AAV Cap gene can result in serotypes with novel targeting and immunogenic profiles[152].

Early researchers recognized that rAAV demonstrated long-term expression *in vitro* and *in vivo*. While some studies saw genomic integration of rAAV, this effect was predominantly seen when the cells were selected for using virally encoded antibiotic resistance. Other studies saw little to no integration in antibiotic-free conditions, and many pieces of evidence suggested that a rAAV episome was responsible for long-term expression[153]. It has since been shown that these circular intermediates arrange in a head-to-tail fashion and are dependent on viral ITRs[154]. These episomes can form as monomers or larger order concatemers[155].

The Development of scAAV

Researchers have further optimized the strengths of AAV by creating a progeny vector: self-complementary AAV (scAAV). The rationale behind scAAV came upon the realization that complementary strand synthesis is a rate-limiting step in the AAV life cycle[156]. Further, it was known that when rAAV genomes were less than half of the AAV carrying capacity, the virus would package either two monomers or one dimer[157, 158].

Accordingly, researchers aimed to design a vector that would package two shorter complementary strands of DNA rather than one longer strand, avoiding second strand synthesis. In this scenario, once the virus is unpackaged, the complementary strands will bind to each other and transcription can begin.

Understanding the mechanisms behind the AAV life cycle elucidated a clear mechanism by which scAAV could be generated. As previously described, Rep78 and Rep68 are responsible for nicking the AAV genome during genome replication. If nicking fails, replication will continue through the left ITR and onto the second strand, creating a single genome encoding both plus and minus strands. It follows that this genome will be twice as long as the original AAV, thus in order to ensure that the virus can properly package, scAAV can only hold one half of the coding capacity of single-stranded AAV. While AAV can hold up to 4.7kb, scAAV is restricted to only 2.3kb[151, 159]. Creating scAAV was technically rather simple; researchers mutated the *trs* site to prevent Rep proteins from nicking the genome[159, 160].

When directly compared, scAAV outperforms rAAV in a number of measurements. The most intuitive difference is that without waiting on second strand synthesis, scAAV can express transgenes much more quickly; rAAV takes approximately 7-10 days while protein from scAAV can be seen in 2-4 days[161]. The delay in expression seems to be more significant than can be explained by simply DNA extension. However, a combination of increased efficiencies by scAAV could better explain this effect. The transgenic genome is more efficient at circularizing, which could explain why transgene expression is more robust and sustained from an scAAV vector[160]. Both rAAV and scAAV integrate at low levels (.1-.5%), however rAAV has shown a preference for

integrating in highly transcribed regions in hepatocytes, while no such effect was seen with scAAV[162, 163].

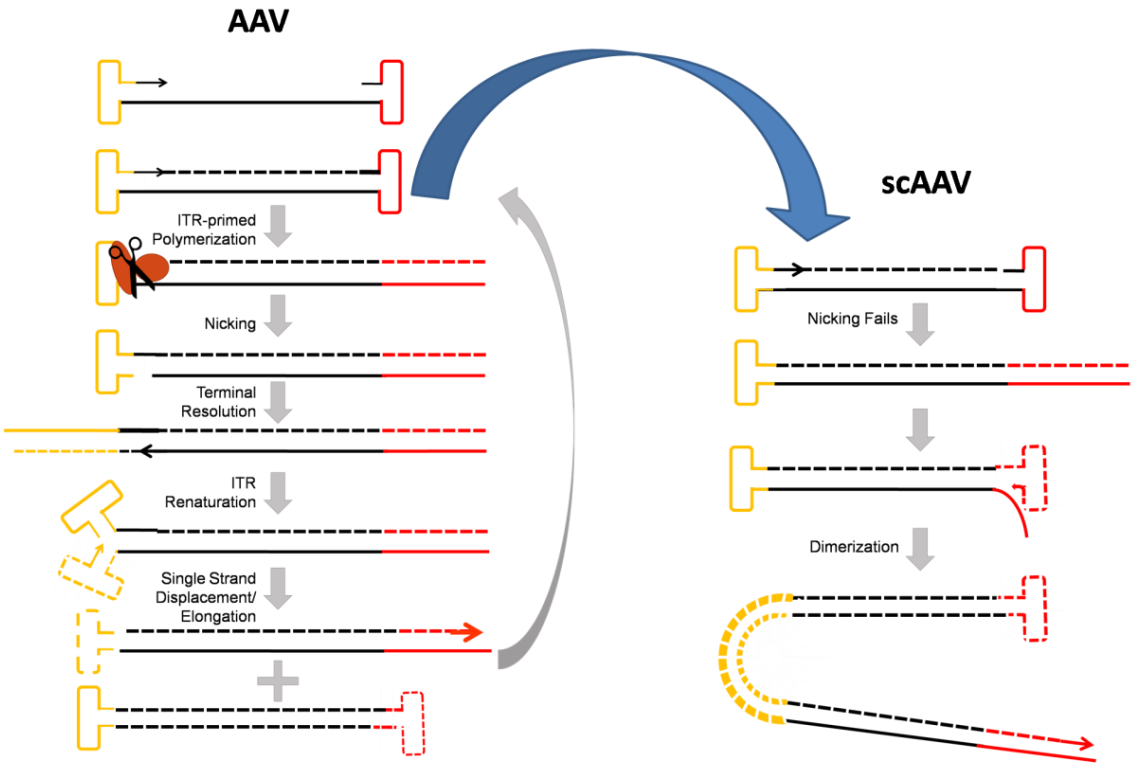


Figure 7: AAV and scAAV genomic replication.

The normal process of AAV second strand synthesis is dependent on Rep-mediated nicking activity at the *trs* site. This allows for the formation of two AAV genomes. To generate scAAV, nicking activity was abrogated which does not allow for the second strand to separate into its own copy of the genome, rather elongation continues by displacing the original strand and extending through the ITR and the remaining strand of DNA. Colored areas represent ITRS. Dashes represent newly synthesized DNA.

2.3 Early Gene Therapy in Human Trials

A robust body of work regarding safety and efficacy of rAAV in a variety of models led to groundbreaking clinical trials in the mid-1990s. The first clinical trial treated patients with Cystic Fibrosis via an aerosol delivery[164]. This was followed by a Hemophilia B trial that utilized an intramuscular (IM) delivery route[165]. Due to the high turnover rate of the airway and blood cells that these trials targeted, increase in the therapeutic gene was temporally limited. Later, the first trials in a neurodegenerative disorder, Canavan disease, delivered AAV to the CNS intracranially to target neurons more efficiently[166]. Neurons represent an ideal target because of their longevity; as long as the cells don't die the transgene will express continuously. However, this can also be a hazard in strategies where prolonged transgenic expression may interfere with the dynamic functions of brain[167]. Nevertheless, several clinical trials have taken place for diseases such as Parkinson's and Alzheimer's diseases. None of these have demonstrated adverse effects and investigation of neurotrophic factors to treat neurodegenerative diseases is still an active field[167].

Each of the therapies that advanced into clinical trials noted no adverse effects. However, follow-up studies revealed the presence of AAV neutralizing antibodies in each study, with CNS delivery yielding the fewest seropositive individuals[167]. In addition to those listed here, close to 100 clinical trials have utilized AAV technology[168]. This robust body of work has helped identify challenges and strengthen the foundation of clinical safety from which trials have begun in SMA.

2.4 SMA Gene Therapy

After the realization that viral delivery of FLSMN could be an attractive therapeutic candidate, a great deal of research went into preclinical optimization of this process.

While clinical trials will require their own degree of optimization, work in animals has already established some key requirements for the delivery of SMN. Results seen in SMA models of mice and pigs as well as non-SMA nonhuman primates are remarkably consistent, lending confidence to upcoming human trials. This process has also created an ideal system for using viral vectors to study disease pathology.

Serotype Selection

scAAV was recognized early within SMA gene therapy as a powerful vector to carry SMN. For general gene therapy, rAAV is a good choice due to its low propensity to illicit an immune response and its ability to infect non-dividing cells. For neurodegenerative diseases targeting long-lived neurons, episomes carrying the transgene are sufficient to provide long-term expression without the additional risk of widespread genomic integration that other viruses utilize. Due to the small size of the SMN coding region, it was recognized that SMA could take advantage of the additional benefits offered by self-complementary AAV.

One of the first decisions that must be made is which scAAV serotype would work best in an SMA context. There are 11 naturally occurring AAV serotypes that have been characterized and more have been established with novel binding properties through *in vitro* directed evolution[169]. As AAV2 is the most commonly studied serotype, it was early established that this vector can enter the CNS through a process of retrograde transport by entering through the distal axon projections[170, 171]. AAV8 was seen to transducer motor neurons in the lower horn at a low efficiency that was not believed to be effective within SMA, however *in vivo* experimentations within mice showed a significant improvement upon delivery of rAAV8 or scAAV8 carrying SMN [172, 173]. This

encouraging result demonstrated that a meaningful effect is possible even with a modest improvement in motor neuron numbers[173].

Thus far, the field has settled on scAAV9 as the vector of choice for SMA. Its ability to cross the blood brain barrier ensures that motor neurons can be targeted even with less-invasive peripheral delivery routes. This effect was shown in young mice, adult cats, and nonhuman primates [128, 174-177].

The use of naturally occurring viral vectors may restrict the available patient population to individuals who have not already contacted this virus and have developed neutralizing antibodies. AAV2 is the most common naturally occurring serotype and accordingly is the most likely to be targeted by the patient's immune system. Even low levels (1:200) of neutralizing antibodies is enough to abrogate delivery of the therapeutic[177]. While young patients such as SMA Type I infants may be less likely to have encountered AAV, all sources of antibodies including mother's breast milk need to be considered. Fortunately, temporary immune suppression to allow transgene delivery is a relatively straight-forward component of gene therapy.

Transgene Expression

Fortunately, SMN is thought of as a safe transgene. Because all patients express low levels of SMN, exogenous protein should not be recognized as a foreign antigen. Further, high levels of SMN are not considered deleterious, and as the protein is ubiquitously expressed, it is not expected to be toxic to any tissue type[178]. Accordingly, SMN vectors are designed to express at high levels in all cell types transduced. The promoter widely accepted for this means is the Chicken β-Actin promoter (CBA)[179]. This chimeric promoter is actually a basal β-Actin promoter with a cytomegalovirus (CMV) immediate

enhancer region. For expression of SMN, it was found that a codon-optimized version of SMN outperformed the native human sequence in animal models[180]. Overall it is expected that robust expression will translate to the maximal benefit in patients.

Route of Delivery

Determining the route of delivery to be used in patients is a balance between the effectiveness and tissue targets reached by each therapy as well as the invasiveness of the injection. Fortunately, viral based gene therapy is designed to be a one-time occurrence, making the invasiveness of the delivery a relatively short-term hurdle. It is notable that means of injection are different between mice models and larger mammals; in young mice delivery into the CNS is through the skull, whereas in larger mammals this is often achieved through the spinal cord. Despite these differences, work in mice has been repeatedly validated in larger mammals, demonstrating how such results should translate to meaningful guidelines for human application.

Intramuscular Delivery

Intramuscular (IM) delivery has been successful in other diseases and is the delivery method used in the first approved gene therapy in Europe. Given the extreme muscle weakness in SMA as well as the observation that scAAV9 could cross the BBB to reach the CNS through peripheral delivery, this could be an attractive option for SMA. The process of retrograde transport is highly important for IM delivery to successfully transducer the CNS, so researchers have investigated AAV as well as other viruses toward this end. AAV has been shown to target the CNS in adults and neonates [181, 182]. It was determined that this was through retrograde transport as the effect can be blocked upon the chemical axonal interference[183]. Motor neurons transduced by this means are shown to be sufficient to provide a therapeutic benefit[170]. However, it is of note that one

study found a portion of the viral particles within the circulatory system, suggesting that the efficiency of transduction through retrograde transport may be skewed by circulating viruses that further promote viral spread[182].

Intravenous Delivery

Intravenous (IV) delivery offers a relatively non-invasive means of delivering therapeutics. Further, accessing the circulatory system allows for widespread delivery. While circulating viruses are usually kept out of the CNS via the blood brain barrier, use of scAAV9 overcomes this hurdle. Transduction of the CNS through this means has been validated in multiple animal models[101, 128, 180, 184, 185]. While motor neurons are considered a highly important therapeutic target, it is established that other organs are vulnerable in SMA[186-188]. Accordingly, using antisense oligonucleotides it has been shown that delivery to the periphery alongside delivery to the CNS delays peripheral defects including distal necrosis compared to CNS delivery alone[189]. The motor neuron defects in SMA are likely so prominent that they present earlier and more severely than defects in other tissue types in patients. Upon overcoming the initial lethality associated with gross motor neuron death, other issues may occur. As SMN is a ubiquitous protein, restoring SMN in a fashion that is as ubiquitous as possible may be important in SMA.

Delivery into the CNS

The most direct route to transducing motor neurons is to introduce therapeutic into the CNS. In mice, this is accomplished by delivering a bolus of therapeutic into the brain using a pulled capillary needle[190]. Known as intracerebroventricular (ICV) delivery, this method targets the ventricular system within the brain, aiming to circulate therapeutic through the cerebrospinal fluid. The soft neonatal skull is easy to penetrate with small needles however this type of delivery is much more invasive once the skull ossifies: holes

must be drilled through the hardened skull. Other entry points for delivery into the CNS are used in larger animals including the cistern magna or intrathecal injection into the spinal cord[191].

Because the blood brain barrier separates the CNS environment from objects floating in the circulatory system, delivery into the CNS is expected to be hidden from the immune system. However, work in nonhuman primates demonstrated that this is not true in practice. Rather, vectors are still susceptible to circulating antibodies and the capsid antigen is still capable of eliciting an immune response[192].

While delivery into the CNS is recognized to efficiently target motor neurons, its benefit could extend to other cell types as well. Recently, the impact of other cells within the CNS have garnered attention of SMA researchers. It has been established that pan-neuronal restoration of SMN is beneficial while restoration within motor neurons alone yields little improvement[122 193-197]. Further, depletion of SMN only in MNs has only a mild deleterious effect[198]. This suggests that other neuronal cell types impact motor neurons within SMA. Myelinating Schwann cells have been found to have intrinsic defects and selective restoration of SMN to these cells improves neuromuscular junction defects[199, 200]. Astrocytes have also been implicated in SMA and selective delivery of SMN to astrocytes provides phenotypic improvement including preservation of motor neurons and life extension *in vivo*[134].Whether SMA is caused by a cell autonomous defect in motor neurons, or whether MN death is caused by a combination of internal defects and/or improper signals from other cell types, delivery into the CNS will be the most efficient means of reaching these important cell types.

Objective Comparison of Delivery Methods

To meaningfully impact the pathology associated with SMA, targeting motor neurons is considered essential. Studies within various SMA animal models indicate that not all motor neurons must be targeted to impact SMA, rather a minimum of 20%-30% of motor neurons transduced can produce a meaningful phenotypic improvement[128, 185, 191]. While both IV and CNS delivery are capable of transducing sufficient quantities of motor neurons, ICV is clearly a more efficient route to accomplish this. Accordingly, suboptimal doses of scAAV delivering SMN results in a significant improvement in survival upon delivery to the CNS compared to IV delivery[184, 191].

An additional benefit that comes with the efficiency of CNS delivery is a decreased dosage required to provide meaningful benefit. Work in NHPs has demonstrated that CNS delivery requires 10x less viral particles to promote the same level of motor neuron transduction[185]. When scaled down to dosages appropriate for an SMA mouse model, this lower dosage delivered ICV provided an indistinguishable rescue effect to the higher IV dosage[185].

Lower dosages are beneficial in a medical sense because fewer viral particles leads to fewer chances for transgene integration. While rAAV integrates only at a low rate, even a small percentage of large doses (~10^{12}viral particles) provides many integration opportunities. While naturally occurring AAV integrates in a site-specific method, removing Rep genes in the gutting process strips rAAV constructs of this safe integration mechanism. From an industrial view, lower dosages are beneficial as well. While scientists are always working on improving the process, large-scale production of rAAV is still an industrial challenge. Efficient treatment utilizing dramatically lower

doses allows for the possibility of treating more patients as well as the ability to properly dose older patients[185].

The first round of viral-based SMA clinical trials is approved to utilize an IV route of delivery. As of this writing, early results indicate improvements in treated patients[201]. No other trials to date have reported such robust improvement in the most severe population of SMA patients. It is anticipated that the company will seek approval to deliver into the CNS using data accumulated from preclinical work as well as this initial clinical trial.

Pre-clinical Optimization of Delivery Techniques

Route of Delivery in clinical application

Recent work has aimed specifically at optimizing the spread of viral particles in nonhuman primates for the purposes of informing envisioned delivery into patients. As there is no NHP model of SMA, these studies utilize scAAV9-CB-GFP in order to visualize the cell types transduced by different methods. The previously described work comparing CNS vs IV delivery utilized similar methods to demonstrate the number of motor neuron transduced by each delivery. More recent publications focused on different locations of CNS delivery. Interestingly, it was found that regardless of which region of the spinal cord received the injection, the highest levels of transduction were found in the lumbar region. However, SMA patients often die due to dysfunction of motor neurons in the upper spinal cord that control respiration. In order to enhance transduction in the upper levels of the spinal cord, researchers reclined the subjects in the Trendelenburg position for 5-10 mintues following the procedure. This is a simple technique where the table is put at a negative incline such that the feet are 15-30 degrees above the head[185].

Indeed, the effects of gravity promoted viral spread such that 55% of motor neurons were transduced in the cerebral spinal cord. These studies also demonstrated that scAAV9 is able to leave the CNS; GFP expression was found in peripheral muscles and organs[185].

Timing of Delivery in clinical application

Pre-clinical results have repeatedly found that early, preferably presymptomatic, delivery is most effective at rescuing the disease[101, 174, 176, 195, 202-204]. Fortunately, it has also been shown that delivery after symptom onset still provides some benefit[202, 205]. This has proven sound in human SMA patients as well. Early results from the clinical trial designed by AveXis show that patients with Type I SMA have gained strength and are living longer than the SMA natural history would predict[201]. Some patients were dosed at 6 months, after SMA symptoms have onset, while others were injected at earlier timepoints. All patients are showing improvement, and those patients who were dosed at higher levels and at earlier times seem to be gaining the most strength[206]

2.4 Viral Delivery to Probe SMA Biology

The use of gene therapy techniques to study the pathology of SMA is well-established. Viral vectors such as AAV and scAAV have deomonstrated wide transduction and long-term transgene expression making this an attractive alternative to the long, expensive, and labor intensive process of creating transgenic models. Using this method, researchers can probe the effects of multiple genes in a relatively short amount of time and can further test them in multiple animal models with no further processing required. Because FLSMN transduced into animal models offers a remarkable rescue of the SMA phenotype, we know that less effective genes could still offer a measurable improvement. Currently the results from scAAV9 delivery of FLSMN demonstrate that patients are

gaining strength and living longer. These results mimic those seen in mice as well as pigs, and serve as an indication that this form of delivery is truly clinically relevant.

scAAV delivery has previously been used to assess the spatial requirements of SMN *in vivo*. One of the most highly debated issues in the SMA field asks whether the defects seen in motor neurons are a result of intrinsic features of SMN depletion within the motor neuron, or whether other cells are responsible for making motor neurons sick. Previous experiments demonstrated that restoration of SMN to motor neurons was not sufficient to rescue the SMA phenotype, but that pan-neuronal expression showed a beneficial effect. Thus it appears that other types of neurons are partially responsible for the MN loss in SMA. To address this question, Rindt *et al.* used a scAAV9 vector to deliver SMN under cell specific promoters. It was found that FLSMN driven by the astrocyte-specific GFAP promoter extended lifespan, weight, and strength of a mouse model of SMA. In conjunction with other results *in vitro* and *in vivo*, the scAAV vector was able to establish a non-motor neuron requirement for SMN[134].

Temporal requirements for SMN have also been addressed using scAAV9. Previously it had been suggested that "earlier is better" for SMA therapeutics. Indeed, earlier and even pre-symptomatic treatments outperform late treatments. Recently, scAAV9 has been used to define the therapeutic window of opportunity within the most thoroughly characterized SMA model—the SMNΔ7 mouse. A step-wise delay in treatment was achieved by delivering SMN driven by a ubiquitous promoter as a single injection on either day 1 through day 8 after birth. Using this method it was confirmed that early is better, however it was also noted that the late delivery on day 8 still had a positive effect for those mice[202]. Viral delivery within the SMNΔ7 proved to be a

relatively easy assay that was able to show even small differences between treatment groups.

3. SMA Clinical Trials

This is an exciting time to be active in the field of SMA due to several promising clinical trials currently underway as even more progressing through the clinical "pipeline." These clinical trials are a symbol of the scientific field committed to SMA as well as the strong and supportive patient community. Due to the high costs of research and relatively small patient populations, disease research is often not pursued as aggressively by large corporations concerned about generating large profits. This type of research then is left largely to academic researchers and in the case of SMA is partially funded by generous nonprofit patient organizations. Indeed, the United States Food and Drug Administration (FDA) and European Medicines Agency (EMA) recognize the unmet demand in rare disease treatments and have developed special programs within the clinical trial process to accelerate the course for qualifying applications[207]. While not all SMA trials qualify for such programs, this illustrates the urgency of developing treatments for Spinal Muscular Atrophy.

Due to the unique genetic cause of SMA, aiming to ameliorate symptoms can take a number of different forms. As SMA can broadly be defined as a neurodegenerative disease and a muscular dystrophy, some drugs are not specific to SMA and aim to protect motor neuron or muscle from dying. As SMA is a monogenic disease, replacing *SMN1* should address the molecular cause of the disease and halt disease progression. Further, *SMN2* serves as a very attractive therapeutic target because it is present in all patients, most often in multiple copies. We know that *SMN2* produces low levels of FLSMN and

high levels of SMNΔ7. The latter is not toxic, and may not be wholly dysfunctional but rather is considered unstable[178]. Therefore to amplify the amount of FLSMN produced therapies could increase promoter activity of *SMN2*, attempt to stabilize FLSMN and/or SMNΔ7, or change the splicing patterns of *SMN2* to include exon 7.

3.1 Ionis/Biogen Nusinersen (formerly Ionis-SMNRx)

The drug that is furthest along in the pipeline toward FDA approval is a genetic modifier of *SMN2* splicing. This strategy is a particularly attractive option as altering *SMN2* splicing does not change the expression pattern of SMN spatially or temporally. While SMN products have never been considered toxic, this adds an additional layer of safety when considering human patients.

This drug is an antisense oligonucleotide (ASO). As the name implies, these are short sequences of nucleic acids or modified nucleic acids that bind to the sense strand of mRNA with high specificity. ASOs designed for SMA must be based on several criteria. These include the ability to efficiently increase exon 7 retention, low toxicity with no off-target effects, long-lasting resistance to cellular degradation, ability to bind to their target with high affinity, and the ability to permeate cells within the CNS. The ASOs utilized in this therapeutic are designed to block intronic splicing silencer N1 (ISS-N1) which has been shown to be a potent negative regulator of exon 7 inclusion. The 2'-O-methoxyethyl (2OMOE) backbone chosen for this sequence makes them more stable than natural RNA sequences. This technology has been highly successful in preclinical applications. A single administration into the CNS extended the lifespan of the SMNΔ7 mouse from ~15 days to >100 days. This increased splicing inclusion of exon 7 within the CNS, however

this effect diminished over time. Accordingly, the trials being conducted by Ionis/Biogen currently utilize an intrathecal delivery method with injections repeated.

This group is simultaneously running several clinical trials testing the same drug. The two Phase 3 trials are called CHERISH and ENDEAR. These trials are both randomized, placebo controlled studies targeting patients with later onset (2-12 years) SMA and infantile SMA accordingly. These trials have almost completed enrollment and primary outcomes are scheduled to be evaluated in May 2017.

Two additional trials are underway called NURTURE and EMBRACE. The former is designed to treat patients genetically identified with SMA before symptom onset, and the latter aims to treat infant-onset and later-onset patients who do not meet the requirements for the CHERISH or ENDEAR trials. NURTURE tests in humans the preclinical notion that presymptomatic treatment yields optimal improvements.

3.2 AveXis-ChariSMA

This trial is based on the system of using scAAV9 to deliver FLSMN as discussed previously. A novel company, AveXis, formed to perform this trial lead by Dr. Jerry Mendell at Nationwide Children's Hospital in Columbus, Ohio. The first trial utilizing this therapy is performed in infants with genetically confirmed Type I SMA. This is a open-label dose escalation Phase 1/2 trial utilizing an IV route of delivery. The trial began in 2014 and as of early 2016 enrollment was completed. In April 2016 the company released data on patient survival and strength as of Dec 31, 2015. Results indicate that patients are benefiting from measurably improved strength and reaching new developmental milestones. Improved strength may be having positive effects on the child's respiration and nutrition, which further promote survival. Currently no "events"

have occurred, meaning no patients have died or needed aggressive respiratory aid. These results are all in contrast to the natural history of untreated SMA Type I patients, who typically lose strength, lose milestones they had achieved, and only 5% of whom are "event free" at two years of age.

The company aims to initiate additional clinical trials. While this trial tests IV route of delivery, trials designed to test intrathecal delivery are also envisioned. Delivery into the CNS has been preclinically shown to be more effective and require less dosage. Further, AveXis has started European subsidiaries, but no announcements have yet been made regarding trials to initiate trials to seek approval in the European Union[208].

3.3 Roche/PTC/SMAF: RG7800

Another splicing regulator has advanced into Phase 1b/2a clinical trials, but is currently suspended. The trial, called MOONFISH, was designed to assess in adults and pediatric SMA patients the safety, tolerability, pharmacokinetics and pharmacodynamics of the drug RG7800. The trial was suspended in April 2015 when a long-term safety study in animals found an "unexpected eye finding." Patients who were dosed prior to the trial's suspension have thus far demonstrated no safety issues. The drug is an orally available small molecule designed to increase fidelity of *SMN2* exon 7 splicing inclusion.

3.4 Novartis-- LMI070

This small molecule has been recognized to enhance exon 7 splicing from *SMN2*. It is orally available and currently in Phase 1 open-label first-in-human trial in Type I patients with the possibility of dose escalation.

3.5 Repligin/Phizer—Quinazoline

A large patient organization, CureSMA initiated the first drug discovery program for SMA and directed the preclinical testing of RG3039. The drug belongs to a group of quinazolines which bind to the SMN promoter and enhance its activity. Drug development was subsequently licenced to Repligen in 2009 and Phizer joined in collaboration in 2012. This relationship terminated and as of 2016 there are no plans for future development between Phizer and Repligen.

3.6 Cytokinetics/Astellas – CK-2127107

Another non-specific treatment for SMA targets skeletal muscle and not levels of SMN. This troponin activator is dosed orally and has been studied for safety, tolerability, bioavailability, pharacodynamecs in previous Phase 1 clinical trials in healthy volunteers. A double-blind, randomized, placebo-controlled Phase II trial in SMA patients designed to test efficacy is currently underway.

3.7Genentech/Roche—Olesoxime (TRO19622)

Olesoxime is one of few drugs within clinical trials for SMA that targets motor neurons in an SMN-independent fashion. This compound is considered a neuroprotectant as it is active in the mitochondria and offsets effects normally caused by oxidative stress. As an orally available compound that can permeate the blood brain barrier, this is an attractive candidate for treating neurodegenerative diseases. While this compound showed efficacy *in vitro* and *in vivo*, a previous clinical trial failed to ameliorate muscle degeneration in patients with Amyotrophil Lateral Sclerosis. Formally run by Trophos, Roche has recently undertaken direction of production and future development of the compound.

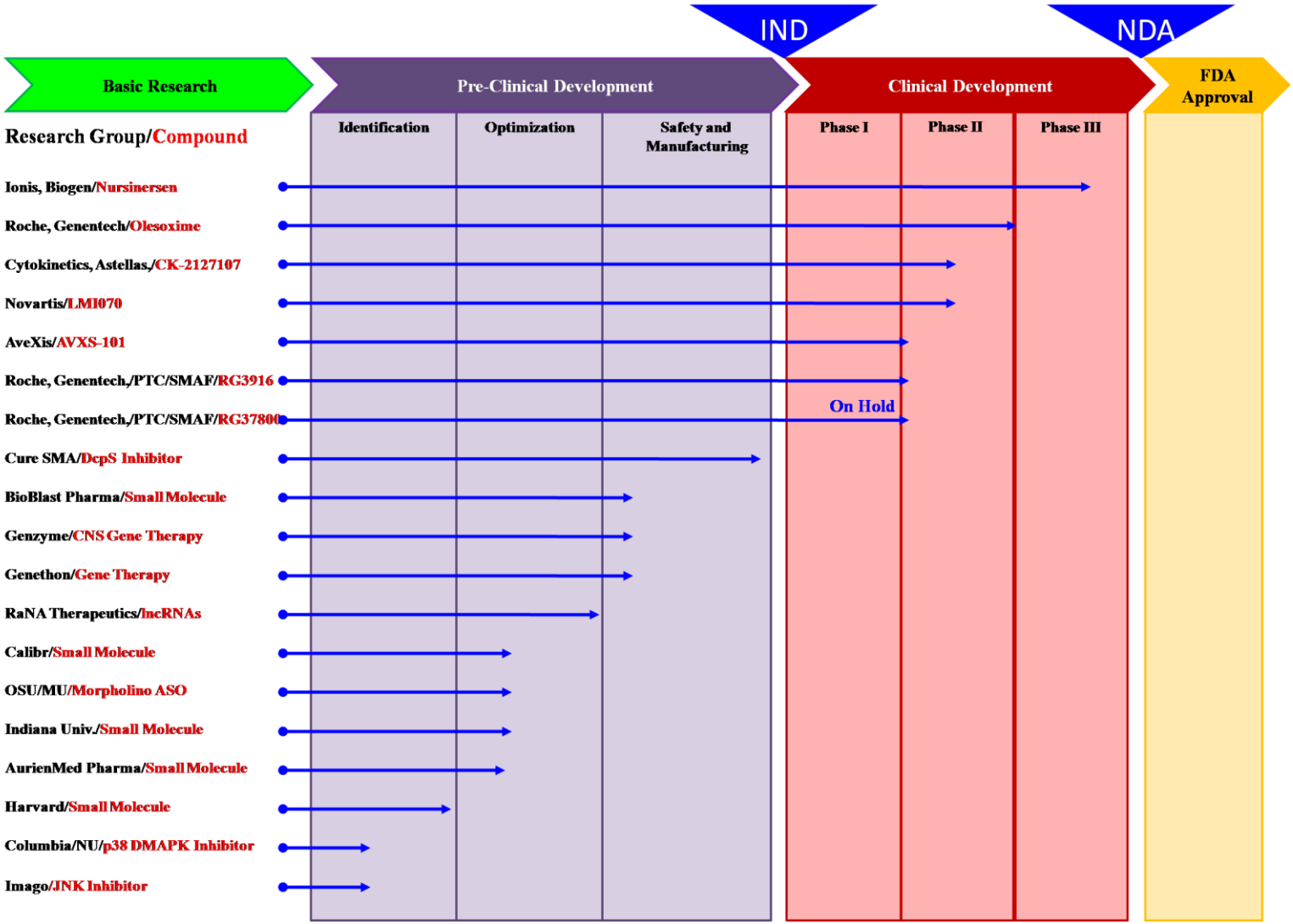

IND
NDA
Basic Research
Pre-Clinical Development
Clinical Development
FDA Approval
Identification
Optimization
Safety and Manufacturing
Phase I
Phase II
Phase III
Research Group/Compound
Ionis, Biogen/Nursinersen
Roche, Genentech/Olesoxime
Cytokinetics, Astellas,/CK-2127107
Novartis/LMI070
AveXis/AVXS-101
Roche, Genentech,/PTC/SMAF/RG3916
Roche, Genentech,/PTC/SMAF/RG37800
On Hold
Cure SMA/DcpS Inhibitor
BioBlast Pharma/Small Molecule
Genzyme/CNS Gene Therapy
Genethon/Gene Therapy
RaNA Therapeutics/lncRNAs
Calibr/Small Molecule
OSU/MU/Morpholino ASO
Indiana Univ./Small Molecule
AurienMed Pharma/Small Molecule
Harvard/Small Molecule
Columbia/NU/p38 DMAPK Inhibitor
Imago/JNK Inhibitor

Figure 8: Drug progress through clinical trials.

There is no approved therapy for SMA but many drugs are advancing through preclinical development and into clinical trials. Before entering clinical trials drug developers must seek Investigational New Drug (IND) approval to test in humans. Upon completion of clinical trials, the New Drug Application must be approved before the product can be commercially offered.

Based on data from CureSMA.org

Chapter Two: The Search for an Evolutionary-Guided Minimal Domain to Identify the Disease-Relevant Regions within SMN

To be published as: Miller MR, Ross JE, Villalon E, Pelizzoni L, Ebert AE, Garcia M, and Lorson CL.

1: Introduction

SMN's function toward motor neuron disease

Spinal Muscular Atrophy is ultimately caused by deletion or mutation of the *SMN1* gene which is only partially ameliorated by the presence of one or more copies of the *SMN2* gene. The key difference between these genes is their propensity to promote differently spliced transcripts: *SMN1* produces 100% full length transcripts resulting in stable SMN protein whereas *SMN2* is spliced such that 90% of transcripts lack exon 7, resulting in the unstable SMNΔ7 protein. SMN protein is ubiquitously expressed and its knockout results in early embryonic lethality in every model tested. The 10% of full length transcripts and accordingly low levels of SMN produced by each copy of *SMN2* are the only means by which SMA patients are able survive past this early lethality. It is established that motor neurons are especially susceptible to low levels of SMN, however the mechanism behind this effect has not reached consensus. Multiple SMN functions have been proposed and within each there is means by which a ubiquitously expressed protein can be responsible for targeted motor neuron loss. It is of great interest within the field to establish which functions of SMN are dysregulated in SMA, however delineating between these functions is quite challenging.

The canonical function of SMN toward snRNP biogenesis is considered important within every cell. In this role, SMN works in combination with the SMN complex of Gemins 2-8 and UNRIP to assemble Sm proteins and allow the heptameric ring of Sm proteins to bind a U snRNA. Once completed, these snRNPs will work in combination to carry out the process of pre-mRNA splicing. Similar processes require SMN in order to assemble snoRNPs as well as other RNP-based machinery. These processes are all considered essential housekeeping functions of SMN. While these functions are essential to life even at a single-cellular level, their function toward the motor-neuron defects seen in SMA are disputed. Dysfunction within this role is suggested to affect motor neurons in an indirect way; improper RNP formation could lead to dysregulation or malformation of downstream products such as mRNA or protein that is disproportionately important in motor neurons. To successfully carry out such functions, SMN would minimally require exons 2b and 6 in order to oligomerize as well as exon 2a to bind Gemin2 and the Tudor domain within exon 3 to bind Sm proteins. Homologous regions to exons 2, 3 and 6 are present in almost all homologs of SMN.

SMN has demonstrated an ability to perform other functions, such as roles in actin dynamics within the CNS. Through interactions with Profilin, SMN is able to promote the polymerization of actin and thereby aid in axonal outbrowth. SMN binds to mRNA binding proteins ZBP, hnRNPQ and hnRNPR and in turn these RBPs bind β-actin mRNA. This complex can be seen fast-trafficking across axons to promote the distal translation of these valuable mRNAs. Yet despite this evidence demonstrating a clear link between SMN and neuronal morphology, evidence is not conclusive that loss of this

function can result in dramatic effects in the motor neuron. For this process, SMN needs its central poly-proline region found in exons 4, 5, and 6.

The field of SMA is in continuous debate as to which role of SMN is responsible for SMA pathology. It is very possible that motor neuron loss occurs from a combinatorial effect of several functions disrupted by the depletion of SMN.

1.1: Domains of SMN

One means of considering the function of SMN is by understanding its structure. To this end, many studies have helped develop a picture of SMN with increased resolution. The domains within SMN have been characterized to various extents based on studies of deletions and patient mutations. Further, evolutionary comparison of these regions provides insights as to which functions were maintained or gained over time. While SMN is conserved in distant organisms such as *S. pombe* and *C. elegans,* not all domains within human SMN are found in such distant species. Starting from the SMN N-terminus we will discuss the features of each domain.

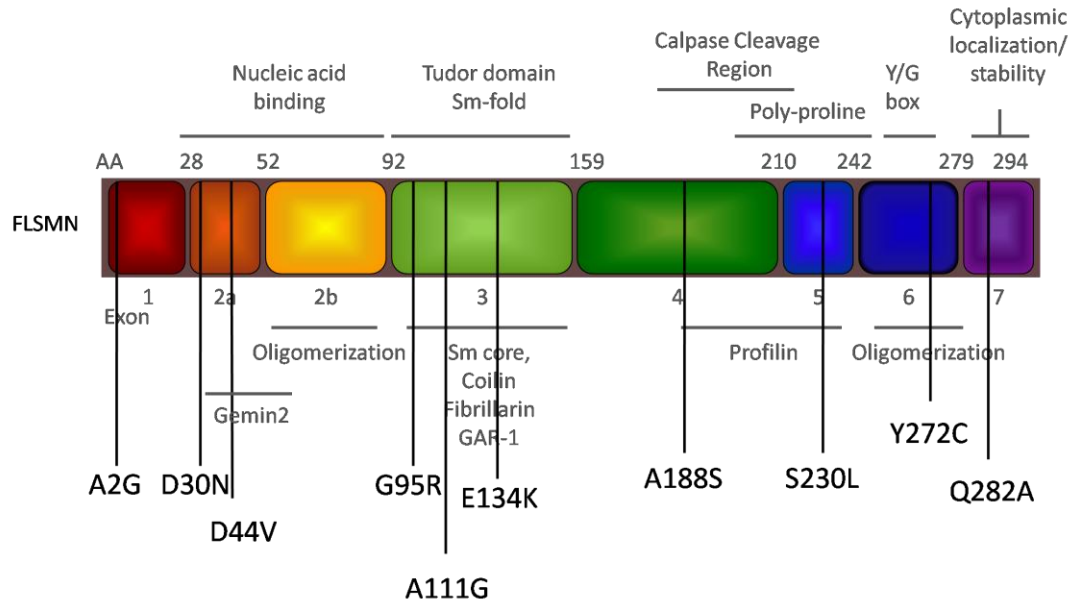


Figure 9: Patient mutations spanning FLSMN.

Patient mutations identified from compound heterozygous patients carrying *SMN1* deletion and one allele with a mutation in *SMN1*. Studies on SMN function disruption by SMA patient mutations are described within the text.

Modified from Coady and Lorson, 2011[36]

Mutation	Exon	*SMN2* Copy #	SMA Type	Ability to assemble snRNP	References
A2G	**1**	**1**	**III**	**++**	116 47, 209
D30N	**2a**	**2**	**II**	**+++**	48, 210
D44V	**2a**	**1**	**III**	**++**	48, 211
G95R	**3**	**1**	**III**	**ND**	48
A111G	**3**	**1**	**Ib**	**+++**	117 210 48 76
E134K	**3**	**2**	**I**	**+**	48, 210 212
A188S	**4**	**ND**	**ND**	**ND**	213
S230L	**5**	**2**	**II**	**ND**	214
Y272C	**6**	**2**	**II**	**+**	76, 210, 215, 216

Table 1: Patient mutations spanning FLSMN have variable effects on symptom severity.

Patient mutation described within the text are listed along with the severity of the patient in which each mutation was identified. Together with the copy number of *SMN2*, the mutation within SMN influences how non-functional SMN is *in vivo*.

Table adapted from Burghes and Beattie 2009[217]

N-Terminus/Exon 1

This region within SMN is not well defined based on an established secondary structure or function. Unlike the SMN C-terminus, the function of SMN does not seem to be disturbed by binding fusion proteins to the N-terminus. This region's importance can be noted due to the finding that the a patient mutation, A2G. This mutation was identified in an SMA patient who displayed the milder Type III of SMA despite having only 1 copy of *SMN2,* thus implying that the mutated protein maintained some function[209 Prior, Am J Hum Genet 1998]. When modeled on a *M.m.Smn* null background, the mutation was not able to prevent embryonic lethality. However, when modeled in a mouse that carries *SMN2,* the transgene was able to rescue the SMA phenotype such that the mouse symptoms were very mild[116, 218].

Deletion studies show that the N-terminal 27 amino acids (all of exon 1) are required for function. Such deletion does not shorten the half-life of SMN[44] but it abrogates SMN function. Addition of SMN lacking these 27 residues causes a dominant negative effect due to mislocalization of snRNPs[219]. SMN variants with this deletion cause the apparition of large aggregates within the nucleus which contain high levels of symmetrically dimethylated arginine proteins, likely coilin[219, 220]. When the entirety of exon 1 is deleted and expressed in NSC-34 cells, SMN localizes into large puncta within the cytoplasm extending throughout the cell body and neurite[221 Androphy, 2013]. Part of this effect is likely due to the clustering of phosphorylation sites in the SMN N-terminus. Phosphorylation has been demonstrated to alter SMN location as well as function and stability which will be discussed shortly. In contrast, wider deletion of the N-terminus (defined in this experiment as exons 1, 2a, and 2b) caused SMN's accumulation in the nucleus within COS-7 cells and fibroblasts derived from a Type I SMA patient[222]. Thus,

it appears that the N-terminus has the potential to change SMN localization. Evolutionarily, this region is poorly conserved. No sequence aligning to exon 1 is found in yeast or *C. elegans*. Within vertebrate species *H. sapiens, M. musculus, D. rerio,* and *X. laevis,* a few residues are conserved toward the end of exon 1. These residues—LFRRG—have not been characterized.

Exon 2a

SMN's ability to bind to nucleic acid has been mapped to a region spanning exons 2a and 2b[36, 107, 223, 224]. Exon 2a encodes the region within SMN which has been shown to bind to Gemin 2[211, 225]. The interaction between SMN residues 21-56 and Gemin 2 residues 95-280 has been characterized using NMR spectroscopy and demonstrates that an amphiphatic helix within SMN fits into a structured groove within Gemin2[226].

Similarly to the SMN N-terminus, deletion of exon 2a resulted in nuclear accumulation of SMN particles in COS-7 cells and fibroblasts derived from a Type I SMA patient[222]. This region is highly conserved throughout most of evolution; this region shares 100% homology with *D.r.Smn,* comes directly from exon 1 of *C.e.Smn* and can also be found in *S. pombe*[107].

Despite its high evolutionary conservation, mutations in this region are relatively well tolerated. D30N and D44V both lie within this region, the latter of which has been found in a Type III patient with 1 copy of *SMN2.* Both strongly bind to FLSMN and are able to promote snRNP assembly[217].

Exon 2b

While the region encoded by exon 2a is primarily responsible for binding RNA, domains encoded by exon 2b and exon 3 are thought to aid in this process[107]. This region

cooperates with other SMN domains as well. Exon 2b encodes a lysine-rich region that resembles the nuclear localization sequence within Coilin. This region is also known to interact with SMN exon 4, and it has been hypothesized that this interaction results in the masking of the nuclear localization sequence within exon 2b[224, 227]. Along with exon 6, a self-association domain has been mapped to residues 52-91 encoded by exon 2b[224, 228 Young PJ., 2006]. As oligomerization is necessary for SMN stability, deletion of SMN exon 2(a &b) moderately decreases the half-life of SMN from ~4.35 hours to 3.1 hours[44].

Without this exon, SMN fails to rescue neurite outgrowth in NSC34 cultures and exhibits diffuse staining throughout the nucleus and cytoplasm[229]. Upon further dissection, deletion of only the first 8 amino acids from exon 2b rescues this phenotype, whereas deletion of a central portion of exon 2b was unable to to rescue. The authors attribute this to the loss of lysines 65-76[229].

SMN exon 2b is significantly less conserved than exon 2a; Human *SMN* and *D.r.Smn* share 38% homology in this region[107]. While homologs of SMN are seen to self-oligomerize, and sometimes oligomerize with human SMN this is accomplished though the C-terminal Y/G box.

Exon 3

Residues 92-144, found within exon 3, comprise a region known as the Tudor domain. SMN was identified as having this region of homology with other proteins involved in RNA interaction in *Drosophila*, however the general function of tudor domains is not established[230]. The Tudor domain mediates SMN interaction with arginine-glycine (RG) motifs in different proteins, including Sm proteins, GAR1, and Fibrillin[103, 231]. The latter two interactions imply that SMN may be involved with small nucleolar RNP processes.

Together with the lysine-rich region found in exon 2a and the YG box found in exon 6, the Tudor domain helps SMN interact with coilin and accumulate in Cajal Bodies[232].

Altering exon 3 has meaningful effect on SMN function. SMN lacking exon 3 showed diffuse localization within the nucleus and cytoplasm and was further unable to rescue neurite outgrowth in NSC34 cells[221]. Further, patient mutations including G95R and A111G are found within exon 3. These mutations do not impact self-oligomerization, but have been demonstrated to decrease SMN's ability to bind to SmB and SmD1[48]. Despite this finding, A111G was found to be capable of complementing low levels of FLSMN in order to restore snRNP assembly and viability in an SMA mouse model[117]. Further, when expressed at high levels, A111G has been found to rescue the axonal phenotype seen in Smn knockdown zebrafish and transgenic SMA mice. This effect was only seen in the presence of FLSMN. While these patient mutations may have an effect on Sm binding, much stronger interactions are found within the C-terminal half of the Tudor domain (residues 120-144)[212]. For this reason, patient mutation E134K has been seen to completely abrogate SMN binding with Sm B and Sm D1 without affecting SMN cellular localization or its ability to bind to itself or to Gemin 2[48, 212].

Exon 4

The entirety of exon 4 is considerably less conserved than other regions of SMN. Vertebrate species have corresponding sequence within this area, however identity is fairly low; mouse and human homologs share only 57% identity[233 2008]. The latter half of exon 4 is derived from exon 4 within *C.e.Smn*, demonstrating that this region is modestly conserved in invertebrates. Deletion of exon 4 had no effect on subcellular localization and performed as well as FLSMN in restoring neurite outgrowth in the NSC34 line[229].

Exon 4 carries the first section of SMN's polyproline region. Spanning exons 4, 5 and 6, the polyproline region is a symmetrical P(5)-X(17)-P(10)-X(17)-P(5) motif in which X represents any amino acid. While no other poly-proline region has been described with this pattern, other proteins with poly-proline regions are involved with actin dynamics. Similarly, it has been shown that through this region, SMN is able to bind to profilin I and profilin II[73]. Profilin I is found in early embryogenesis and then ubiquitously at low levels in later development. Profilin II is located at high levels primarily in the CNS. As is true with most poly-proline motifs, SMN binds more strongly to profilin II[73]. Mutation within the poly-proline region disrupts profilin II binding without affecting subcellular localization. Similarly, patient mutation S230L, located near the poly-proline region abrogated binding with SMN *in vitro*[214].

Exon 4 also encodes part of a calpain cleavage region (CCR). It has been seen in flies and mice that SMN localizes to Z-discs and that this interaction can be broken by addition of the protease calpain[234]. This region of interest was mapped to residues 176-224 of SMN, located within exons 4 and 5 and the cleavage sites were determined to be after S192 and F193. This region includes the polyproline region, however alteration of prolines into alanines did not affect calpain cleavage—suggesting that secondary structure resulting from the poly-proline region is not necessary for cleavage. Several patient mutations were seen to inhibit cleavage; D44V, an N-terminal mutation abrogated cleavage as well as a mutation near the CCR, A188S. SMN was seen to be cleaved by cytosolic, but not nuclear calpains. As calpains are considered regulatory enzymes rather than degradative, it is hypothesized that this could be a spatial regulatory mechanism[235].

Serine 187 is a residue of interest within exon 4. This residue has been shown to be a target of phosphorylation by PKA[236]. A S187A mutation abrogated phosphorylation at this site. While this activity is not thought to influence SMN localization, it does increase SMN's ability to form higher-order complexes and therefore increases SMN stability[236]. Though alignment within this region is less confident than areas of high homology, this residue is not consistently conserved among diverse organisms (Figure 13).

Exon 5

SMN1 and *SMN2* are known to express isoforms of SMN that do not include exon 5. No function has been assigned to this SMNΔ5 isoform. Conservation within this region is fairly low; invertebrate species have a noticeable vacancy when compared to human SMN exon 5. Among vertebrates, there are considerable levels of similarity, but apart from short conserved motifs, multiple alignment does not demonstrate high levels of identity. The reason behind this evolutionary addition of structure has not yet been investigated.

Selective deletion of exon 5 does not alter the levels of SMN within the nucleus or cytoplasm, but did result in a more diffuse staining, rather than normal puncta seen with FLSMN in NSC34 cells. Despite this abnormality, this construct was able to restore neurite outgrowth compared to control SMN-depleted cells [229]. In other cell lines, this construct had no effect on SMN localization[222].

Exon 5 also houses parts of the polyproline tract and is considered part of the profilin-interacting domain. The middle stretch of prolines that is found to be necessary for binding profilin is found within exon 5.

Exon 6

Exon 6 is home to the highly conserved triplicated YXXG box found in all species from yeast to humans[230]. Further, the region surrounding the dodecapeptide region maintains a high degree of similarity. This high level of conservation emphasizes the regions importance in combination with the fact that at least 12 patient mutations have been identified between residues 260 and 274. Mutations Y272C and G279V are both shown to bind with low affinity to FLSMN and to promote snRNP activity in the presence of FLSMN, however they do not rescue vertebrate models of SMA[217].

Deletion of this exon leads to diffuse nuclear and cytoplasmic staining. This construct is unable to rescue the stunted neurite outgrowth or morphology seen in SMN-depleted NSC34 cells[229].

It was established early that exon 6 was required for SMN self-interaction and that multimerization is required for SMN function[215]. Accordingly, the region has been of great interest. As one of the few domains within SMN that lends itself amenable to crystallization, the structure of the Y/G box and surrounding region has been solved. Initially the region appeared to take a glycine zipper conformation, but further investigation showed that this could not be true[237, 238]. This region of oligomerization stabilizes SMN protein; deletion of exon 6 decreases SMN half-life from ~4.35 hours to 2.1 hours. This effect was seen by point mutagenesis of residues in the YG box including G279V(1.5 hours) and Y272G (1.25 hours)[44].

The region of exon 6 is mostly conserved. Apart from the highly conserved Y/G box, the 5' end of exon 6 shows high levels of similarity across vertebrate species, and part of this region is similarly conserved among invertebrates.

Exon 7

This region has been highly investigated due to the high levels of SMNΔ7 in SMA and is considered to affect localization and stability of FLSMN. Exon 7 was concluded to confer stabilization upon FLSMN due to the instability of the protein in its absence. Exon 7 also contributes to SMN localization, promoting a cytoplasmic localization. This was mapped to a short "QNQKE" motif within exon 7[239]. Interestingly, these effects seem to be relatively non-specific; addition of short (4-6 amino acid) sequences onto the SMN C-terminus can affect both localization and stability[239-241].

It was originally thought that exon 7 contained a region that promoted stability[35]. Indeed, deletion of exon 7 can shorten the SMN half-life from 4.35 hours to 2.21 hours[44]. However, this hypothesis has been further refined to suggest that the *absence* of exon 7 *promotes* instability. Notably the fusion of exon 6 with exon 8 has been identified as a degradation signal known as the "degron." [37].

The SMN C-terminus is also known to interact with Gemin 3. Neither SMNΔ7 nor SMNY272C are capable of binding Gemin 3 *in vitro*[242].

It may be of further interest to study the structure and function of SMN as it has changed throughout evolution. As the SMN sequence has increased in complexity, so too has its repertoire of binding partners. Analysis of the SMN complex illustrates this well: the human SMN complex is complete with Gemins2-8 and UNRIP, however members of this complex first appeared at different times in evolutionary history. Within *S.pombe*, the SMN complex was merely SMN and Gemin2. While this simple two-member complex is sufficient to meet the needs of a single-cellular organism, evolutionary pressures have selected additional specialized members that in turn can manage higher demand. Indeed

addition of these members likely contributed to the evolutionary changes within the SMN coding sequence.

1.2 Post-translational modifications of SMN

Phosphorylation

FLSMN is known to be phosphorylated[243]. This modification is thought to affect the localization, binding affinity, and function of SMN. The phosphorylation patterns of SMN differ between the cytoplasm and the nucleus. Within the nucleus, SMN is in a hypophosphorylated state which is shown to be inactive in snRNP assembly[244]. Two residues within the Gemin 2 interaction region (S28 and S31) were determined to be phosphorylated and mutation of these residues was able to change their function. Mutation to alanine or glutamic acid to abrogate phosphorylation prevented SMN from releasing Sm proteins or assembled snRNPs, while mutation to aspartic acid to mimic constitutive phosphorylation showed an increased activity in snRNP assembly[244]. These mutations do not affect the binding affinity of SMN with other members of the SMN complex, rather the function of the complex is altered by phosphorylation.

SMN phosphorylation and localization is mediated by several known cofactors. The nuclear phosphatase PPM1G phosphorylates SMN as well as Gemin 3—a second main phosphoprotein within the SMN complex. Knockdown of PPM1G was correlated with disappearance of SMN and its binding partner Gemin 2 from nuclear CBs. This effect can be ameliorated upon reintroduction of catalytically active PPM1G or with concomitant knockdown of UNRIP[245]. Thus it is thought that PPM1G and UNRIP have antagonistic functions in localizing SMN to the nucleus or cytoplasm, respectively.

Protein phosphatase PP1γ is shown to interact with the SMN complex as well as Gemin 8 and its presence is required for the formation of gems. In PP1γ knockdown conditions, there is a significant shift in SMN localization resulting in fewer gems and more abundant CBs. This also results in hyperphosphorylated SMN in nuclear extracts[246 2012].

Protein Kinase A (PKA) is also thought to influence SMN through phosphorylation. Early evidence suggested that addition of cyclic-AMP (cAMP) increases levels of SMN. Accordingly, researchers investigated whether this effect was due to cAMP activation of PKA. Several PKA sites have been identified in the SMN protein[247] and it has also been shown that incubation with PKA results in phosphorylated SMN, resulting in an extended SMN half-life[44]. Upon investigation of which residues are responsible for this effect, it was found that mutation of the canonical PKA sites, including S28 and S31, did not abolish the PKA-dependent phosphorylation suggesting that PKA acts on some non-canonical sites in SMN[44]. Additional sites of phosphorylation by PKA have been identified at serines 4, 5, 8, 187, and threonine 85. While phosphorylation at these sites did not alter SMN localization as seen with serines 28 and 31, it did enhance SMN binding with other Gemin proteins to form a higher-order complex[44, 247]. Together this suggests that quarternary structure may be the mechanism behind PKA-dependent SMN stabilization[44].

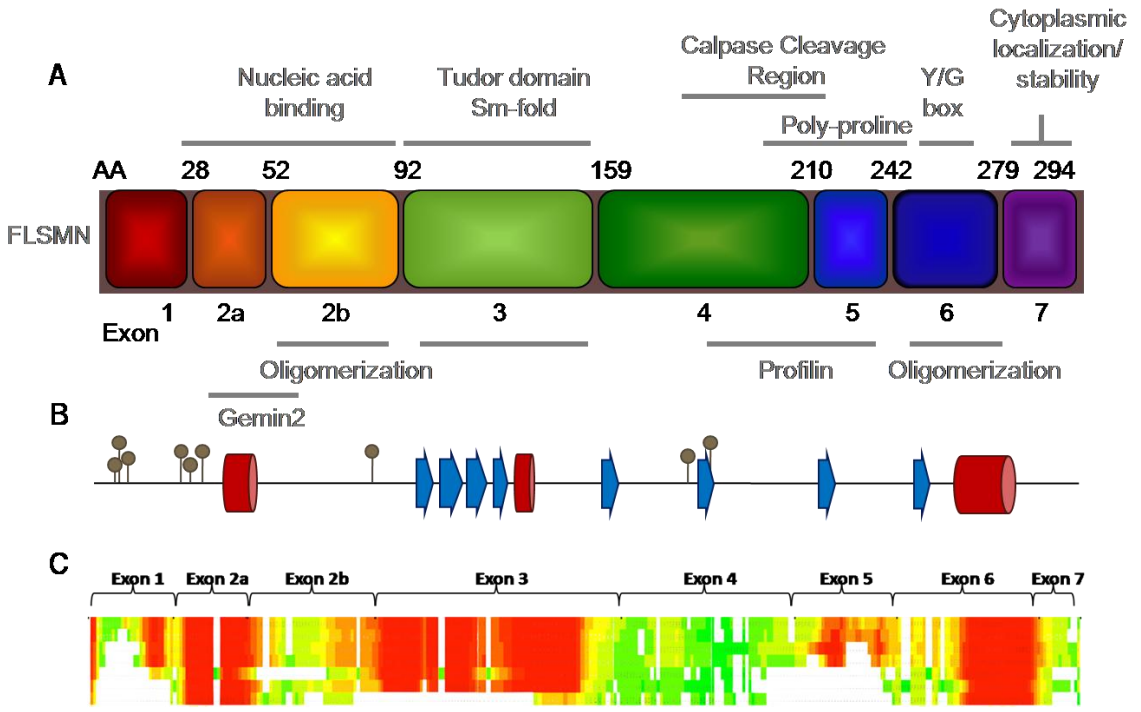


Figure 10: SMN secondary structure and phosphorylation in SMN.

A) Protein map of SMN and interacting partners. B) Phosphorylation sites marked in green dots: S4, S5, S8, T25, S28, S31, S85, S187. Secondary structure as determined by NMR and crystallography. α-helixes are red cylinders from residues 38-48 (PDB: 2LEH) and 138-140 (PDB: 4A4E), β-sheets are blue arrows residues 97-101, 108-117, 122-127, 133-137 (PDB4A4E). All sites are from experimental data and published on RCSD Protein Data Base. C) Multiple alignment of SMN from *H. sapiens, M. musculus, D. rerio, X. laevis, C. elegans, D. melanogaster, S. pombe* to demonstrate areas of conservation. Full sequences are shown in Figure 13 and Figure 14.

Ubiquitination

SMN is known to be ubiquitinated and degraded via the proteasome[44, 248]. Indeed, treatment with proteasome-inhibiting drugs can increase levels of SMN[248, 249]. The E3 ubiquitin ligase Mind bomb1 mediates transfer of ubiquitin onto SMN and knockdown of this gene has also proven to increase SMN levels and improve the SMA-related condition in *C. elegans*[250].

Proteomics studies suggest that SMN ubiquitination occurs on multiple lysines including residues 41, 51, 186, and 209. Experimentally it has been shown that mutation of all FLSMN lysines is able to abrogate ubiquitination, however, reintroduction of one or more lysine is sufficient to cause the proteins monoubiquitination. Thus, ubiquitination within SMN is a promiscuous process that is not dependent on a specific subset of lysines[251].

Deubiquitination is performed by Usp9x. This enzyme is found in the cytoplasm and stabilizes FLSMN via removing ubiquitin tags on its 22 lysine residues. This enzyme does not deubiquitinate SMNΔ7, presumably because the two proteins are subcellularly separated: SMNΔ7 lacks a cytoplasmic localization signal and is thus primarily within the nucleus, whereas Usp9x is found in the cytoplasm[251].

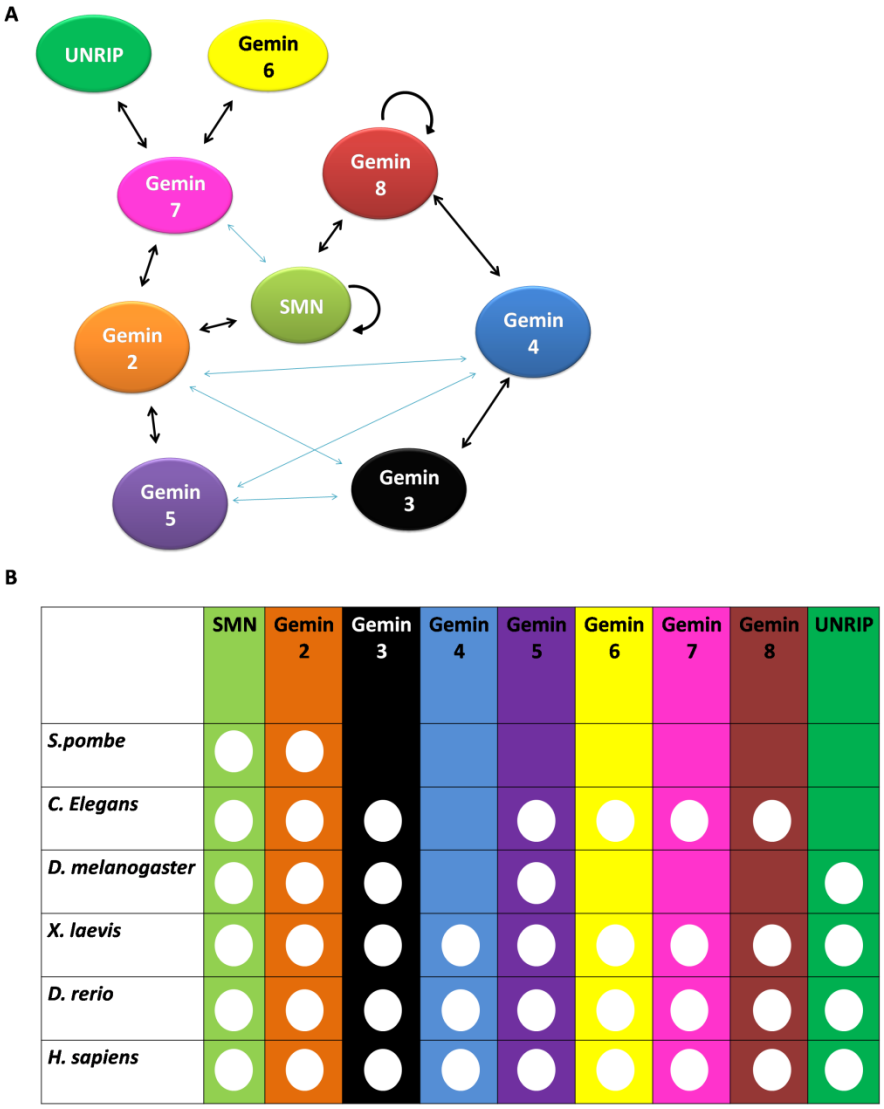


Figure 11: The SMN complex has gained members throughout evolution.

A) Interactions within the human SMN complex are mapped according to *in vitro* interaction studies. Interactions shown by two methods are shown as black arrows. Interactions shown through only one method are shown in light blue. B) Homologs of the SMN complex interacting partners are variably conserved throughout evolution. Presence of a homolog in each species is demonstrated by a white dot.

Some regions and binding partners associated with SMN are proposed to be essential for its function in humans, however these components had not yet evolved in earlier species. While all organisms between humans and yeast have respective homologs of SMN, these homologs can contain considerable divergences. A key difference can be found in genetic structure of SMN homologs. Each of the invertebrates discussed before had unique sequences as well as intron/exon divisions. *Xenopus SMN* represents the first SMN homolog to be encoded by 9 exons and 8 introns, similar to *D. rerio Smn* and human SMN. Comparison between *C.elegans* and *D. rerio* SMN structures elucidates this evolution in which exons 1-4 of *C.e.Smn* are conserved in different regions of *D.r.Smn* and entire swaths of coding region are added into the vertebrate homologs. This evolutionary conservation further extends to include other genes. Synteny between *Homo sapiens* and *Xenopus tropicalis* includes NAIP and GTF2H2[104].

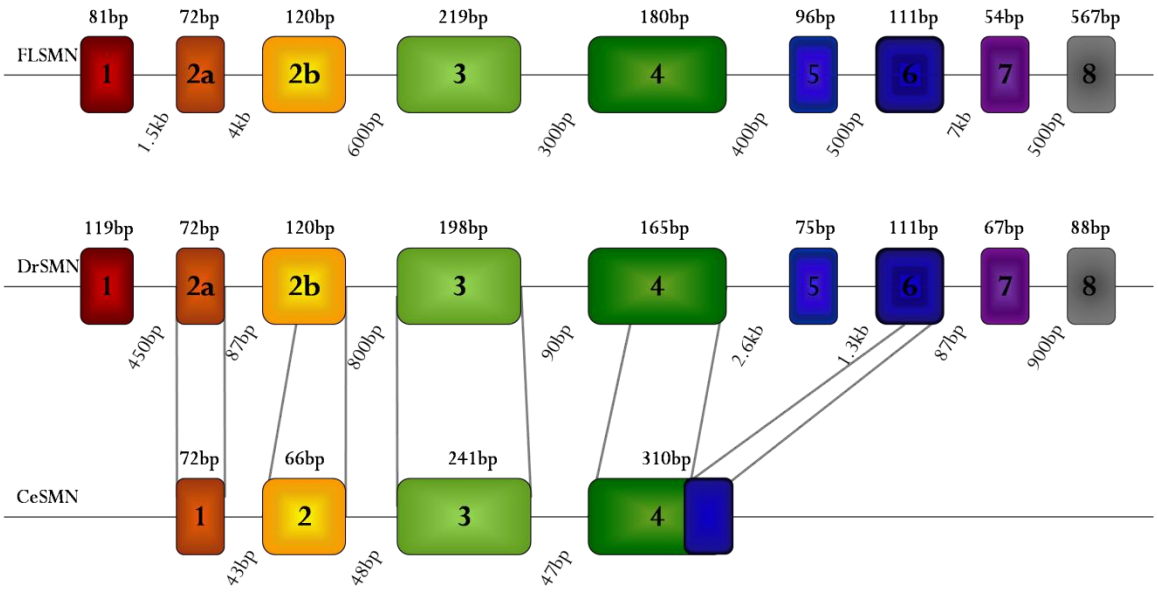


Figure 12: Genomic regions of SMN maintain homologous exonic coding regions and gain additional regions.

Comparison between *C.e.Smn* and *D.r.Smn* demonstrate high levels of conservation between these homologs. The main structure of SMN is conserved from the zebrafish to human genes.

Figure adapted from Bertrandy *et al.* 1999[107]

2: Methods

Genotyping and Mouse Handling

Animals were handled in accordance with protocols approved by the University of Missouri Animal Care and Use Committee. Mice heterozygous for *mSmn* ($Smn^{+/-}$;$SMN2^{+/+}$;$Smn\Delta7^{+/+}$) were bred to obtain experimental knockout mice ($Smn^{-/-}$;$SMN2^{+/+}$;$Smn\Delta7^{+/+}$). Tail clips were obtained within 24 hours of birth and used for genotyping as previously described[252]. The day of birth was considered PND1. Knockout pups were kept with at least 2 healthy heterozygous siblings. Wild type and additional heterozygous animals were culled to maintain a litter size of 4-6 pups. Experimental mice alive at 21 days were weaned from their mother and kept in cages with their siblings according to sex.

2.1: Viral Production

scAAV9 viral vectors were created in-house as described previously[253]. Briefly, HEK293T cells are triple transfected with plasmids carrying AAV Rep/Cap genes, AAV helper gene, and ITR-flanked transgene. 48 hours post-transfection, cells are harvested, lysed, and vector is purified using 3 sequential cesium chloride density gradient ultracentrifugation steps. Purified virus is dialysed against a HEPES buffer (20mM HEPES, 100mM NaCl) and quantified using quantitative PCR with a SYBR Green detector. All transgenes are driven under the control of the chicken beta actin promoter unless otherwise stated.

2.2: Real-time PCR (qPCR)

Quantification of viral genomes was performed using SYBR® Green and primers to amplify the chicken β-actin promoter region, forward (5′-CCGGTGGTGGTGCAAATCAAAGAA-3′) and reverse (5′-

AGCAGAAGTAACACTTCCGTACAGGC-3′). The absolute quantitation method using a standard curve was utilized on the Applied Biosystems® 7500 Real-Time PCR System using Applied Biosystems 7500 Sequence Detection Software Version 1.3 Viral fractions were diluted 1:1000 and the PCR cycle was as follows: 50°C 2 min,95°C 10 min, 40 cycles (95°C 15 s), 60°C 1 min. A standard curve was obtained using serial dilutions of the transgene-containing plasmid (10^{10}–10^{5}) to calculate melting curves of each sample. The viral fractions containing the highest titer were dialyzed with HEPES buffer (100 mm NaCl, 20 mmHEPES). Following dialysis, qPCR was performed to obtain the final titer of the virus-containing solution to be used for injections into the mice.

2.3: In Vivo Injections

Unless otherwise specified, on PND 2, experimental mice were injected with viral particles $1x10^{11}$ particles as determined by qPCR. Each aliquot to be injected was colored 1:50 with filtered food quality green dye immediately prior to injection. Upon both IV and ICV injection, dye localization was used to determine the success of the injection and subjects that received sub-par injection were not used as data points.

IV delivery was performed using a 33 gauge 0.25 inch hypodermic needle attached to a 100µl Hamilton syringe as previously described[190]. Neonates were secured to a transilluminator to visualize the facial vein. Injections were performed on PND2 in a single injection up to 60µl of virus plus dye.

ICV delivery was performed using a sterilized glass pulled capillary needle as previously described[190]. The needle tip was broken to allow for approximately 2 mm of penetration into the neonatal skull. Upon injection, each mouse was monitored to visualize dye spread indicative of a successful injection. Each injection consisted of up to 6µl total

volume. Due to this variability in viral titer, some constructs were injected up to 3 times to achieve $1x10^{11}$ viral particles. In such instances, injections were spaced such that a minimum of 1-4 hours were allowed for uptake of the previous injection before administration of the next.

2.4: Motor Function Analysis

Time to Right (TTR) was measured every day from PND6-PND18 on SMNΔ7 experimental animals. These animals show a measurable difference in strength at this early timepoint whereas animals characterized as "intermediate" SMA models show no appreciable difference in strength before symptom onset (unpublished observation). Mice were placed on their back and were given a maximum of 30 seconds go successfully turn themselves onto their paws. Mice that right themselves and fall over again within 2 seconds were not considered to have successfully righted themselves. After 30 seconds has expired, mice that did not right were considered to have failed the test.

2.5: RNA analysis

The total RNA was isolated using Trizol (Thermo Fisher Scientific, cat. no. 15596-026) followed by digestion with RNase-free DNase I (Thermo Fisher Scientific, cat. no. AM2222). For mRNA analysis, mixture of oligo-dT primers and random hexamers was used to generate cDNA using Advantage RT-for-PCR kit (Clontech) and 1 μg of total RNA following the manufacturer's instructions. For spliceosomal snRNA analysis, snRNA-specific reverse primers were used in the RT reaction according to a previous study. All primers used in RT-qPCR experiments were previously described.

2.6: Neuromuscular Junction Analysis

All NMJ procedures were performed as previously described. Mice were anesthetized by inhalation of isofluorine and fixed using cardiac perfusion of phosphate buffered saline

followed by 4% paraformaldehyde (PFA). Mice were submerged in PFA for 2 hours and then kept in cold PBS until dissection. *Splenius capitis,*and *longissimus capitis* muscles were dissected. In animals >2 weeks of age the muscles were gently teased apart to promote antibody penetration. Nerve was stained using chicken anti-neurofilament, heavy (1:2000, Millipore, Temecula, CA). Presynapse was stained using rabbit anti-synaptophysin (1:200, Invitrogen Corporation, Camarillo, CA). Secondary antibodies were Alexa Fluor 488-conjugated Goat Anti-Chicken (1:200, Invitrogen Corporation, Camarillo, CA) and DyLight 488-conjugated anti-rabbit (1:400, Jackson ImmunoResearch Laboratories, West Grove, PA). Postsynapse was stained using TRIT-C conjugated α-bungarotoxin(1:200). Post-staining, samples were whole mounted in Citifluor (Catalog number 19470, Ted Pella, Inc). Fluorescent images were taken on a Leica, using LAS-X software. Confocal imaging was performed using a Zeiss LSM 510 META confocal microscope. Z-stacked images were taken at 1µm intervals and deconvolved using Metamorph software. Images presented are maximum projections of Z-stacked images.

3: Results

Toward a larger goal of establishing a minimal domain that is capable of restoring necessary functions of SMN *in vivo*, we aimed to determine whether evolutionarily conserved domains could sufficiently function to rescue an SMA model. It is well established that scAAV9 delivery of the human SMN cDNA is effective for rescuing the severe phenotype of the SMNΔ7 mouse model. Accordingly, we utilized this modality to deliver SMN homologs from a panel of model organisms. Representative sequences include homologs from *D. rerio*, *X. laevis*, *C. elegans, D. melanogaster*, and *S. pombe.*

Sequences from these organisms were selected because they represent a broad range of evolutionary distances and because each sequence has been previously characterized within the field of SMA so that no hypothetical protein sequences were used.

3.1: SMN homologs represent divergent sequences

Initial sequence analysis demonstrated that these homologs display significant differences in their amino acid sequences. However, as is expected, certain domains within SMN are more highly conserved than others, creating "hot spots" of conservation. The most highly conserved regions are the N-terminal Gemin 2 binding domain, the central Tudor domain and the C-terminal YG box. Interestingly, Smn from *S. pombe* is the only homolog that does not have a tudor domain. As this domain is considered essential for SMN to bind Sm proteins and facilitate snRNP assembly, it is presumed that this function would also be valuable even in a single-celled organism such as yeast. Notably, fission yeast have homologs of SMN and Gemin 2, but no other recognizable members of the SMN complex. Some data suggests that yeast SMN still binds to Sm proteins in a tudor-independent manner, through interaction with the YG box[87].

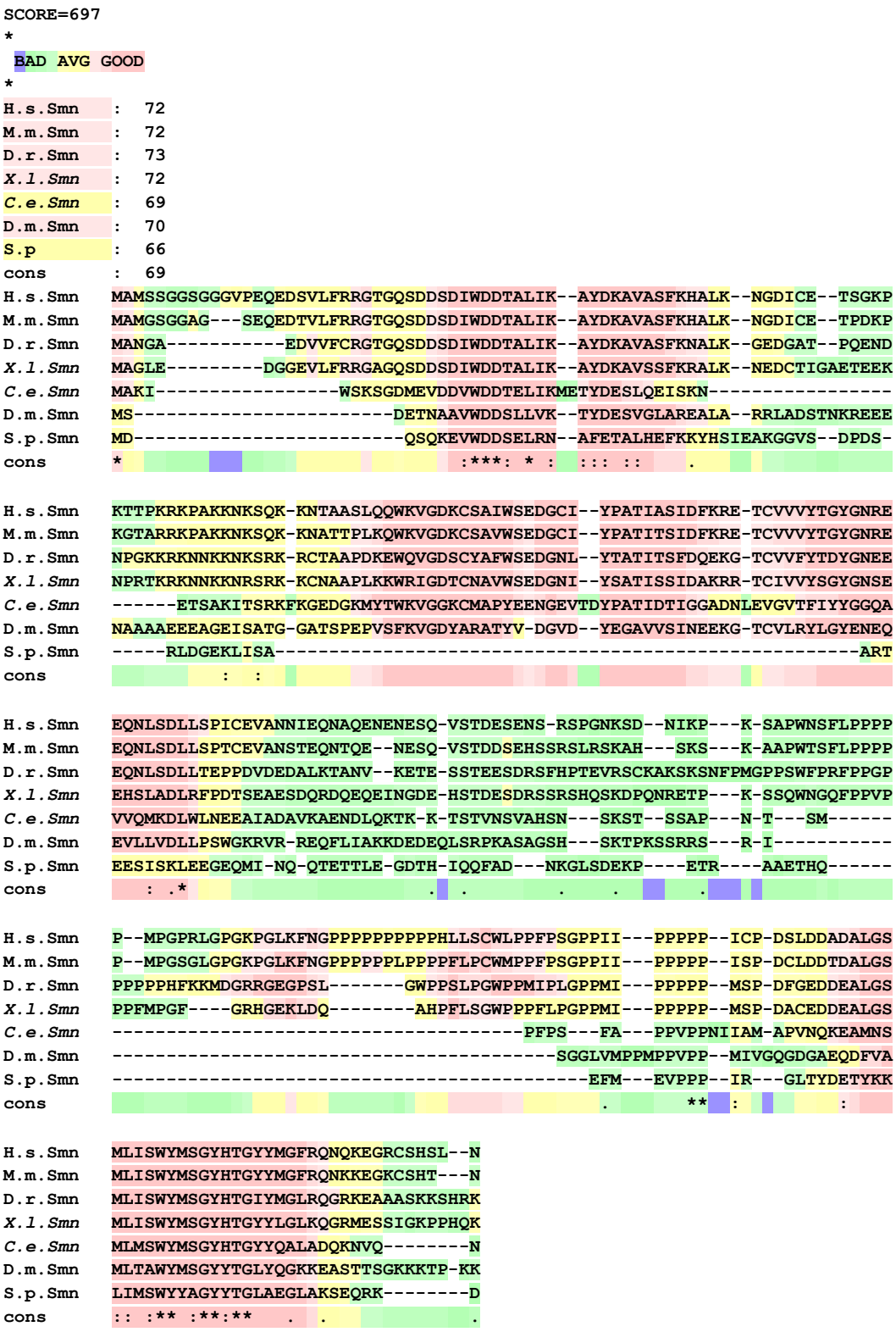
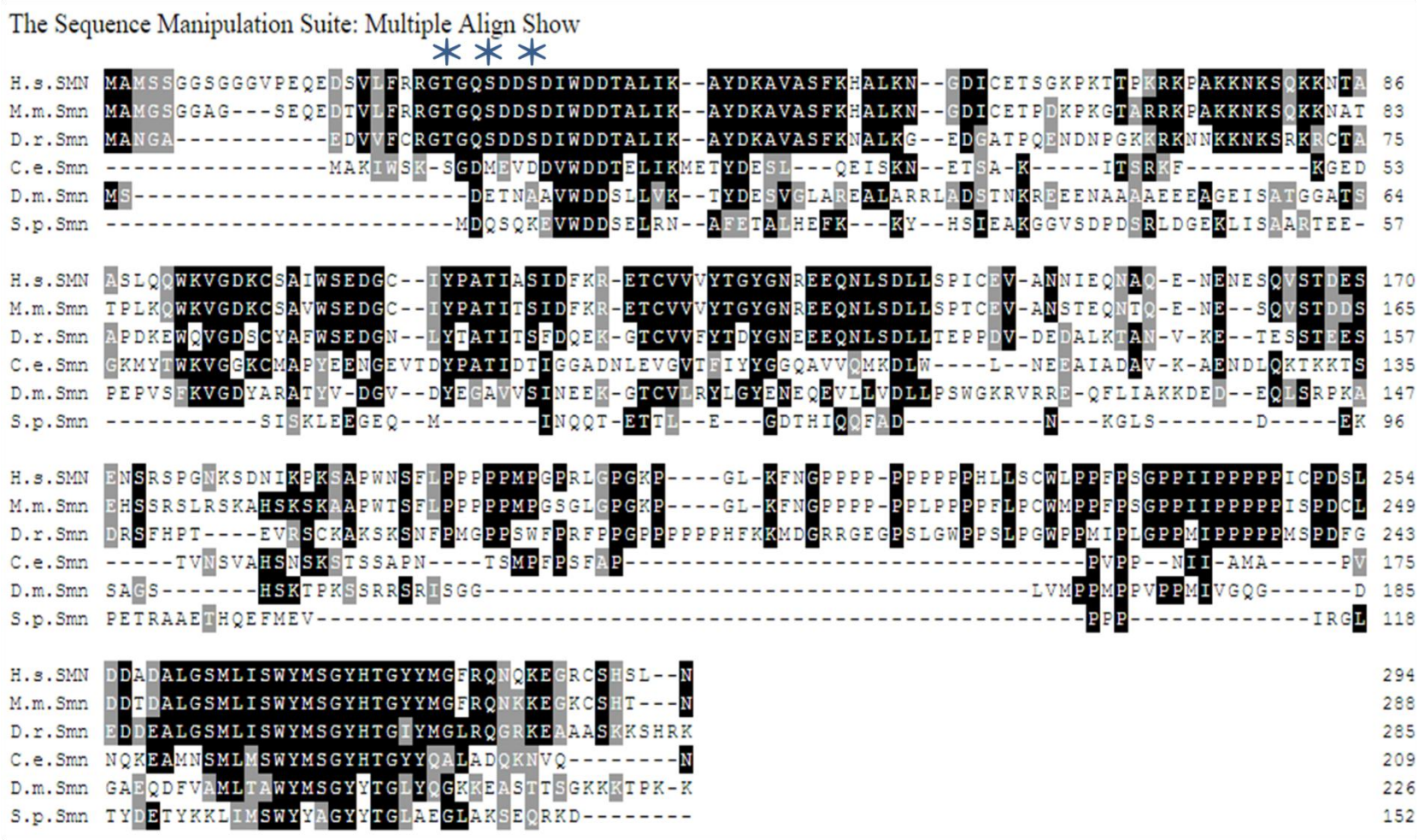

SCORE=697
BAD AVG GOOD
H.s.Smn : 72
M.m.Smn : 72
D.r.Smn : 73
X.l.Smn : 72
C.e.Smn : 69
D.m.Smn : 70
S.p : 66
cons : 69
H.s.Smn MAMSSGGSGGGVPEQEDSVLFRRGTGQSDDSDIWDDTALIK--AYDKAVASFKHALK--NGDICE--TSGKP
M.m.Smn MAMGSGGAG---SEQEDTVLFRRGTGQSDDSDIWDDTALIK--AYDKAVASFKHALK--NGDICE--TPDKP
D.r.Smn MANGA----------EDVVFCRGTGQSDDSDIWDDTALIK--AYDKAVASFKNALK--GEDGAT--PQEND
X.l.Smn MAGLE---------DGGEVLFRRGAGQSDDSDIWDDTALIK--AYDKAVSSFKRALK--NEDCTIGAETEEK
C.e.Smn MAKI----------------WSKSGDMEVDDVWDDTELIKMETYDESLQEISKN----------------
D.m.Smn MS------------------------DETNAAVWDDSLLVK--TYDESVGLAREALA--RRLADSTNKREEE
S.p.Smn MD------------------------QSQKEVWDDSELRN--AFETALHEFKKYHSIEAKGGVS--DPDS-
cons
H.s.Smn KTTPKRKPAKKNKSQK-KNTAASLQQWKVGDKCSAIWSEDGCI--YPATIASIDFKRE-TCVVVYTGYGNRE
M.m.Smn KGTARRKPAKKNKSQK-KNATTPLKQWKVGDKCSAVWSEDGCI--YPATITSIDFKRE-TCVVVYTGYGNRE
D.r.Smn NPGKKRKNNKKNKSRK-RCTAAPDKEWQVGDSCYAFWSEDGNL--YTATITSFDQEKG-TCVVFYTDYGNEE
X.l.Smn NPRTKRKNNKKNRSRK-KCNAAPLKKWRIGDTCNAVWSEDGNI--YSATISSIDAKRR-TCIVVYSGYGNSE
C.e.Smn ------ETSAKITSRKFKGEDGKMYTWKVGGKCMAPYEENGEVTDYPATIDTIGGADNLEVGVTFIYYGGQA
D.m.Smn NAAAAEEEAGEISATG-GATSPEPVSFKVGDYARATYV-DGVD--YEGAVVSINEEKG-TCVLRYLGYENEQ
S.p.Smn -----RLDGEKLISA---ART
cons
H.s.Smn EQNLSDLLSPICEVANNIEQNAQENENESQ-VSTDESENS-RSPGNKSD--NIKP---K-SAPWNSFLPPPP
M.m.Smn EQNLSDLLSPTCEVANSTEQNTQE--NESQ-VSTDDSEHSSRSLRSKAH---SKS---K-AAPWTSFLPPPP
D.r.Smn EQNLSDLLTEPPDVDEDALKTANV--KETE-SSTEESDRSFHPTEVRSCKAKSKSNFPMGPPSWFPRFPPGP
X.l.Smn EHSLADLRFPDTSEAESDQRDQEQEINGDE-HSTDESDRSSRSHQSKDPQNRETP---K-SSQWNGQFPPVP
C.e.Smn VVQMKDLWLNEEAIADAVKAENDLQKTK-K-TSTVNSVAHSN---SKST--SSAP---N-T---SM------
D.m.Smn EVLLVDLLPSWGKRVR-REQFLIAKKDEDEQLSRPKASAGSH---SKTPKSSRRS---R-I-----------
S.p.Smn EESISKLEEGEQMI-NQ-QTETTLE-GDTH-IQQFAD---NKGLSDEKP----ETR----AAETHQ------
cons
H.s.Smn P--MPGPRLGPGKPGLKFNGPPPPPPPPPPHLLSCWLPPFPSGPPII---PPPPP--ICP-DSLDDADALGS
M.m.Smn P--MPGSGLGPGKPGLKFNGPPPPPPLPPPPFLPCWMPPFPSGPPII---PPPPP--ISP-DCLDDTDALGS
D.r.Smn PPPPPHFKKMDGRRGEGPSL-------GWPPSLPGWPPMIPLGPPMI---PPPPP--MSP-DFGEDDEALGS
X.l.Smn PPFMPGF----GRHGEKLDQ--------AHPFLSGWPPPFLPGPPMI---PPPPP--MSP-DACEDDEALGS
C.e.Smn --PFPS---FA---PPVPPNIIAM-APVNQKEAMNS
D.m.Smn ---------------------------------------SGGLVMPPMPPVPP--MIVGQGDGAEQDFVA
S.p.Smn --EFM---EVPPP--IR---GLTYDETYKK
cons
H.s.Smn MLISWYMSGYHTGYYMGFRQNQKEGRCSHSL--N
M.m.Smn MLISWYMSGYHTGYYMGFRQNKKEGKCSHT---N
D.r.Smn MLISWYMSGYHTGIYMGLRQGRKEAAASKKSHRK
X.l.Smn MLISWYMSGYHTGYYLGLKQGRMESSIGKPPHQK
C.e.Smn MLMSWYMSGYHTGYYQALADQKNVQ--------N
D.m.Smn MLTAWYMSGYYTGLYQGKKEASTTSGKKKTP-KK
S.p.Smn LIMSWYYAGYYTGLAEGLAKSEQRK--------D
cons

Figure 13: Multiple alignment of SMN homologs demonstrates variable similarities.

Amino acid sequences from SMN homologs were aligned and visualized using a ClustalW all against all pair-wise alignments combined with Lalign all against all local alignments via the T-Coffee webserver[254-256]. Residues are scored on a scale of 1-10 based on the confidence of alignment and colored from blue to red as indicated by the color reference at the top. Red areas demonstrate regions of high alignment confidence, whereas green indicates low confidence. Consensus is demonstrated in the bottom row. Asterisks indicate residues consistent among all homologs, colons represent conservation between groups of strongly similar properties whereas periods represent conservation between groups of weakly similar properties as scored by the Gonnet PAM 250 matrix.


The Sequence Manipulation Suite: Multiple Align Show
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M.m.Smn MAMGSGGAG---SEQEDTVLFRRGTGQSDDSDIWDDTALIK--AYDKAVASFKHALKN--GDICETPDKPKGTARRKPAKKNKSQKKNAT 83
D.r.Smn MANGA-----------EDVVFCRGTGQSDDSDIWDDTALIK--AYDKAVASFKNALKG--EDGATPQENDNPGKKRKNNKKNKSRKRCTA 75
C.e.Smn ----------------MAKIWSK-SGDMEVDDVWDDTELIKMETYDESL---QEISKN--ETSA-K-----ITSRKF---------KGED 53
D.m.Smn MS------------------------DETNAAVWDDSLLVK--TYDESVGLAREALARRLADSTNKREEENAAAAEEEAGEISATGGATS 64
S.p.Smn -------------------------MDQSQKEVWDDSELRN--AFETALHEFK---KY--HSIEAKGGVSDPDSRLDGEKLISAARTEE- 57
H.s.SMN ASLQQWKVGDKCSAIWSEDGC--IYPATIASIDFKR-ETCVVVYTGYGNREEQNLSDLLSPICEV-ANNIEQNAQ-E-NENESQVSTDES 170
M.m.Smn TPLKQWKVGDKCSAVWSEDGC--IYPATITSIDFKR-ETCVVVYTGYGNREEQNLSDLLSPTCEV-ANSTEQNTQ-E-NE--SQVSTDDS 165
D.r.Smn APDKEWQVGDSCYAFWSEDGN--LYTATITSFDQEK-GTCVVFYTDYGNEEEQNLSDLLTEPPDV-DEDALKTAN-V-KE--TESSTEES 157
C.e.Smn GKMYTWKVGGKCMAPYEENGEVTDYPATIDTIGGADNLEVGVTFIYYGGQAVVQMKDLW----L--NEEAIADAV-K-AENDLQKTKKTS 135
D.m.Smn PEPVSFKVGDYARATYV-DGV--DYEGAVVSINEEK-GTCVLRYLGYENEQEVLLVDLLPSWGKRVRRE-QFLIAKKDED--EQLSRPKA 147
S.p.Smn -----------SISKLEEGEQ--M-------INQQT-ETTL--E---GDTHIQQFAD----------N---KGLS-------D-----EK 96
H.s.SMN ENSRSPGNKSDNIKPKSAPWNSFLPPPPPMPGPRLGPGKP----GL-KFNGPPPP-PPPPPPHLLSCWLPPFPSGPPIIPPPPPICPDSL 254
M.m.Smn EHSSRSLRSKAHSKSKAAPWTSFLPPPPPMPGSGLGPGKP----GL-KFNGPPPP-PPLPPPPFLPCWMPPFPSGPPIIPPPPPISPDCL 249
D.r.Smn DRSFHPT----EVRSCKAKSKSNFPMGPPSWFPRFPPGPPPPPPHFKKMDGRRGEGPSLGWPPSLPGWPPMIPLGPPMIPPPPPMSPDFG 243
C.e.Smn -----TVNSVAHSNSKSTSSAPN----TSMPFPSFAP--------------------------------PVPP--NII-AMA-----PV 175
D.m.Smn SAGS-------HSKTPKSSRRSRISGG---LVMPPMPPVPPMIVGQG------D 185
S.p.Smn PETRAAETHQEFMEV---PPP-------------IRGL 118
H.s.SMN DDADALGSMLISWYMSGYHTGYYMGFRQNQKEGRCSHSL--N 294
M.m.Smn DDTDALGSMLISWYMSGYHTGYYMGFRQNKKEGKCSHT---N 288
D.r.Smn EDDEALGSMLISWYMSGYHTGIYMGLRQGRKEAAASKKSHRK 285
C.e.Smn NQKEAMNSMLMSWYMSGYHTGYYQALADQKNVQ--------N 209
D.m.Smn GAEQDFVAMLTAWYMSGYYTGLYQGKKEASTTSGKKKTPK-K 226
S.p.Smn TYDETYKKLIMSWYYAGYYTGLAEGLAKSEQRKD-------- 152

Figure 14: Homologs of SMN colored by residue similarity.

Homologs were aligned using T-Coffee webserver and colored using Multiple Align Show (bioinformatics.org). Residues that are identical are colored in black, residues that are similar are colored n gray. 50% of sequences must meet the similarity requirements in order to be colored. Residues considered similar were classified into groups as follows: ILV, FWY, KRH, DE, GAS, P, C, TNQM. Stars represent sites known to be phosphorylated in human, mouse, and rat SMN but which are not maintained across the conservation threshold. Phosphorylation data transferred from phosphosite.org.

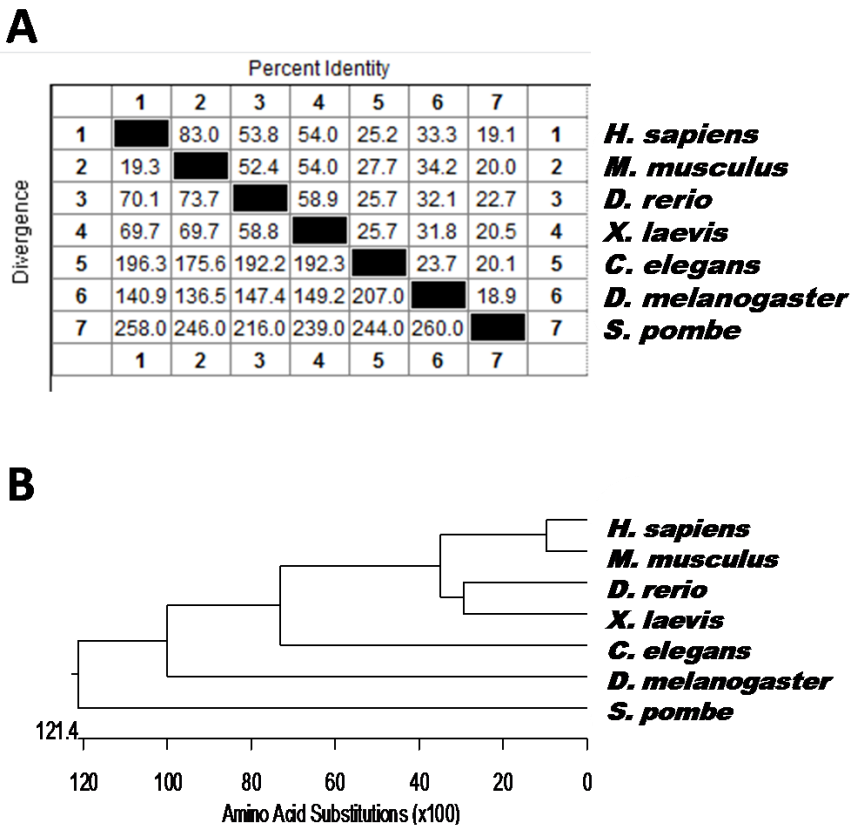

Figure 15: SMN homologs demonstrate significant divergence.

A) Conservation relationships among homologs are depicted in percent identity which is calculated from amino acid differences as well as divergence which includes information established from the phylogenetic relationship between species. B) Phylogenetic tree derived from the amino acid sequences of SMN homologs. Amino acid substitutions include both residue changes as well as gap penalties. Calculations and graphics were generated using MegAlign (DNASTAR Lasergene).

3.2: D. rerio and X. laevis homologs extend survival in SMNΔ7 mice

scAAV9 vectors containing the cDNA from each of the SMN homologs were prepared and $1x10^{11}$ viral particles were injected into newborn SMNΔ7 pups. An intracerebroventricular (ICV) method of delivery was chosen due to its enhanced ability to rescue SMA defects as previously shown[184]. Survival was monitored daily and demonstrated a drastic difference between treatment groups. Pups injected with *D.r.Smn,* the most highly conserved homolog, survived beyond 80 days. At this time point, one animal demonstrated cardiac difficulties including labored breathing and subcutaneous fluid buildup. To ease the animals suffering, it was euthanized at this point. Two of the other animals in this cohort were euthanized at the same time to preserve muscle tissues. The three remaining animals lived to 120 days with no complications, at which point the experiment was terminated. Phenotypically the mice were active and mobile, had well maintained fur coats and no observable kyphosis. Distal necrosis was seen primarily in the tail, as is common for long-lived SMA mice.

Pups injected with *X.l.Smn* also lived measurably longer than their untreated counterparts. The earliest death within this cohort occurred at day 28—twice as long as the typical SMNΔ7 lifespan. Several animals died within a few days, however 3 of 6 animals survived past day 40 and one long living animal survived past day 60. Within the first two weeks, mice appeared healthy. At later timepoints these mice demonstrated considerable defects by simple visual assessment. Distal necrosis was exaggerated on the tail and ears, their fur coats were unkempt, and their spine demonstrated considerable kyphosis. They demonstrated tremors when walking and were less mobile than healthy animals. These symptoms are all consistent with late stage phenotypes observed in

“intermediate” mouse models of SMA or mice treated with other SMN targeting therapeutics.

Survival results from cohorts injected with *C.e.Smn, D.m.Smn* and *S.p.Smn* demonstrated no appreciable improvement in lifespan. A few interesting findings arose from these cohorts, although none of the observations reached significance. First, *C.e.Smn* seemed to provide a short lived benefit in mice. Within a week of injection, several mice in this cohort gained a healthy amount of weight and were mobile. Interestingly, this effect did not occur in every mouse injected with *C.e.Smn*, but rather half of the subjects gained no weight and appeared identical to uninjected mice. Given this healthy weight gain, it was surprising to find that these individuals all died suddenly on either day 9 or 10, experiencing no drop in weight prior to death. Injected animals that experienced no exceptional weight gain died after losing weight on days 9-14 in a pattern typical of uninjected mice. This virus was remade, and the second round of injected mice saw no appreciable weight gain, rather this group all phenotypically resembled uninjected mice.

As *S.p.Smn* is the most divergent from human SMN and as this cohort saw several early deaths, *S.p.Smn* was assessed for overt toxicity in healthy animals. Heterozygous animals were injected with the standard dosing of *S.p.Smn* and monitored for several weeks. Of the 3 healthy injected animals, none displayed noticeable negative symptoms (data not shown). Presumably then, these constructs are not harmful in mice, however they are insufficient to prolong life in a severe model of SMA.

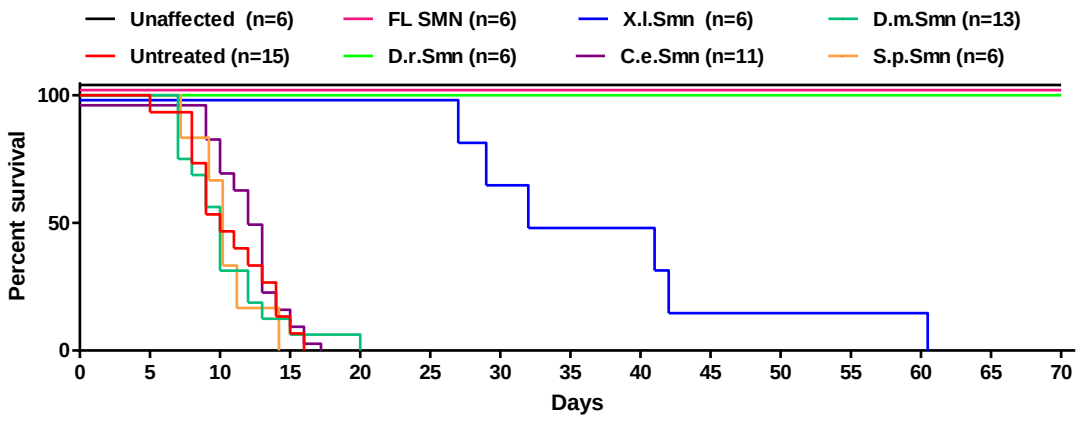

Figure 16: SMN homologs provide variable lifespan extension.

Kaplan-Meyer survival chart demonstrates that mice injected with *D.r.Smn* (green) and *X.l.Smn* (blue) lived significantly longer than uninjected animals (red). Other, more distantly related homologs fail to extend survival. Curves are significant as determined by Log-rank (Mantel-Cox) test ($P<0.0001$).

3.3: D. rerio and X. laevis homologs promote weight gain SMNΔ7 mice

The same animals used for survival studies were simultaneously monitored for weight gain. Similarly to survival data, *D.r.Smn* promoted weight gain in mice, however these mice did not gain as much weight as mice injected with FLSMN. This suggests that while *D.r.Smn* is beneficial in mice, some differences between these homologs render *D.r.Smn* less effective at preventing SMA. Notably, animals injected with *X.l.Smn* lagged behind their counterparts injected with *D.r.Smn* for a considerable length of the experiment. However, around day 30, the *X.l.Smn* line approaches the *D.r.Smn* line because the weakest mice from the *X.l.Smn* group died and no longer affected the group average. Long-lived mice in both of these groups reached a robust adult weight that was still below that of their healthy counterparts.

All SMN homologs that did not extend survival also failed to gain appreciable weight. Typical SMNΔ7 SMA mice do not breach 5 grams of weight. This was true of animals treated with SMN homologs with a few exceptions: one cohort of 8 animals previously described injected with *C.e.Smn* reached a range of 3.5-5.8 grams and died suddenly on day 9 or 10. Further, one long-lived animal injected with *D.m.Smn* reached 6.5 grams and survived to day 20. While these robust individuals may indicate a slight benefit from homologous SMN function, upon repeating the experiment, neither of these atypical results were seen again.

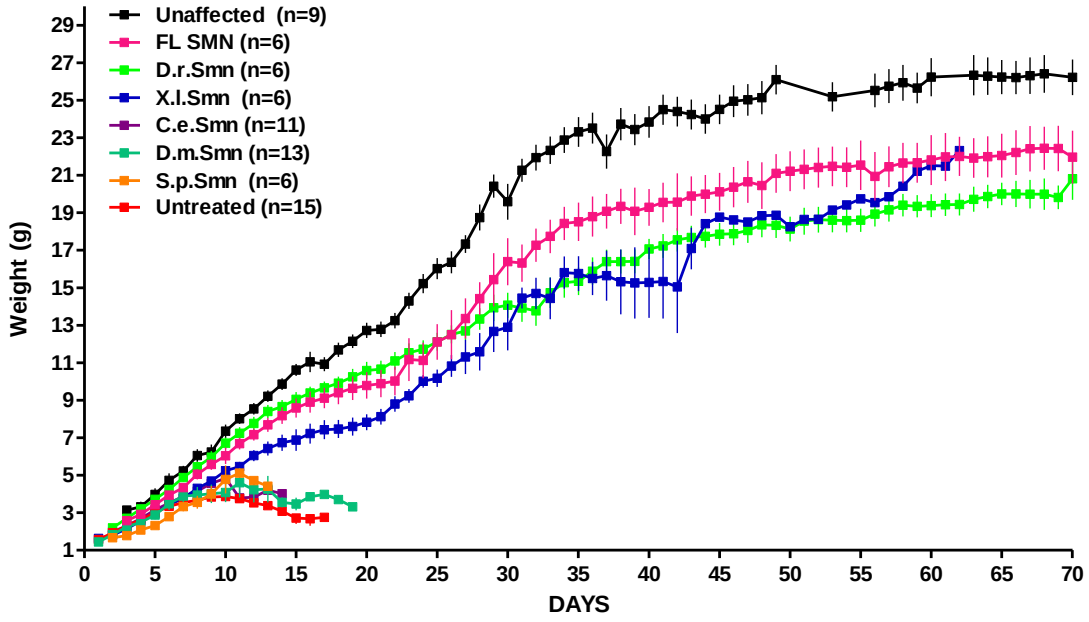

Figure 17: Homologs of Smn induce healthy weight gain in SMNΔ7 animals.

D.r.Smn (green) and *X.l.Smn* (blue) are the only homologs which demonstrate significant weight gain over time. Weight gain in these cohorts is less than healthy animals but approaches that gained upon injection with FLSMN.

3.4: D. rerio and *X. laevis* homologs demonstrate increased motor function

In order to determine the gross motor function and strength of these animals, the time to right assay (TTR) was employed. This simple test demonstrates a young mouse's ability to right itself once placed on its back. Starting at approximately one week of age, healthy animals are slow to right themselves, however by week two they can right themselves immediately. SMNΔ7 animals are never able to complete this task faithfully: after 30 seconds of trying to right themselves, the animal is considered to have failed the test. Consistent with survival and weight gain patterns, animals injected with *D.r.Smn* and *X.l.Smn* performed well on this test. However, there is a lag in which these animals are slower than their healthy counterparts. Between *D.r.Smn* and *X.l.Smn,* there is a slight trend in which *X.l.Smn* injected animals are able to right themselves more quickly than their *D.r.Smn* counterparts, however no conclusions have been drawn based on this slight inter-group difference. By day 17, all animals in these test groups were able to right themselves instantly.

In contrast, only a few animals injected with *C.e.Smn* were able to right themselves. The animals that did right remained slow at this task for the entirety of their shortened life. Animals from *D.m.Smn,* and *S.p.Smn* cohorts displayed little motor function and did not successfully right.

From the entirety of these gross phenotypic analyses, we determined that *D.r.Smn* and *X.l.Smn* are able to rescue the SMA phenotype in SMNΔ7 mice, while treatments of *C.e.Smn, D.m.Smn,* and *S.p.Smn* are unable to rescue. Further, there is a measurable difference in efficacy between the rescue effects afforded by *D.r.Smn* and *X.l.Smn.* We identified a significant conservation threshold that exists between *Xenopus* and *C.*

elegans homologs. Therefore, we set to investigate which regions of SMN could be responsible for these discrepancies.

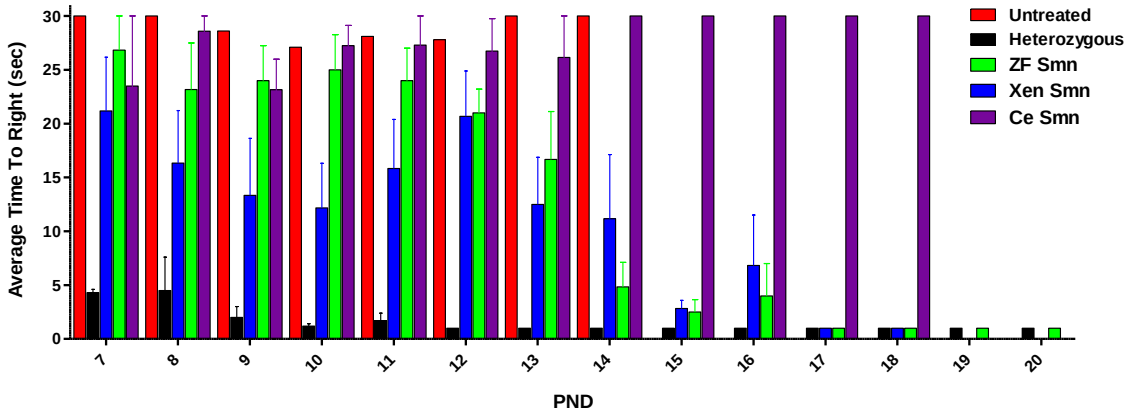

Figure 18: Effective treatments are able to improve gross motor function.

The righting reflex was tested in mice injected with SMN homologs as a measurement of motor function. Untreated SMNΔ7 animals are either unable to right (given 30 seconds) or very slow to right whereas heterozygous healthy animals right quickly. *D.r.Smn* (green) and *X.l.Smn* (blue) are delayed in acquiring the ability to right. *C.e.Smn* does not consistently right quickly.

3.5: C. elegans Smn exhibits a lack of function in vivo

To assess whether the homologs of SMN are functional at a molecular level, we examined relative mRNA levels of disease-relevant transcripts. Several mRNA processing changes have been identified as SMN-dependent[46]. Accordingly, the panel of transcripts we assessed are designed to represent a range of SMN functions.

As the canonical function of SMN is considered its role in snRNP assembly, we analyzed splicing of a U12 intron-containing gene *Stasimon*, which has been found to be improperly spliced in SMN-deficient environments[52]. The proper splicing of *Stasimon* is expected to be restored upon delivery of functional SMN.

SMN is also required in assembly of U7 snRNP, which functions in 3' processing of histone mRNAs. SMN deficiency results in an accumulation of U7 pre-mRNA, a decrease in U7 protein, and disruption of histone mRNA 3'-end processing. Accordingly our panel contains *histone H1c*, a transcript which is found to be increased in an SMA context[46].

Several mRNAs are found to have altered stability in a low SMN context. Several SMN-associated RBPs regulate stability of mRNAs through AU-rich elements in their 3' UTR. Cyclin-dependent kinase inhibitor 1A (Cdkn1a) is a well described example of this. It is antagonistically regulated by KSRP and HuD, and in SMA motor neurons this mRNA accumulates due to increased stability. *Chondrolectin, (Chodl)*, a neuron-specific gene is expressed at lower level in SMA mice even at presymptomatic stages. This gene has been implicated in the outgrowth and elongation of neuronal processes[257]. These transcripts were included in the mRNA panel, ensuring that it represents a broad range of downstream effects of various SMN functions.

Based on the survival and weight gain data, we chose to analyze the two constructs that are most closely related but which fall on opposing sides of the conservation threshold: *Xenopus* and *C. elegans.* We aimed to look more closely into the phenotype changes afforded by these homologs. Analysis of the neuromuscular junction (NMJ) provides a disease-relevant readout of SMA severity. It is well established that NMJs are disrupted in SMA, as will be discussed at length in Chapter 4. Briefly, NMJ pathology in SMA can take the form of presynaptic neurofilament accumulation, postsynaptic immaturity, or denervation of motor endplates. Normal NMJ maturation proceeds through stages characterized at the motor endplate: these stages include plaque, perforated, C-shaped, branched, and pretzel-like shapes. NMJs are disrupted in multiple mouse models of SMA; the most commonly used animals have a characterized set of muscles that are affected at disease end stage.

In accordance with the denervation profile of the SMNΔ7 mouse, we analyzed the splenius capitis at PND12 for morphological abnormalities at the NMJ. Upon observing NMJs within animals treated with *C.e.Smn,* it was apparent that the major defect in these mice was at the motor endplate. While many NMJs demonstrated a "pretzel-like" presynapse, these elegant nerve endings contacted postsynaptic regions that did not have the "pretzel-like" postsynapse that accompanies a mature NMJ. Most of the motor endplates seemed to be in earlier morphological stages of development displaying plaque and perforated morphologies. In contrast, animals treated with *X.l.Smn* had an NMJ profile that more appropriately matched healthy, developing animals. Not all NMJs were mature in this model, however the presynaptic and postsynaptic levels of intricacy were

more evenly matched: most pretzel-like nerve endings synapsed onto mature, pretzel-like postsynapses.

In order to determine if this finding demonstrated statistical significance, we quantified the maturity of motor endplates between treated and healthy animals. NMJs were individually analyzed and categorized into groups based on the number of perforations within each endplate: 0 perforations represented the most immature, plaque-like endplates, 1-2 perforations indicated that the NMJ was maturing into the perforated stage, and 3 or more perforations was considered to be developmentally advanced. To avoid skewing results by including denervated endplates, units were counted only if both the presynapse and postsynapse could be visually identified.

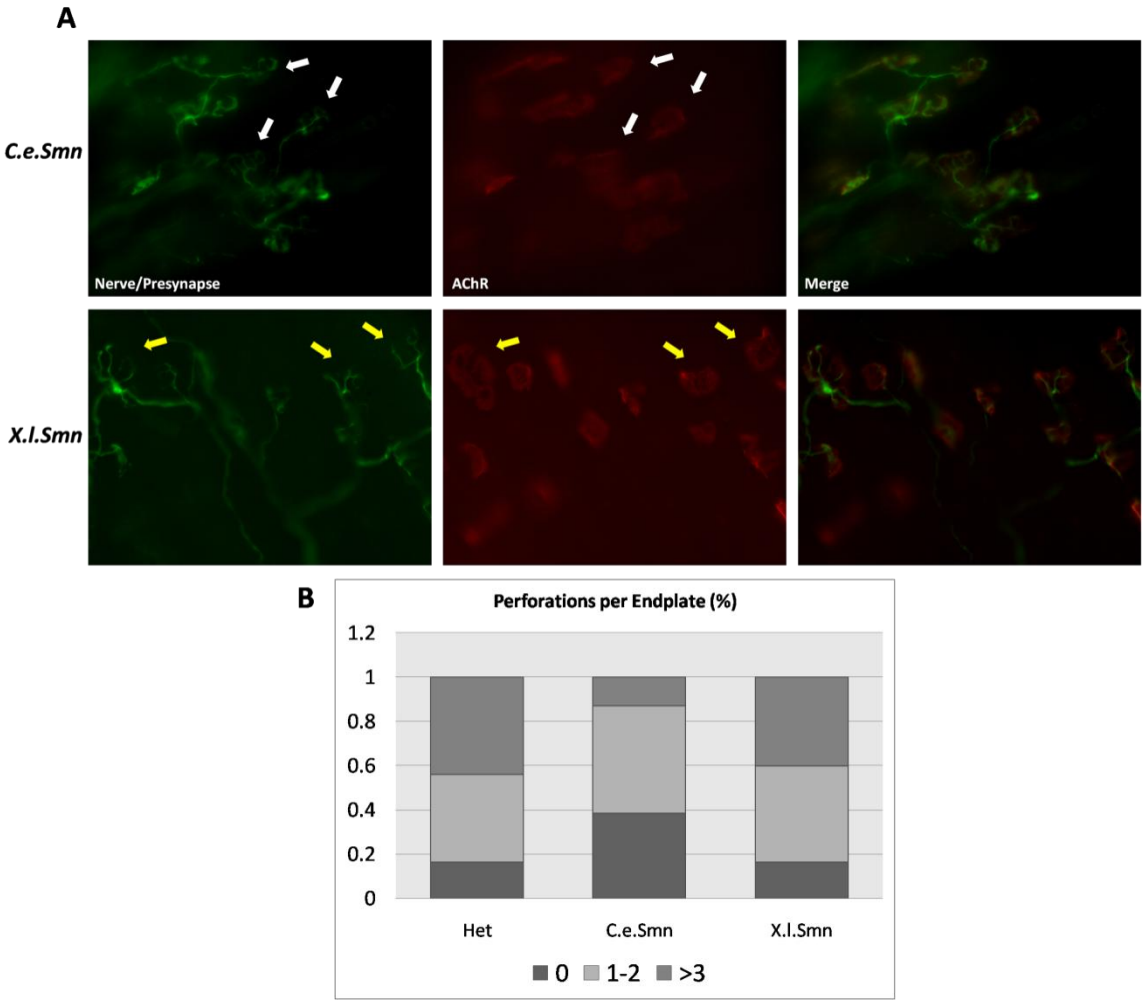

Figure 19: Endplates from C.e.Smn demonstrate less maturity

A) Representative images from animals treated with *C.e.Smn* and *X.l.Smn*. White arrows demonstrate a branching presynapse coupled with an immature postsynapse in *C.e.Smn* treated mice. In *X.l.Smn* treated mice, branching presynapses are paired with pretzel-like postsynapses and are noted with yellow arrows B) Quantification of the endplates divided into maturity stages based on the number of perforations (0, 1-2, >3) per endplate. Numbers are represented as a percentage NMJs counted within the entire splenius capitis.

After establishing that defects can be seen in animals treated with *C.e.Smn* at the gross phenotypic level as well as at a physiological level, we aimed to determine if these defects were due to dysfunction of *C.e.Smn* at a molecular level. To this end, there is an established panel of mRNAs that can serve as indicators as to whether SMN is able to perform RNP-related functions. To perform this analysis, animals that received each treatment were harvested on PND12 and their spinal cords were used to assess mRNA changes. The results of SMN's molecular function matched the phenotypic outcome of the animals. *X.l.Smn* was able to restore proper splicing of *Stasimon*, keep levels of *H1c* and Cdk-1a low, and increase levels of *Chodl* compared to their untreated counterparts. Each of these improvements were comparable with those afforded by FLSMN. Alternatively, *C.e.Smn* did not yield any mRNA changes *in vivo* compared to SMNΔ7 mice.

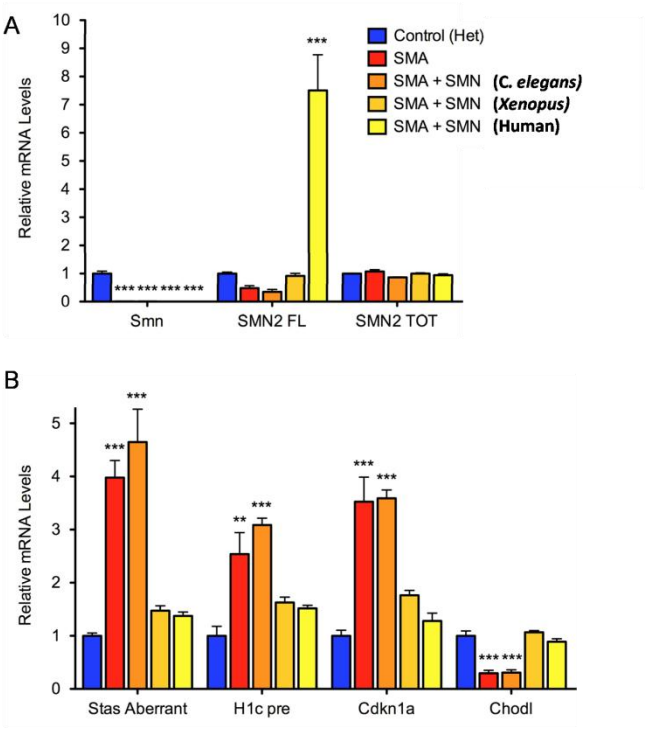

Figure 20: SMN homologs demonstrate different abilities to participate in RNA modifications.

Total mRNA levels from mouse spinal cord (n=3) were assayed by qPCR. Relative mRNA levels are normalized and compared to heterozygous untreated animals. A) Levels of *M.m.Smn,* full-length *SMN* derived from *SMN2* and total transcripts from *SMN2* demonstrate that injected mice express no unexpected levels of *SMN.* B) A panel of transcripts indicate whether certain SMN-related roles are being fulfilled in relation to splicing, 3'-end processing and stability. *C.e.Smn* (dark orange) is not able to restore these mRNAs more effectively than no treatment (red). Conversely, *X.l.Smn* (gold) restored mRNA levels to a similar degree as treatment with FLSMN (yellow).

This work was performed by L. Pellizzoni, Columbia University

3.6: Sequence comparison reveals a missing region that correlates with rescue effect

We next sought to determine which regions of SMN could be responsible for the discrepancy between those homologs that rescue and those that do not. Upon multiple sequence alignment, it becomes immediately apparent that each of the homologs which did not rescue (*C. elegans*, *Drosophila*, and *S. pombe)* encode no sequence homologous to exons 4 and 5 of human SMN. These regions are known to house the polyproline region of SMN which, in turn, is responsible for SMN's interaction with profilin. To determine whether this region was responsible for the differential rescue effects seen with different homologs, we probed this region more specifically. First, we created a virus carrying a version of human SMN carrying the mutations P219-224A. The disruption of these central prolines has been shown to abrogate SMN binding to profilin IIa. Whereas delivery of FLSMN provides a robust rescue in mice, this mutated version afforded no improvement over SMNΔ7 knockout animals. This striking contrast to human FLSMN will be further investigated to ensure the viral delivery was effective. However, this result seems to indicate that binding to profilin is necessary for ameliorating SMA *in vivo.*

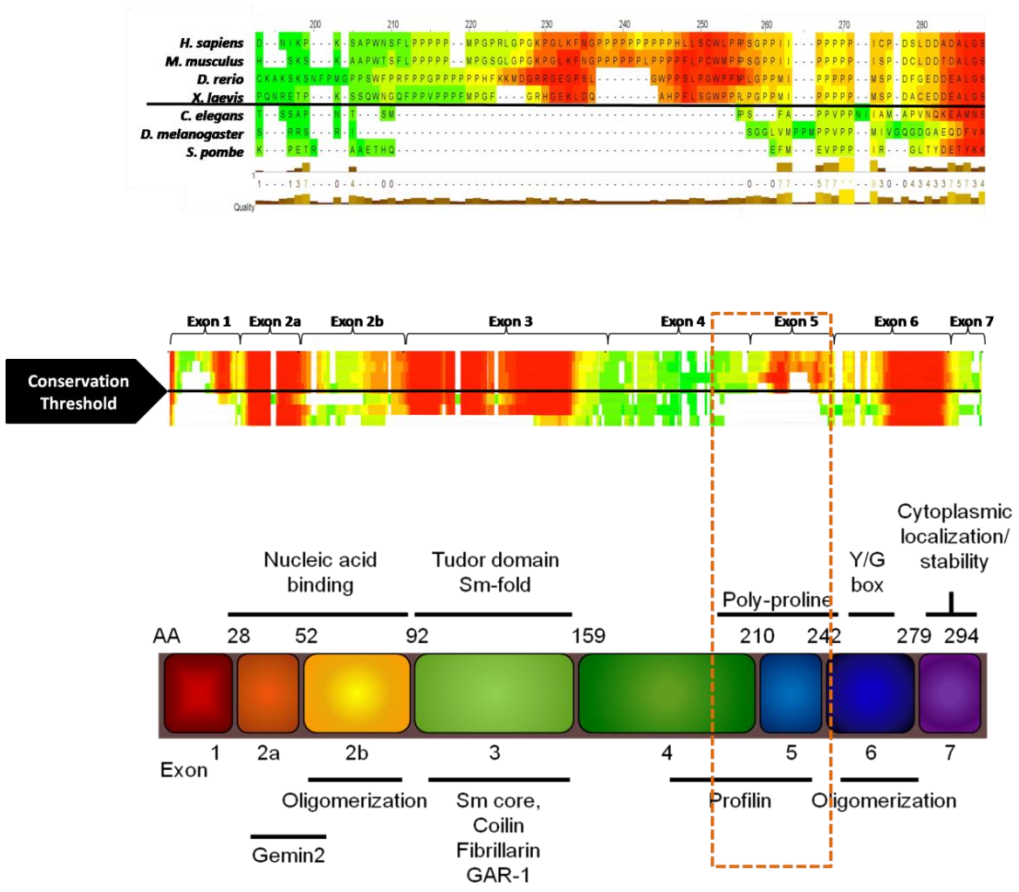

Figure 21: Identification of an *in vivo* conservation threshold identifies regions of interest in aligned SMN homologs.

Multiple alignment produces a heat map of conservation among SMN homologs from similar residues (red) to dissimilar residues (green). White spaces indicate a gap assigned to that location. The black line indicates the conservation threshold. Homologs above the threshold demonstrate phenotypic improvement in mouse models of SMA while those below do not. Homologs below the threshold all have gaps in the proximity of exons 4 and 5 while those above the threshold demonstrate high similarity. This region corresponds to the SMN polyproline region and Profilin interacting domains. Alignment and heat map image rendered by T-Coffee.

3.7: Profilin-interacting domain may be necessary, but not sufficient to restore rescue ability in C.e.Smn

We then aimed to determine if the polyproline region is sufficient to restore rescue ability to an otherwise non-functional SMN homolog. For this, we chose *C.e.Smn,* as it is the most closely related homolog that failed to rescue in our initial trials. We created a chimeric sequence by inserting the human polyproline region into *C.e.Smn* guided by sequence alignment. Notably, *C. elegans* does have a few conserved prolines. However, rather than continuous stretches of prolines, the *C.e.Smn* motif is interrupted by a valine. To most closely match the human SMN polyproline region, this native sequence was replaced by the continuous proline pattern of human SMN. This chimeric sequence is referred to as $C.e.Smn^{HumProf}$. When injected into mice, this construct did not perform better than its native counterpart, nor uninjected SMNΔ7 animals. Therefore it seems that the critical differences that prevent *C.e.Smn* from rescuing mice are more expansive than the poly-proline region alone.

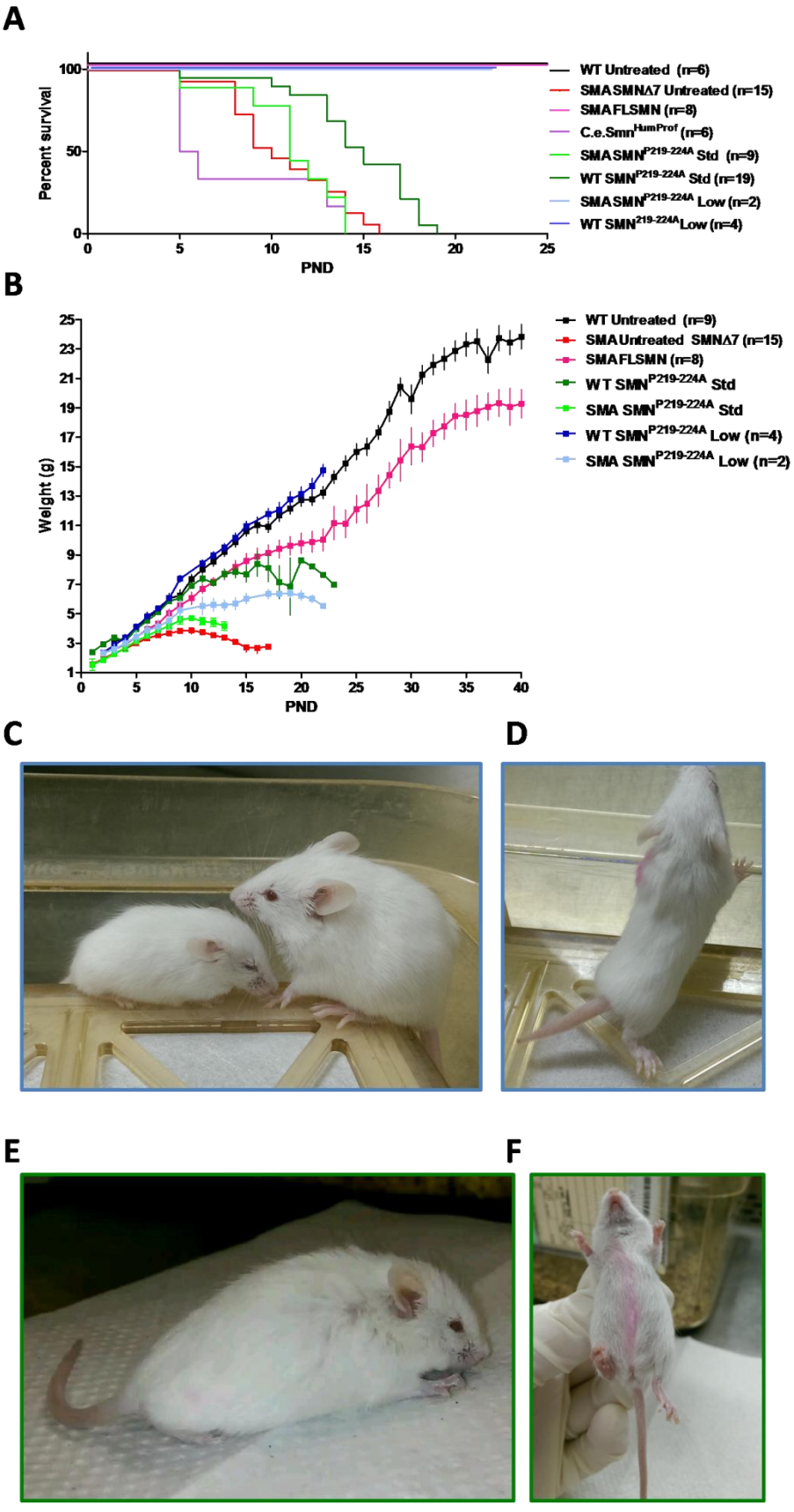
A
Percent survival
PND
WT Untreated (n=6)
SMASMNΔ7 Untreated (n=15)
SMAFLSMN (n=8)
C.e.Smn HumProf (n=6)
SMASMN P219-224A Std (n=9)
WT SMN P219-224A Std (n=19)
SMASMN P219-224A Low (n=2)
WT SMN 219-224A Low (n=4)
B
Weight (g)
PND
WT Untreated (n=9)
SMA Untreated SMNΔ7 (n=15)
SMAFLSMN (n=8)
WT SMN P219-224A Std
SMA SMN P219-224A Std
WT SMN P219-224A Low (n=4)
SMA SMN P219-224A Low (n=2)
C
D
E
F

Figure 22: The profilin-interacting domain is required *in vivo.*

A) Survival analysis of SMNΔ7 animals, both WT (includes *mSmn+/+; SMN2; SMNΔ7* and *mSmn +/-*; *SMN2; SMNΔ7*) and SMA (*mSmn-/-; SMN2; SMNΔ7*) animals were injected with standard and low doses of $SMN^{P219\text{-}224A}$. Low dose animals were still alive at the time of writing. B) Weight obtained by treatment groups vs controls. Low dose $SMN^{P219\text{-}224A}$ is beneficial to SMA animals but does not affect healthy animals. Standard dose $SMN^{P219\text{-}224A}$ does not greatly affect SMA animals, but has a highly detrimental effect on healthy animals. C) SMA (left) and WT (right) animals treated with low dose $SMN^{P219\text{-}224A}$ at PND 20. Mouse tails cannot be seen due to a crevice in the holding container. D) SMA mouse injected with low dose $SMN^{P219\text{-}224A}$ is able to climb independently at PND 20. Tail necrosis typical of treated SMA animals can be seen. E) WT animal treated with standard dose $SMN^{P219\text{-}224A}$ demonstrates a SMA-like phenotype on PND 18. Animal displays kyphosis, poorly kept fur coat, rapid labored breathing, tremors while walking, stunted gait and little ambulation. F) WT animal treated with standard dose $SMN^{P219\text{-}224A}$ demonstrates weak leg muscles that are unable to splay and clenched digits. Distal symptom severity is asymmetric.

Due to the nature of this work, many differences exist between human SMN, the homologs that rescue, and those which do not. In order to test the importance of binding Profilin 2 in a more precise manner, we decided to abrogate Profilin binding alone in the context of human SMN. The human Profilin interacting domain overlaps the polyproline region. As Profilins have been known to bind to polyproline regions previously, this binding has been characterized within SMN. Three polyproline stretches exist within human SMN. The overall motif contains P(5)-X(17)-P(10)-X(17)-P(5). It has been shown that upon mutation, the first and last span of prolines do not abrogate interaction with Profilin. However, mutating the central span disrupts binding; the mutated motif p219-224a fails to bind profilin *in vitro*[214]. Accordingly, we performed these same mutations in human SMN and delivered the construct into mice. At a standard dosage of 1e11 vg, this construct failed to rescue sick SMNΔ7 mice and also exerted a dramatic negative effect on healthy animals: both heterozygous and wild type animals developed SMA-like symptoms and died before weaning. As seen by weight gain, these animals were comparable with their healthy untreated counterparts until day 8 at which point they lost weight until death. Phenotypically they develop a slight kyphosis, appear to be overweight, and suffer from labored breathing. Some symptoms such as leg and foot splay are asymmetric in severity which could be attributed to incomplete viral spread. It is interesting that there was no negative difference in sick animals treated with this construct. This may indicate that when delivered at high levels, $SMN^{P219\text{-}224A}$ acts as a dominant negative and that the effects are either less potent or more delayed than the molecular progression of SMA in the sick SMNΔ7 animals.

In order to determine whether this negative effect was due to a contaminant within this viral preparation or whether it correlates with the viral genomes injected, we delivered a dosage of the same volume from a sample within the same viral preparation that was less concentrated in viral genomes. While the standard dosage is $1x10^{11}$ vg, this low dose was almost 10-fold lower, at $1.7x10^{10}$ vg.. This lower dosage did not adversely affect healthy animals, suggesting that the deleterious effects at the higher dosage were due to the transgene rather than a non-specific effect of the viral preparation. Of further interest was that this lower dosage provided benefit to the sick SMNΔ7 animals. At the time of this writing, two confirmed knockout animals are alive and highly ambulatory at day 21. They right themselves instantaneously, climb quickly and without difficulty and are able to keep their fur coats well maintained. They are notably smaller than their healthy counterparts: sick animals have weight around 6-7 grams steadily for the previous 4 days whereas healthy animals that were also injected weigh approximately 12 grams. In summarly we see that that SMN with the P219-224A mutation is beneficial to SMA mice at a low dosage, yet harmful to even healthy animals at a high dosage. While this seems counterintuitive, it fits within the context of SMN as a multimer. Multiple copies of SMN

Interestingly, however, although sequence is found homologous to exon 4 within *D.r.Smn* and *X.l.Smn* and these homologs demonstrate high localized similarity in this vicinity, the central proline region is not ultimately conserved. Each of these homologs, *D.r.Smn* and *X.l.Smn*, have a conserved region that lacks these prolines, and yet are able to remarkably improve the SMA condition in mice. These data together have not yet been resolved. The means by which alanine substitution within human SMN is able to abrogate its naturally robust rescue ability, yet *D.r.Smn* and *X.l.Smn* still function without these

residues requires further testing. Protein analysis to determine the proper expression of p219-224a will help confirm that these preliminary results are valid.

3.8: The Tudor domain is not the only defective region of C. elegans Smn

As SMN's role in snRNP assembly is considered crucial to its function, we decided to look at the effect that altering the Tudor domain could have on SMN function *in vivo.* It is known that patient mutations within the Tudor domain (E134K) can have deleterious effects on SMN function. To get a better structural understanding of the differences between the homologous Tudor domains, we ran protein folding predictions through ITASSER. In order to match the crystallized human SMN Tudor domain (PDB ID: 1MHN), sequences were cropped at the residue corresponding to SMN L89 and E147. ITASSER recognized this sequence as homologous to 1MHN and threaded it onto this frame. Due to variations in the Tudor domain of *Xenopus* and *C. elegans*, the protein prediction software was not able to recognize and recapitulate all secondary structures identified in the crystallized human protein segment. Despite this visual discrepancy however, it is believed that all homologs of SMN carry Tudor domains which fold similarly. This is essential for the binding of Sm proteins which is in turn seen to be essential for each species discussed.

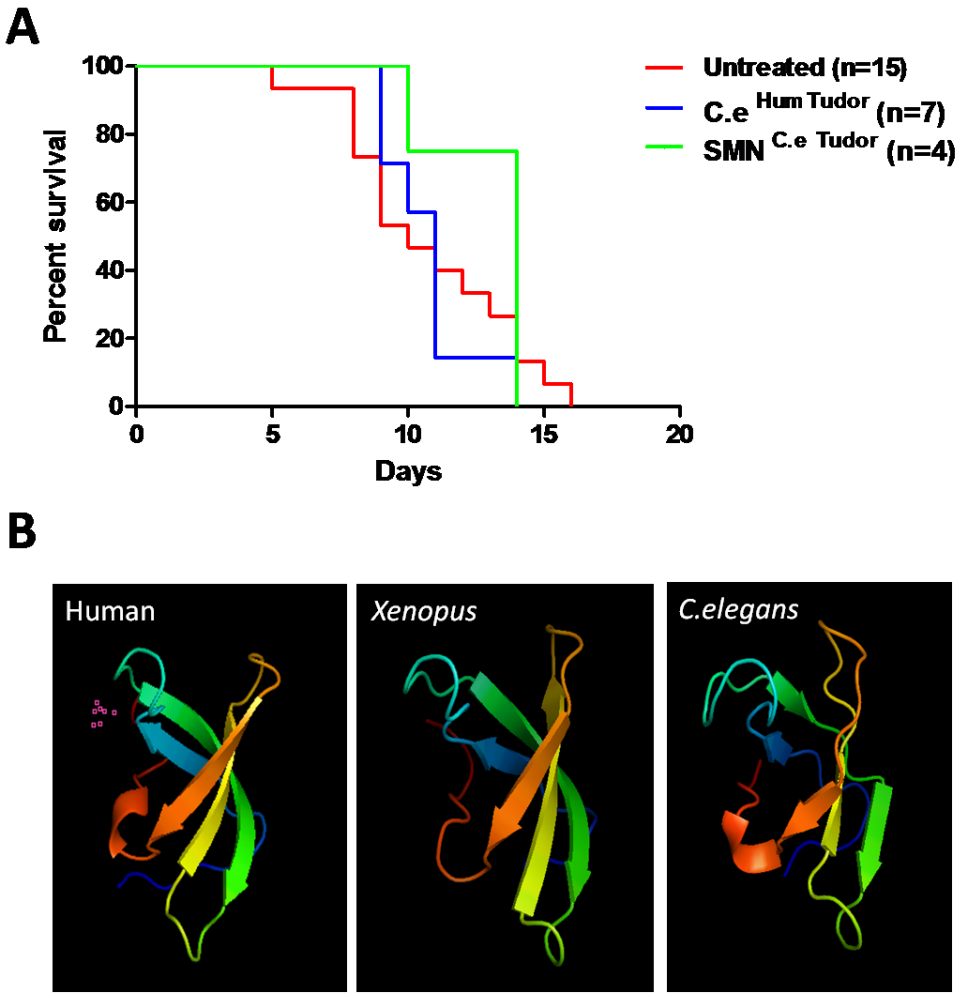

Figure 23: The *C.elegans* Tudor domain may be one reason that *C.e.Smn* does not function in vivo.

A) Despite high levels of amino acid similarity, structural prediction of SMN Tudor homologs does not recognize all structures within *Xenopus* and *C. elegans* Smn. An N-terminal helix is missing from *X.l.Smn* and beta sheets within *C.e.Smn* are truncated. Prediction by ITASSER. B) Chimeric domain swap sequences are unable to rescue SMNΔ7 mice. The human Tudor domain was not sufficient to restore rescue to *C.e.Smn* (blue). Conversely, *C.e.Smn* Tudor rendered human SMN non-functional (green).

Therefore, we aimed to test whether differences within the Tudor domain were responsible for the poor function of *C.e.Smn*. We designed two chimeric proteins: the first was *C. elegans Smn* with a human SMN Tudor domain and its reciprocal was human SMN with a *C. elegans* Tudor domain. If the *C. elegans* Tudor domain is indeed insufficient to perform the functions of RBP assembly in mammals, we expect that delivery of human SMN with this defective Tudor domain would not rescue the mice. Indeed, this sequence failed to improve lifespan in SMNΔ7 mice. Notably, it did not seem to have any overt harmful effects when delivered into healthy animals. If the *C. elegans* Tudor domain was the region which prevents the overall function of this homolog in mammals, we would predict that *C. elegans* with the human Tudor domain would now be sufficient to rescue SMA mice. The evidence demonstrated that this is not the case. This construct also failed to provide improvement *in vivo* and also had no dominant negative effect when delivered to healthy animals. This evidence suggests that the *C. elegans* Tudor domain is defective in a mammalian system, but that this domain is not solely responsible for the overall dysfunction of *C.e.Smn*.

3.9: Rescue ability is not due to specific amino-acid changes among SMN homologs

As noted before, this work is complicated by the many differences that exist between the homologs of SMN. It is possible that the key differences in rescue ability are due to small mutations within the genes that disallow key interactions with homologous binding partners. For example: if *C. elegans* contains all the proper domains required for SMN function, but cannot bind to human snRNPs and Gemins, then this will result in a false negative conclusion. In order to test whether conserved domains are indeed sufficient to act as a minimal functional domain for SMN while maintaining the human SMN amino acid sequence, we created a synthetic, human SMN-based sequence. In this we chose to

include only SMN exons 2a, 2b, 3, and 6. Not only are these regions the most highly conserved, but they are responsible for the most thoroughly characterized requirements for SMN function: they contain the regions responsible for SMN oligomerization as well as binding to Gemin2 and RNA. Thus we aimed to determine if this same rescue would translate into a mammalian model. Utilizing the same mechanism by which we tested the homologs of SMN, we delivered *SMN2*36 into SMNΔ7 animals via scAAV9. Because we are primarily concerned with whether these domains are functional, rather than whether this synthetic construct could be stable, we fused EGFP onto the SMN236 N-terminus. GFP-SMN has previously been shown to be much more stable than other SMN modifications[44], thus we sought to enhance SMN236 stability and increase its opportunity to function *in vivo*. This construct demonstrated no benefit to weight gain, but seemed to prevent some of the earlier deaths within this severe model. While robust therapeutics are able to rescue this model, modest therapeutics sometimes are not sufficient to overcome the rapid decline seen in SMNΔ7 animals. Thus, we tested this construct in a milder model of SMA, the $Smn^{2B/-}$ mouse. Once again this treatment provided perhaps a slight improvement. The earliest deaths were delayed by 9 days (PND19-28) by treatment with *SMN2*36. While a difference in the sample size must be considered, still by PND 28 approximately 40% of untreated animals had already died. As in previous studies utilizing the $Smn^{2B/-}$ model, these data included a few animals that overcame the normal $Smn^{2B/-}$ endpoint and lived an extended amount of time. These animals account for most of the weight gained above uninjected counterparts. This modest gain in life is intriguing, however it does not imply that *SMN2*36 is sufficiently able to reproduce the necessary functions of SMN *in vivo*.

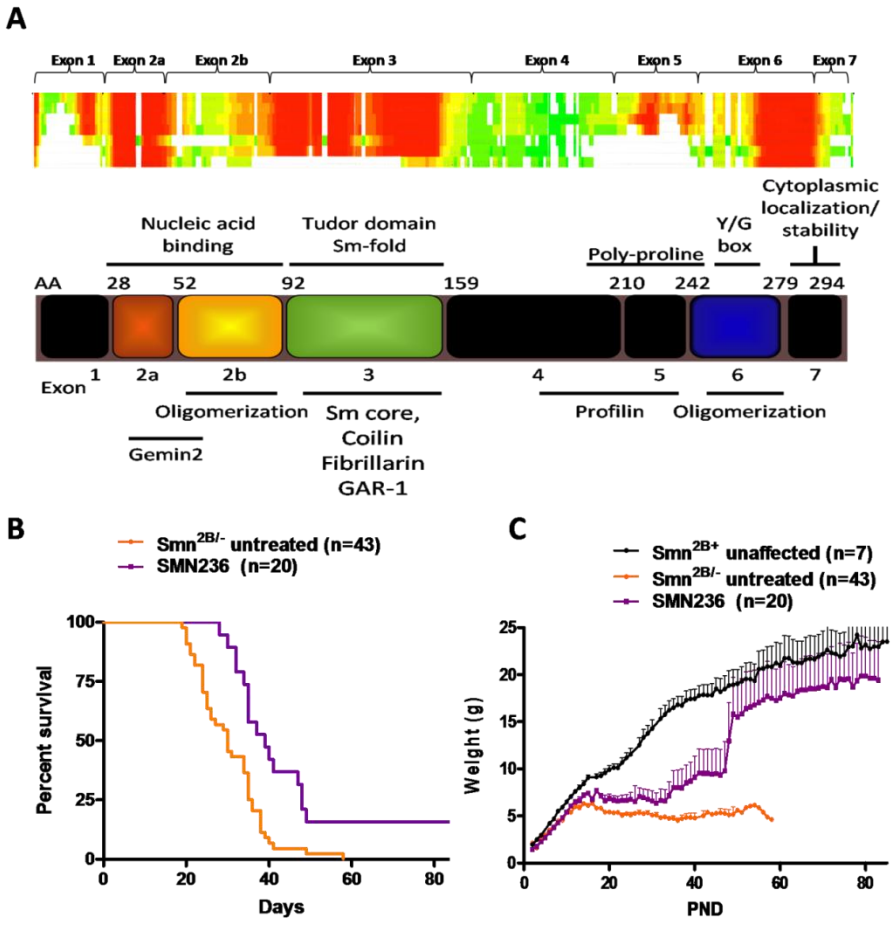

Figure 24: A human-derived fusion protein representing the most highly conserved domains yields modest effects in vivo.

A) A heat map based on SMN homolog alignment demonstrates regions of high conservation in the equivalent of human SMN exons 2a, 2b, 3 and 6. These domains contain essential oligomerization domains as well as sequence to allow binding for RNA, Gemin 2, and other RNP-related partners. B) *SMN2*36 delivered into *Smn*$^{2B/-}$ mice delays early deaths by 9 days. C) Treatment with *SMN2*36 has the greatest weight gain effect in long-lived animals.

4: Discussion

To our knowledge, this work is unique in taking an evolutionary approach to establishing a minimal functional domain. Based on sequence conservation, one might hypothesize that exons 2, 3, and 6 are the most important to SMN function and possibly that they are sufficient for SMN rescue. Indeed, these regions are involved in SMN oligomerization and scaffolding the formation of various RNPs. When disrupted, these regions cause a loss of SMN function. However, these functions are known to be so important for cell survival that they are considered the reason behind the dramatic lethality associated with knockout of SMN in other models and the low rate of birth among patients expressing very low levels of SMN. Here, we demonstrate that the functions afforded by these regions of SMN are not sufficient to rescue a mammalian model of SMA.

Whereas there are small difference between the SMN homologs even in these highly conserved areas, the fusion protein of SMN-236 maintained all appropriate human SMN residues, yet still did not function *in vivo. SMN2*36 represents the most highly conserved regions of SMN that are also thought to be most critical for its function. However, this construct was still unable to rescue a severe or intermediate model of SMA. We have therefore shown that not only the specific sequences, but the domains within SMN that were required in early evolutionary history are not sufficient to provide overt benefit in a mammalian system. This implies that functions found within exons 1, 4, 5, or 7 may contain key elements for SMN function in SMA. The noticeable absence of the profilin-interacting domain in those species that failed to rescue led us to investigate whether this was the missing region responsible for dysfunction of distantly related homologs. While our *C.e.Smn*^{Prof} chimera did not demonstrate increased survival, we

found that a simple mutation in this region can abrogate function of FLSMN. Together these data implicate the Profilin-interacting domain, and therefore SMN's roles in actin dynamics, among the *disease-relevant* functions of SMN. While RNP assembly may be an essential housekeeping function, it may not be solely responsible for the motor neuron specific display of SMA symptoms. However, mRNA analysis of *Xenopus* and *C. elegans* Smn implied that *C.e.Smn* was not able to properly carry out any of the RNP-related functions that FLSMN and *X.l.Smn* performed.

Together, this with previous evidence suggests that there could be differential requirements for SMN function between vertebrates and invertebrates. Specifically consider the *Drosophila* model of SMA. The endogenous *D.m.Smn* contains similar domains to *SMN2*36 and while published results demonstrate that restoration of *D.m.Smn* appropriately rescues the fly model, *SMN2*36 is purportedly able to improve this phenotype as well. The ability of these genes to rescue flies then contrasts with our findings in two mouse models. Of additional interest is the fact that human SMN is shown to have a dominant negative effect when overexpressed in a *D.m.Smn* mutant fly. To our knowledge the mechanism behind this effect has not been investigated. However previous findings coupled with our own demonstrate that these homologs are not interchangeable in a functional sense.

Further analysis could identify patterns aside from the simple conservation threshold. First, there is a striking difference between the efficacy of *D.r.Smn* and *X.l.Smn* that we did not further investigate. Unlike the binary effect seen across the conservation threshold, the rescue effect with these species falls on a gradient. It would be of interest to compare another non-mammalian vertebrate species to see whether the

rescue effect afforded also falls within this gradient. Unfortunately no reptilian SMN sequence has been characterized and therefore this work would relay on a predicted protein sequence. The sequential differences responsible for the differential rescue effect are not immediately apparent from sequence comparison. These differences likely do not represent a lack of function and would require more sensitive analysis to tease apart.

Another interesting suggestion within our data is the discrepancy between *X.l.Smn* and FLSMN. The SMN homologs from human, mouse, share only 54% and 58% identity with *Xenopus*, respectively, thus the differences in their effect *in vivo* are not overly surprising. The rescue effect of *X.l.Smn* was indeed considerable, but not nearly as effective as rescuing with human SMN. However according to our mRNA-based assays, the *Xenopus* homolog was able to produce proper levels of each mRNA *in vivo*, implying that its function involved with various RNP complexes was intact. This is supported by previous findings that *X.l.Smn* is capable of restoring Cajal Body formation in an SMN knockdown environment[258]. CB formation can only be restored by the proper import of nascent snRNP complexes[41]. It can then be inferred that the difference in the efficacy with *X.l.Smn* is not due to an overt loss-of-RNP function, but a loss or decrease in function or binding affinity in another aspect of SMN function.

One aspect which could affect the rescue ability of SMN homologs is the ability of each to localize within the nucleus or cytoplasm. This localization has been partly attributed to phosphorylation of specific residues in SMN[258]. In these experiments, versions of SMN that are unable to be phosphorylated as well as phosphomimetics are able to alter the subcellular localization of SMN. However, these mutations do not influence SMN's capacity to oligomerize and to assemble into the SMN complex[258].

Interestingly, phosphorylation sites in human SMN are most heavily clustered at the N-terminus which is notably less conserved than other areas of SMN. A few sites are known to be phosphorylated in human, mouse, and rat SMN and these sites are maintained in zebrafish and xenopus but lost on the other side of the conservation threshold. Residues homologous to human SMN T25, S29, and S31 could therefore contribute to the difference in rescue efficacy seen in on either side of the conservation threshold. These residues are noted in Figure 14. In other instances where one homolog has an altered residue, the alterations do not correlate with the observed conservation threshold that we identified. For this reason, the possibility of residues S4, S5, S18, S28, T37, T62,

This effect seen with *.X.l.Smn* is modest in comparison to the survival outcomes of the *D.r.Smn* cohort, however within the field of SMA this is still a large improvement in a severe animal. Prior to the viral delivery of FLSMN, non-transgenic treatments rarely improved survival in this mouse by more than a few days. Indeed, researchers performing the first scAAV9-CBA-FLSMN were so surprised the treated lifespan that they re-genotyeped to ensure they were assaying SMA animals.

It is important to note that these experiments were not performed with sequences that were codon-optimized for translation within mice. This was chosen in order to remain consistent between sequences; some sequences were cloned directly out of model organism cDNA and thus even synthesized sequences were kept in their native nucleotide sequence.

Chapter Three: Contributions of Astrocytes to the Motor Neuron Presentation of SMA

1: Introduction

1.1: SMA as a non cell-autonomous disease

Due to the selective loss of motor neurons is Spinal Muscular Atrophy, this has long been considered a motor neuron disease. Indeed, low levels of SMN result in motor neuron loss or dysfunction in many different contexts. However, a growing body of research suggests that the disease phenotype is not neuron-autonomous. Initial evidence included the fact that motor neuron-specific ablation of SMN imparted only minor phenotypic abnormalities and did not shorten lifespan[198]. Conversely, selective restoration of SMN in these cells alone caused only a modest phenotypic improvement[193, 194, 197]. Use of the prion protein promoter to drive pan-neuronal expression provided a robust rescue effect, however it was noted that this promoter causes leaky transgene expression in other cell types, including astrocytes. Thus, it is clear that while motor neurons are affected in SMA, this may not be an intrinsic or cell-autonomous disease feature as was once thought[122].

1.2: Astrocytes' role in maintence of the CNS

As the most abundant cell type within the CNS, astrocytes serve essential roles in CNS function. These cells are responsible for maintenance of the blood brain barrier and act as the connection between the circulatory system and the CNS by delivering oxygen, growth factors and metabolic substrates. They are intimately connected with the NMJ as part of the tripartite synapse in which they are responsible for uptake and recycling of extrasynaptic glutamate[259].

1.3: Astrocytes under oxidative stress and pathology

Under diseased, stressed or injured conditions, astrocytes become reactive and undergo distinct morphological restructuring such that cell process are retracted and branching is reduced. They dramatically increase expression of intermediate filaments such as glial-fibrillary acidic protein (GFAP). Activated astrocytes secrete pro-inflammatory cytokines which can trigger apoptotic cascades in motor neurons[260].

Astrocytes have a role in mitigating oxidative stress within the CNS. Reactive oxygen species (ROS) are generated as part of normal cellular metabolism and these potentially dangerous molecules are eliminated by antioxidant enzymes such as superoxide dismutase (SOD), glutathionine peroxidase (GPX) and catalase. Chronic oxidative stress can result in DNA damage which ultimately triggers cell death.

One mechanism by which astrocytes handle situations of oxidative stress is through the NRF2 pathway. Nuclear factor erythroid 2–related factor 2 (Nrf2) is a basic helix-loop-helix transcription factor. Under normal conditions, NRF2 is sequestered in a cytoplasmic complex with KEAP1. Cysteine residues on KEAP1 allow for the interaction of Cullen 3 which is responsible for ubiquitinating NRF2 and triggering its degradation. Thus under normal conditions, the NRF2 half-life is approximately 20 minutes[261]. However, as reactive oxygen species rise within the cytoplasm in diseased or stressed conditions, the cysteines on KEAP1 no longer allow for interaction with Cullen3. Barring its degradation causes NRF2 to accumulate within cells and transport into the nucleus. Here, it binds to antioxidant response elements (AREs) and causes upregulation of a number of antioxidant genes.

1.4: Astrocytes in neurodegeneration

Due to their intimate interactions with motor neurons, astrocytes specifically have been of interest within a neurodegenerative disease context. Both *in vivo* and *in vitro* studies in the field of amyotrophic lateral sclerosis (ALS) demonstrate that astrocytes expressing mutated SOD1 are able to impart increased motor neuron death[262-265]. Further, other neurodegenerative diseases are caused by ubiquitously expressed genes and were once thought to caused by motor neuron intrinsic defects. A growing set of literature suggests that diseases such as ALS, spinocerebellar ataxia (SCA), and Huntington's Disease (HD) may be caused by toxic effects in neurons and/or non-neuronal cell types[266].

Due to its effects on oxidative stress and as a neuroprotectant, NRF2 has been shown to be beneficial in the context of several different disease models. In ALS, this was demonstrated to protect against neurodegeneration in two different mouse models of the disease[267]. This effect was seen when NRF2 was restored in astrocytes, but not when selectively restored to neurons or muscle[268]. NRF2 appears to upregulate genes that ameliorate symptoms of Alzheimer's disease[269]. Within a fly model of Parkinson's disease, genetic activation of NRF2 can ameliorate the animal's locomotor phenotype[270].

1.5: Astrocytes in SMA

In an SMA context, it is clear that astrocytes are in a reactive state. Autopsy results from SMA patients note gliosis within the spinal cord in Type I [271], Type II[272] and Type III[273]. Recently astrogliosis is found to be primarily located within the grey matter of the ventral horn where α-motor neurons are known to reside[134]. Severe SMA mice (*M.m.Smn*$^{-/-}$*; SMN2*$^{+/+}$*)* which die by PND7 are born displaying increased levels of GFAP within the spinal cord[274]. A similar finding demonstrated that GFAP staining within spinal cord sections of SMNΔ7 mice becomes stronger as the disease progresses[134]. Further within

these conditions, astrocytes are shown to have thick, short processes and significant upregulation of GFAP. Interestingly, this effect was seen at both pre-symptomatic (PND3) and post-symptomatic (PND9) time points[132]. Similar phenotypes were found in SMA iPSCs differentiated into astrocytes[132]. Co-culture experiments demonstrate that SMA iPSC-derived astrocytes are able to incite reactive morphology in healthy astrocytes in a manner that is contact dependent[133].

Astrocytes have been shown to have intrinsic defects in the context of low SMN. As the interactions between astrocytes and other cell types are critical for astrocyte function, these are of interest in SMA. Indeed, SMA astrocytes make fewer connections than wild type astrocytes, possibly due to their retracted processes. Astrocytes have the ability to negatively or positively influence their neighboring cells. Data from embryonic stem cells differentiated into specific cell types demonstrates that healthy astrocytes become reactive when cultured among SMA astrocytes[133]. Co-culture of primary cells from SMNΔ7 animals demonstrates that while SMA astrocytes are able to promote motor neuron survival, they are less efficient at promoting motor neuron synapse formation compared to their healthy counterparts[275]. In both of these studies, the ability of astrocytes to influence neighboring cells is contact dependent.

Oxidative stress has been implicated in the context of SMA. Mitochondrial defects and an increase in free radical production correlate with a progressive knockdown of Smn in mouse motor neuron-like cell culture[276] and in human embryonic stem cells[277] [2013] . Within these experiments, addition of antioxidants ameliorates neuronal phenotypes. Further, in cell culture SMA motor neurons show some improvements upon addition of cucumin and reservatrol, polyphenol antioxidants that can be found in food sources[278, 279]

[2014].Very recent findings suggest that oxidative stress exacerbates the splicing exclusion of SMN exons 5 and 7 across many tissue types *in vitro* and *in vivo*[280, 281]. This implicates that the role of astrocytes in protecting the motor neuron from oxidative stress may result in changes of SMN levels in the motor neuron.

Recent evidence contradicts the suggestion that oxidative stress is a part of SMA pathology. Findings in astrocytes and in motor neurons differentiated from SMA patient iPSCs indicate that oxidative stress is not the cause of the reactive phenotype in SMA astrocytes. Neither low levels of SMN nor restoration of SMN dramatically changed mitochondrial respiration or expression of oxidative stress markers[133]. Further evidence will be required to understand these differential findings.

1.6: Restoration of SMN benefits astrocytes

Restoration of SMN to astrocytes is seen to have a beneficial effect not only for the astrocytes, but for motor neurons as well. *In vitro* this can be seen in iPSCs derived from SMA patients. SMN-deprived cells differentiated into astrocytes normally show characteristics of reactive astrocytes including increased GFAP expression, retracted processes and increased levels of reactive oxygen species (ROS). Upon viral re-expression of SMN, these reactive characteristics are mitigated[133]. Viral restoration of SMN in SMA mouse astrocytes also had therapeutic effects. This delivery provided a substantial improvement in lifespan and weight gain in both SMNΔ7 and *Smn*$^{2B/-}$. Importantly, the number of motor neurons was not changed by this result, however the number of synapses that each motor neuron made was improved[134].

2: Results

2.1: Restoration of SMN to astrocytes dramatically improves SMA mouse phenotype

Previous research has utilized genetic means to selectively restore SMN, however the use of viral vectors to establish long-term transgene expression has proven to be a powerful and remarkably reproducible means to ask similar questions. Previous reports have examined the effect of selectively ablating and restoring SMN in motor neurons. Studies on astrocyte restoration have thus far only been performed *in vitro*, or by viral means *in vivo*[134]. Thus, a head-to-head comparison of the efficacy of SMN restoration in motor neurons vs. astrocytes has not been demonstrated. Such comparison would give new insight as to the relative contribution each cell type has on the manifestation of SMA. Viral-directed gene therapy is currently underway in patients utilizing a robust, ubiquitous promoter. This is certainly the right choice for this type of SMA therapeutic given that SMN is depleted in all cell types, has not been shown to be toxic in any cell types, and SMA is being proven to be a multi-tissue disorder. However, without fully in understanding the ramifications of SMN depletion in other cell types, there could be alternative therapeutic avenues left uninvestigated. Indeed, similar work investigating the roles of other CNS cells in the field of ALS lead researchers to identify key antioxidants that ameliorate disease symptoms *in vivo.* This has since influenced some patients to alter their diet in attempt to promote these antioxidant effects. While these diet fads are not yet scientifically supported, the case serves to illustrate that implication of other cell types in the disease progression of ALS has quickly lead to creative strategies attempting to mitigate the disease.

To establish the relative role that motor neurons and astrocytes play toward the SMA phenotype, we created viral vectors capable of selectively restoring SMN to these cell types. scAAV has been repeatedly demonstrated to be an effective means of

delivering transgenes in an SMA context. However, this virus has an extremely limited carrying capacity. AAV, its naturally occurring predecessor, demonstrates the same characteristics of tropism and long-term expression, however this gene has double the carrying capacity of scAAV. Even this capacity is quite limited, allowing for only 4.7kb of sequence that can be packaged. Thus, this means of delivery is limited to relatively small promoter and gene combinations. While the astrocyte-specific GFAP promoter is small enough to fit into the context of scAAV(~700bp), no motor-neuron specific promoter is this short. However in 2005 a minimal enhancer was identified which was responsible for driving strong HB9 expression in motor neurons. This HB9 enhancer in front of a β-globin promoter altogether occupies approximately 2.6kb, making it too big for scAAV, but small enough to fit into rAAV. Therefore, we created different AAV constructs driving FLSMN under either GFAP or HB9 promoters.

Whereas scAAV can express in as little as 3 days by avoiding the rate-limiting step of second-strand synthesis, rAAV can take up to 9 days for robust expression. This is well after symptom onset in the SMNΔ7 model, and only 4-5 days before the animal's predicted death. Therefore, we opted to use an intermediate model of SMA to give the transgene time to express robustly before the rapid disease onset. This SMN-independent work did not require the presence of human *SMN2*, so we utilized the $Smn^{2B/-}$ model. This model is advantages because it displays intermediate SMA phenotypes living on average 28 days and also that expected Mendelian outcome of each litter yields 50% affected pups.

One standard protocol for preclinical gene delivery in SMA is to inject $1x10^{11}$ viral particles via ICV delivery. This CNS delivery is an established means for robust

expression within the brain and spinal cord. One of our primary interests was to determine whether the effects of scAAV-GFAP-SMN could be replicated using rAAV-GFAP-SMN. Therefore, using ICV injection, we delivered GFAP-SMN and compared this to an identical construct encoding GFP rather than SMN. As measured by weight and longevity, this treatment had a strong rescue effect on the *Smn*$^{2B/-}$ phenotype. Notably, within the GFAP-GFP treated group, weight gain mirrored that of uninjected animals. However, 2 of 5 animals treated with GFAP-GFP lived past 30 days: one animal died at 45 days while the other died at 67 days. With a relatively small sample size it is difficult to assess whether this effect was due to treatment with GFAP-GFP as the *Smn*$^{2B/-}$ mice are known for having a low percentage of animals that are long lived. However, it is notable that of 7 mice injected with GFAP-SMN, all grew to healthy levels and lived past 90 days. The phenotypic difference between animals treated with SMN and those treated with GFP was easily observable within a few days of disease onset.

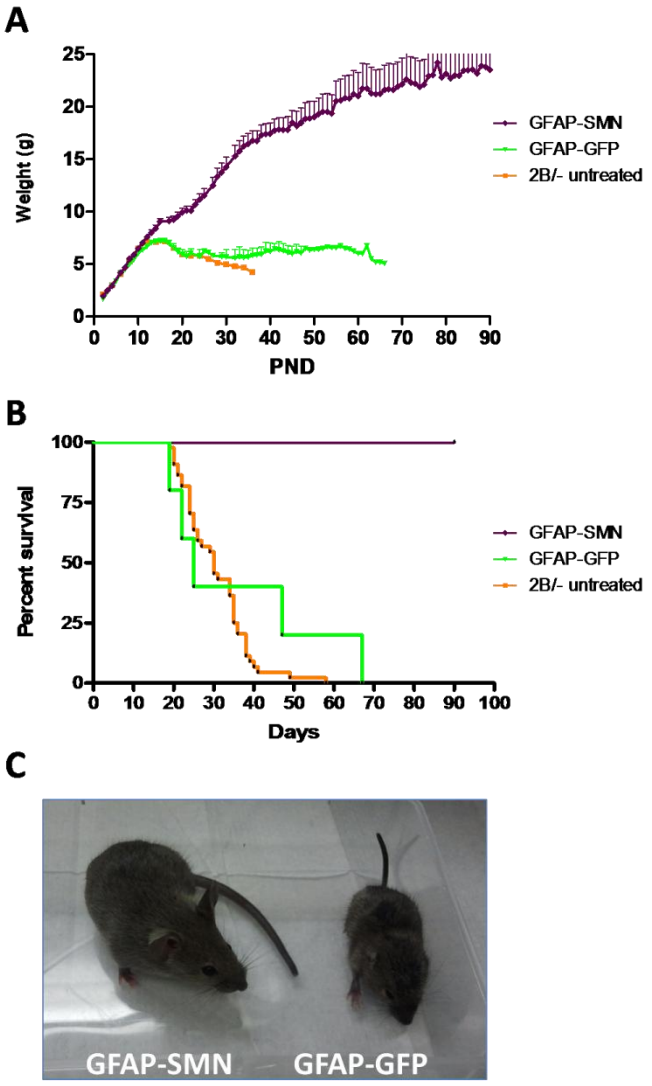

Figure 25: ICV delivery of GFAP-SMN provides robust rescue in *Smn*$^{2B/-}$ mice.

A) Animals dosed with 1x10^{11} viral particles of GFAP-SMN all lived past 90 days whereas animals injected with GFAP driving GFP had only a few long lived animals that died by day 70. B) Treated animals all displayed a uniform improvement in weight. C) Animals treated with therapeutic are phenotypically healthy compared with those treated with GFP.

2.2: SMN driven by GFAP and HB9 promoters both benefit the SMA condition

For direct comparison between GFAP- and HB9-driven SMN, we chose an intravenous route of delivery for a few reasons. First, because ICV delivery involves delivering a large bolus of liquid directly into the brain, we hypothesized that this high, localized concentration of virus may result in higher levels of leaky expression within motor neurons. This is in line with previous findings that scAAV-GFAP-GFP injected ICV can be found in ChAT+ cells (unpublished observation). Second, as viral restoration of SMN is such a powerful expression tool, it is sometimes preferred to use suboptimal dosing or delivery in order to detect meaningful differences between treatment groups.

Thus, $Smn^{2B/-}$ mice were injected IV with $1x10^{11}$ viral particles of either HB9- or GFAP-SMN. Differences between treatment groups were difficult to discern by examining longevity. While median lifespan and log-rank Mantel-Cox statistical tests indicate that mice treated with HB9-SMN outlived those treated with GFAP-SMN($p=0.0001$), the small sample sizes make biological significance difficult to ascertain. However, the difference in weight gained in these two groups is considerable. According to this measure, GFAP-SMN treated mice were much healthier than their HB9-SMN injected counterparts. Both groups had 3 very long lived individuals that reached a steady weight by day 70. While motor neuron restoration allowed mice to gain upwards of 16 grams, astrocytic restoration yielded mice of approximately 17-24 grams by PND90. Together these results imply that astrocytic restoration of SMN is beneficial *in vivo* and that relative to motor neurons, these cells could have a significant contribution to the SMA phenotype.

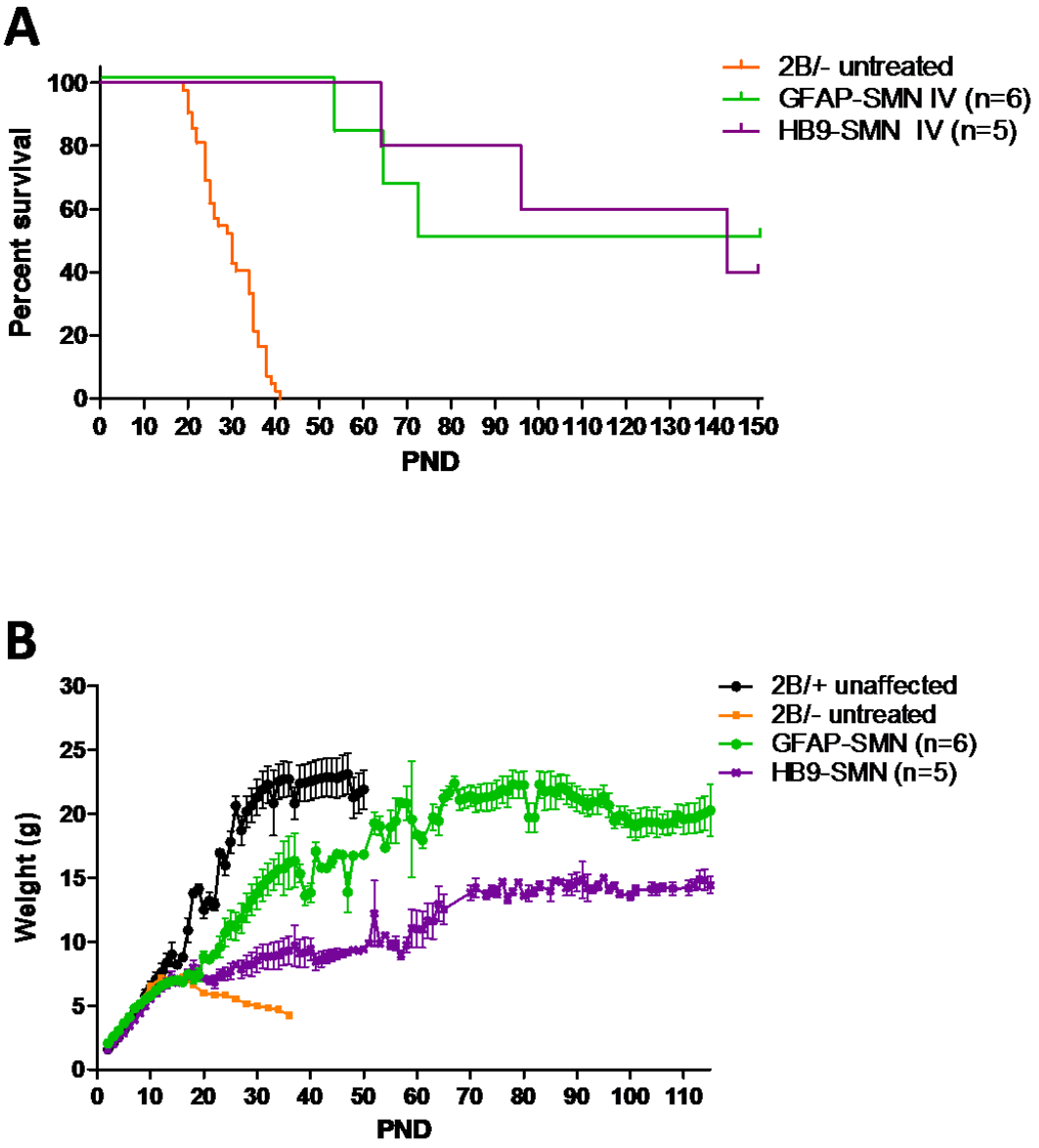

Figure 26: IV delivery of SMN under astrocytic and motor neuronal promoters improve the condition of intermediate mice.

A) Lifespan of treated mice compared to untreated *Smn*$^{2B/-}$ mice demonstrate that both treatments result in 50% of animals living past 70 days. Healthy animals do not die within the timeframe depicted. B) Both treatments improve weight gain. Mice treated

with GFAP-driven SMN (green) gain more weight than those treated with HB9-driven SMN (purple).

2.3: Expression of NRF2 is not sufficient to ameliorate the SMA phenotype in vivo

While we and others have now seen that enhancing health of the astrocyte population can have positive effects on SMA, we sought to identify SMN-independent means of promoting astrocyte health. It is known that one way in which astrocytes maintain a healthy CNS environment is through management of reactive oxygen species. Left unchecked, high ROS levels can damage proteins, lipids and DNA. Accordingly, cells have oxidative stress responses that become activated upon increased ROS levels. As described earlier, NRF2 is considered a "master regulator" of oxidative response genes. Astrocytes are the primary source of NRF2-mediated neuroprotection[282]. In these cells, mild levels of oxidative stress can activate NRF2 pathways, triggering the upregulation of many ARE-containing genes [283]. We investigated whether this same pathway could improve the SMA condition.

Expression of NRF-2 in an intermediate model of SMA did not have an overt effect. The earliest deaths in this cohort were delayed by 4 days, and there were two long lived animals. However, the *Smn*$^{2B/-}$ survival curve is not as tight and predictable as other SMA models such as the SMNΔ7 mice. In these instances, long-living mice still manifest an SMA-like condition including reduced weight gain, kyphosis, and tremors. Delivery of NRF0--2 did not improve this condition, as seen by the slight weight of the long living mice: the oldest mouse weight hit a plateau of approximately 13 grams before the experiment was terminated.

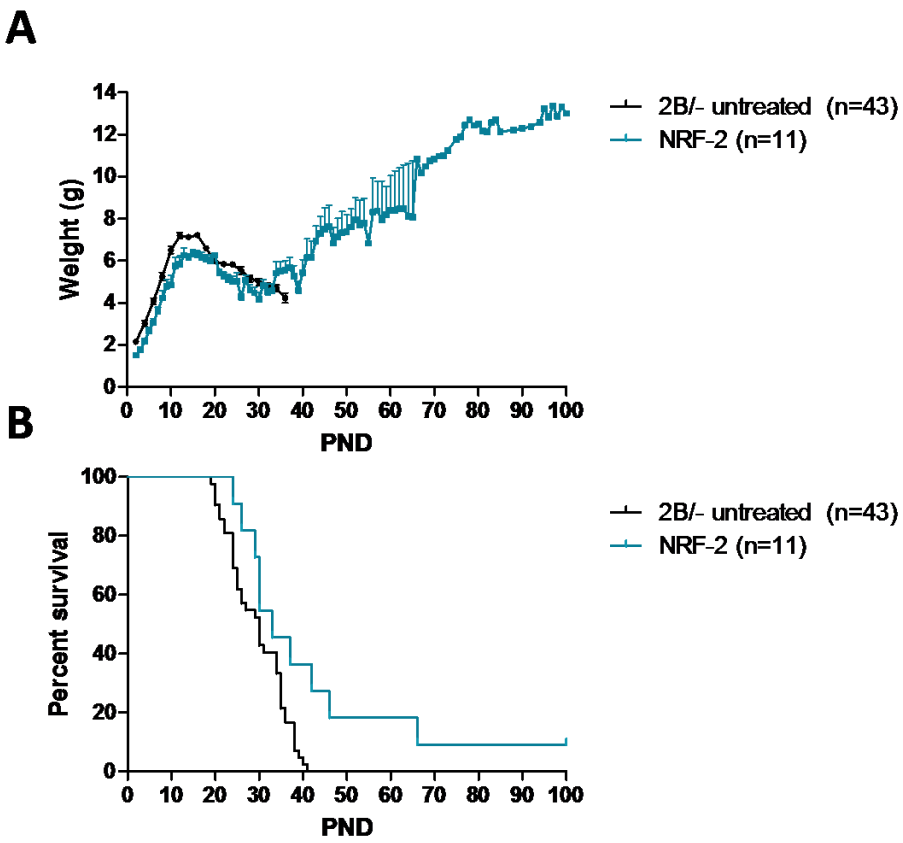

Figure 27: Expression of NRF-2 is unable to benefit *Smn*$^{2B/-}$ mice.

A) A survival curve comparing NRF-2 treated mice with untreated counterparts demonstrates little difference in lifespan. B) Weight gain achieved by these two cohorts also demonstrates no difference in the majority of animals treated: two long-living mice gained increased weight and the oldest mouse gained up to 13 grams.

3: Discussion

Through this work we have helped contribute to the identification of disease-relevant tissues within SMA. While it has been repeatedly demonstrated that other cell types must be involved in the motor neuron loss in SMA, the field has not concluded which cell types are involved and how. Here we first examine the effects of delivering AAV-GFAP-SMN. When delivered via ICV, this therapy resulted in 100% survival past 100 days. However, upon switching to an IV delivery, several animals died before reaching that age. This mimics previous research that finds that delivery into the CNS provides better rescue than does IV delivery[190]. In the case of our experiments, this could represent two scenarios; delivery into the CNS could simply target more astrocytes, providing a more robust benefit in this population, or this high concentration of virus flooding the brain could increase the likelihood that motor neurons are transduced one or more times, in turn leading to higher likelihood of unwanted motor neuronal expression. To avoid the latter, it would be helpful to design constructs containing microRNA sites to ensure their degradation if expressed in alternate cell types. Indeed this method was effective at producing astrocyte-specific expression in scAAV [134].

Next, we directly compared astrocytic delivery to motor neuron delivery and found that SMN expression under a GFAP promoter can result in long-term benefits with increased weight gain over HB9-driven SMN. Further experimentation would be required to determine the root behind the phenotypic difference afforded by astrocytic and motor neuronal restoration of SMN. First, tissue-specific expression from these tissues should be confirmed. Despite presence of a tissue-specific promoter, transgenes can sometimes have leaky expression in other cell types. Indeed, previous observations have found

GFAP-driven transgene expression in motor neurons (unpublished observation). The robust benefit provided by restoration of HB9 is in contradiction to multiple previous findings which showed modest improvement via genetic restoration of SMN to motor neurons. This suggests that our viral delivery may have resulted in leaky expression in non-motor neurons and would require further verification. Next, it would be beneficial to determine the level of protein expression within the CNS. Astrocytes are far more abundant than motor neurons within the CNS. Thus in these experiments there is likely more SMN being expressed as a whole in the astrocyte-driven population. This is not a weakness of the experiment which should be controlled for, but rather this could indicate that part of the influence of astrocytes on SMA can be contributed to a strength in numbers. Finally, the number and health of motor neurons should be compared in this context. A logical hypothesis would suggest that HB9-driven SMN would result in more and/or healthier motor neurons, however this would need to be tested. Delivering scAAV9-GFAP-SMN into SMNΔ7 mice proved to cause life extension as well as improve the number of synapses on motor neurons without improving the number of motor neurons.

In order to expand on the implications of this data, it would be valuable to determine if differences in strength between these two cohorts exist. A combination of weight gain along with improvement in strength may indicate that improving the health of astrocytes has an ability to increase quality of life rather than just lifespan. While it is believed that primary therapeutics for SMA should be broadly targeting, supplemental therapies or diets could be designed to enhance astrocyte function if these cells continue to demonstrate disease relevance in SMA.

Finally, we sought to better understand the mechanism through which astrocytes improve the health of the CNS and motor neurons in SMA. Toward this end, it has been suggested that oxidative stress plays a role in SMA pathology. While several mechanisms are in place to handle oxidative stress, NRF2 is known as a master regulator of oxidative response genes. This pathway has been shown to be stimulated upon oxidative stress in astrocytes and these cells are known to aid in oxidative stress in motor neurons. Further, NRF2 has shown to have gross phenotypic effects in mouse models of other diseases. We delivered NRF2 under a ubiquitous promoter and saw only small benefits to lifespan. This is confounded by a few long-living animals, which may be a trait of the Smn $^{2B-}$ model rather than an effect of the treatment. It is notable that ALS studies in which NRF2 provided improvement were performed by enhancing NRF2 in astrocytes. Upregulation in motor neurons or in muscle had no effect. Therefore it is possible that we chose the wrong promoter to drive our transgene. However, these previous studies also demonstrated that there is no deleterious effect associated with overexpression of NRF2 in other cell types. Therefore the ubiquitously strong expression afforded by the CBA promoter would have allowed us to see a benefit if one can be provided by NRF2 expression. Indeed we saw no obvious benefit from NRF2 expression.

While SMN-dependent therapeutics such as viral restoration of SMN and ASO-based splicing correction provide remarkable phenotypic improvement in preclinical trials, many researchers believe that long-term strategies will include a combination of treatments. Other therapeutics such as repurposed drugs or small molecules and even diet changes tend to have less robust life extension in preclinical work, however these small improvements may help supplement a primary therapy such as viral delivery. The

advantage of these methods is that orally-available compounds are non-invasive and can be dosed regularly. While motor neurons will benefit greatly from the episomal expression of SMN afforded by AAV therapy, this type of DNA cannot be expressed long-term in cells that are regularly dividing. In order to treat the apparent primary concern within SMA (the CNS) and prevent peripheral defects from manifesting later, a combinatorial approach could be best. Indeed it has been shown that peripheral delivery (intraperitoneal) of ASO alongside a CNS delivery does not improve lifespan, but dramatically improves distal necrosis that arises in long-living SMA mice[189]. Further, it is known that the highest requirements of SMN are within the developmental period before the maturation of NMJs, thus implying that therapies that may have side effects if taken chronically can be restricted to the most critical developmental stages and then halted or decreased upon crossing that developmental hurdle. Continued work will be required to identify which tissue types contribute to the pathology of SMA and further which functions these cells carry out which can be exploited through therapeutic means.

Chapter Four: Neuromuscular Junctions as an Assessment of SMA Pathology and Rescue

1: Background

1.1: Neuromuscular Junctions

Neuromuscular junctions (NMJs) are specialized synapses responsible for transmitting impulses from the nervous system to the muscular system. Accordingly, they are a logical region of interest for neurodegenerative diseases like SMA where defects in the nervous system present with a muscular phenotype. Indeed, abnormalities in the NMJ are seen in patients and are represented in models of SMA.

The NMJ must first be properly formed and then maintained through appropriate use. The interaction between acetylcholine and acetylcholine receptors (ACh and AChRs) is integral to a functional NMJ and accordingly play a role in its development. On the muscle fiber side, AChRs cluster in a random or prepatterned manner prior to MN connection[284]. Initially, motor neurons will innervate many myofibers in an overlapping fashion such that each myofiber is poly-innervated. Through a selection process involving synaptic activity at each connection point as well as competition between the nerves innervating a myofiber, all but one nerve will retract, leaving the myofiber mono-innervated[284]. This process of pruning occurs before birth in humans, however mice finish pruning in postnatal stages.

The level of NMJ maturity can be visually assessed by looking at the post-synapse. In the immature state AChRs are evenly spread across the diameter of the endplate in a "plaque" structure. This patterning becomes more specific as AChRs cluster

with increasing specificity around the nerve endings. The endplate goes through "perforated" and "fragmented" stages of morphology, finally resulting in a "pretzel-like" shape in the mature NMJ[285]. Maturity can be quantitatively assessed by visually counting the number of perforations within an NMJ, or by staining for mature NMJ markers[286]. Five different subunits, α,β,γ,δ and ε make up each AChR. Most of these subunits are expressed throughout development, however, the γ and ε subunits are reciprocally expressed. The γ subunit is expressed in fetal muscle and begins to decrease around PND5 whereas the ε begins to be upregulated at this timepoint. In healthy mice this switch is almost complete by PND 14[287].

1.2: Neuromuscular Junctions in Spinal Muscular Atrophy

It has been repeatedly shown that while NMJs initially form in SMA, this pathology is one of the earliest measurable symptoms in SMA mice. In *M.m.Smn*$^{-/-}$*; SMN2* mice, NMJs were present, although morphologically abnormal as early as PND2. Toward end-stage (PND5-6) of this model, endplates were significantly vacant compared to littermates. Similarly, in the SMNΔ7 mouse, early defects followed by later denervation were seen at varying degrees in different tissues[275, 288].

Rather than the possibility that NMJ pathology is a downstream effect of overall dying neurons, a growing list of publications implicate that SMN is directly involved in the NMJ specifically. Early evidence indicated that when making mouse models based on patient mutations or otherwise low levels of SMN, some models exhibit no gross phenotype, but solely a significant difference at the level of the NMJ. While it has been known that requirements for SMN are high in young animals and decrease with age, the reason behind these different temporal requirements was unknown. Recently, the age at

which mice become resistant to SMN depletion has been more precisely mapped. It was found that depletion had highly deleterious effects at all times in mice harboring only one copy of *SMN2*. However, mice with two copies of SMN 2 were vulnerable to depletion on day 12, but resistant to depletion on day 15. This tamoxifen method of depletion was shown to reduce SMN to approximately 5% after 5 days of delivery. Therefore, a significant hurdle is overcome between day 17 and 20 which leaves mice resistant to overt SMA. Notably, this timeframe matches the maturation of young mouse NMJs. Further, cohorts of mice that were SMN-depleted in adulthood appeared overall healthy, but were unable to properly regenerate NMJs upon damage caused by nerve crush or ageing. Upon further investigation, it was found that neuronal SMN expression is increased following nerve crush in healthy tissue, which was not possible in SMN depleted mice[289]. Finally, suboptimal SMN restoration to motor neurons has been seen to improve parts of neuromuscular junction pathology without contributing to motor neuron survival or overall longevity. This indicates that NMJs are highly sensitive to alterations in SMN level[194].

Morphological Deformities

Within both the human condition and murine models of SMA, NMJs demonstrate immaturity. Whereas healthy mice switch to the mature AChR, various muscles in SMA mice remain arrested, expressing the fetal AChR complex. In SMNΔ7 animals at PND11, levels of AChRγ were most highly expressed in abdominal muscle, however gastrocnemius, quadriceps, and triceps all showed abnormally high levels of fetal AChRs[290]. In patients, a comparison of SMA diaphragms to those from healthy controls demonstrated that SMA patients expressed much higher levels of fetal AChRs. For example, a 10 year old patient presented more fetal AChRs than an healthy child of only

4 years old[286]. Further, SMA endplates are seen to be significantly smaller in SMNΔ7 mice and fewer perforations are observed[275, 285, 290, 291].

NMJ neurofilament (NF) accumulation and denervation are further represented by mouse models of SMA as well as the human condition Neurofilament accumulation appears as thick and swollen staining of the nerve terminal. These presynapses fail to form the fine arbors typical of the pretzel-like NMJs structure. Neurofilament accuulation occurs within the pre-terminal axon as well as the nerve terminals in severe SMA mice[290]. This accumulation occurs in early stages of disease progression (PND2 of *M.m.Smn*$^{-/-}$*; SMN2* mice) and prior to denervation in SMNΔ7 as well as *Smn*$^{2B/-}$ mice[61, 275, 292].

Denervation can be caused by failure of the NMJ to form or the retraction of a previously formed NMJ. While defects in axonal pathfinding as well as growth cone defects have been established in SMA, NMJ pathologies represent nerves that have detached from a previously innervated muscle, as will be discussed shortly.

Defects in NMJ morphology associated with SMA are accompanied by functional deficiencies as well. Synaptic transmission is seen to be dysfunctional as endplate currents in SMNΔ7 mice were reduced[293]. Upon further investigation, this effect was attributed to a marked decrease in vesicle density at the NMJ[293].

Functional Deficits

Electrophysiological means such as motor unit number estimation (MUNE) and maximum compound motor action potential amplitude (CMAP) can be used to assess

NMJ function *in vivo*. CMAP measures the output of motor units supplying a particular muscle or group of muscles whereas MUNE measures the number of motor units supplying such muscle.

In SMNΔ7 mice, CMAP and MUNE both demonstrate a significant difference between control and sick animals starting at day 6 in concordance with disease onset. These scores correlated well with overall disease progression in mice[294]. However, muscles that showed significant difference in electrophysiological measures did not always overlap with previously reported NMJ morphological defects[275]. Currently no in-depth analysis has been performed that can link these functional defects with the available data regarding NMJ morphological defects.

Electrophysiological data in mice faithfully recapitulated that seen in the human condition[295, 296]. A single Type I patient demonstrated consistently descending CMAP scores in correlation to standardized strength assessment[296]. These measurements have been shown to correlate to SMA severity based clinical diagnosis and *SMN2* copy number. Due to the fidelity by which they represent the SMA severity and importantly the noninvasive nature of these measures, they are included as biomarkers to assess the efficacy of some SMA clinical trials[295].

2: Characterization of a Novel SMN2-based Intermediate Mouse Model of SMA.

Parts of this work have been published as "Cobb MS, Rose FR, Rindt H, Glascock J, Shababi M, **Miller MR**, Osman EY, Yen PF, Garcia M, Martin B, Wetz MJ, Mazzasette C, Feng Z, Ko CP, and Lorson, CL. Development and Characterization of an *SMN2*-based intermediate mouse model of Spinal Muscular Atrophy. Hum. Mol. Genet. 2013 Feb;22(9):1843-55."

2.1: Introduction

SMA mouse models have been extremely valuable for understanding the biology and pathology underlying SMA, as well as serving as an in vivo context to assess therapeutic strategies. The two most widely examined models are referred to as "*SMN2*" (*SMN2*$^{+/+}$;Smn$^{-/-}$) and "SMNΔ7" (*SMN2*$^{+/+}$;SMNΔ7;Smn$^{-/-}$) SMA mice, with life expectancies of ~5 or ~14 days, respectively [110, 297]. These models include the human genomic *SMN2* transgene and are therefore amenable to therapeutic strategies that target *SMN2*, such as splice-altering antisense oligonucleotides. An important less severe model, called *Smn*$^{2B/-}$, has been used to examine SMN-independent therapeutic approaches such as the modulation of actin dynamics by administering the Rho-associated protein kinase inhibitor Y-27632 [75, 115]. This model is based upon a knock-in mutation within the endogenous *Smn* gene. The *Smn*$^{2B/-}$ mutation reduces full-length expression and results in an exon-skipped product, effectively mimicking the molecular phenotype of the typical human *SMN2* pre-mRNA splicing patterns. However, the *Smn*^{2B} mutation is not equivalent to the *SMN2* sequence, nor are the flanking intron sequences homologous. The mutation, "2B", is not analogous to the human C/T transition since it disrupts the Tra2β1-responsive, AG-rich enhancer within the central portion of exon 7 [298,

[299] This does not diminish the importance of the *Smn*$^{2B/-}$ model, however *SMN2*-based therapeutics cannot be examined in the *Smn*$^{2B/-}$ model because it lacks *SMN2*. Currently, the field lacks an intermediate model that includes the human *SMN2* genomic transgene and results in an extended, yet still significantly shortened life span compared to wildtype animals.

In vitro and cell-based assays have demonstrated that the C-terminus of SMN is relatively flexible regarding the primary amino acid sequence of SMN exon 7. While a cytoplasmic localization signal is present within this region [58, 300], a variety of heterologous sequences can also confer cytoplasmic localization as well as increased activity in snRNP assembly assays, axonal extension assays, and SMN protein stability[239, 241, 301, 302]. In the full-length *SMN* mRNA, the translational stop codon resides in exon 7. The stop codon in the SMNΔ7 mRNA resides at the beginning of the normally untranslated exon 8. Aminoglycosides can induce translational stop codon read-through and, in the case of SMNΔ7, generate a certain level of extended SMNΔ7 protein. This SMN read-through protein can then enter into SMN complexes with the existing full-length protein. This theory is supported by previously published data, demonstrating a small increase in SMN-FL protein after treatment, without increasing *SMN2* transcription or altering exon 7 splicing[240]. While several read-through inducing compounds can elevate SMN levels in SMA cells, aminoglycosides G418 and a novel compound, TC007 have been shown to decrease the severity of animal models of SMA[301, 303-305]. Additionally, SMN levels were increased in motor neuron cultures as well as spinal cord extracts from treated animals, while modest, but statistically significant changes were

also observed in the severity of disease, including the ability of the treated mice to right themselves, perform in a "tube" test, and an increase in life span.

Based upon our previous analysis of the SMN protein and the evaluation of compounds that induce a translational read-through within the SMNΔ7 mRNA, we and others have shown that the inclusion of a small number of amino acids at the carboxyl terminus confers a greater degree of functionality to the SMNΔ7 protein [239-241, 301-304]. As a means to create an *SMN2*-based SMA model that still presented with an overt phenotype, but was less severe than the SMNΔ7 model, we introduced a transgene expressing the SMNΔ7 read-through product into the *SMN2* genetic background. Here we provide evidence that the novel SMN^{RT} transgenic mouse model is significantly less severe than the commonly used SMNΔ7 model and therefore may serve as an important intermediate model of disease that is still principally based upon the *SMN2* transgene.

2.2: Methods

Standard methods

All protocols for NMJ imaging were performed as described within chapter two.

SMN^{RT} Transgenic Mice

The SMN^{RT} transgene consisted of 3.4 kb of promoter from the human *SMN1* gene [306], the complete coding sequence of the SMNΔ7 cDNA, and the 3'-UTR of *SMN1* [116]. The first stop codon in exon 8 of SMNΔ7 was altered from TAG to TAT using overlapping extension PCR to create the read-through mutation. Transgenic founders were generated at the University of Missouri transgenic core facility by pronuclear injection of fertilized oocytes from FVB/N mice, and 13 lines were established by breeding founders to non-transgenic cohorts. All experiments involving mice were carried out in accordance with

protocol #7138 approved by the Animal Care and Use Committee of the University of Missouri. Mice were housed pathogen-free in a microisolator facility. The SMN^{RT} transgene was then bred onto the background of the "severe" *SMN2* model (*SMN2*$^{+/+}$;Smn$^{+/-}$, Jackson Laboratories stock number 5024). Control mice hemizygous for SMNΔ7 (*SMN2*$^{+/+}$;SMNΔ7$^{+/-}$;Smn$^{-/-}$) were produced by breeding mice homozygous for SMNΔ7 (*SMN2*$^{+/+}$;SMNΔ7$^{+/+}$;Smn$^{+/-}$) with *SMN2* stock mice (*SMN2*$^{+/+}$;Smn$^{+/-}$), both purchased from Jackson Laboratories. To standardize litter size, experimental litters were culled to include all SMA pups and two unaffected littermates. To supplement the experimental mice during weaning, Nutrical dietary gel was given daily beginning at P15 and ending at P50. To prevent dehydration, P15-P50 SMA animals received daily subcutaneous phosphate buffered saline (PBS) injections consisting of 0.5 ml PBS per gram body weight. If a SMA mouse had difficulty reaching the food and water in the cage, food pellets and a container of water were placed on the bottom of the cage.

Genotyping was performed by PCR using tail biopsies prepared according to the HotShot method. The following primers were used: SMN^{RT} forward, (5'-CTATCATGCTGGCTGCCTCCATTT-3'); SMN^{RT} reverse (5'-ACAATGAACAGCCATGTCCACCAG-3'); mouse Smn wildtype forward (5'-TCTGTGTTCGTGCGTGGTGACTTT-3'); mouse Smn wildtype reverse 5'-CCCACCACCTAAGAAAGCCTCAAT-3'); mouse Smn knockout allele forward (5'-CCAACTTAATCGCCTTGCAGCACA-3); mouse Smn knockout allele reverse (5' AAGCGAGTGGCAACATGGAAATCG-3).

Transgene copy numbers of SMN^{RT} lines were determined using qPCR with SMN cDNA exon 5 forward primer (5'-TGCTGGCTGCCTCCATTT-

3'), SMN cDNA exon 6 reverse primer (5'-GCATCATCAAGAGAATCTGGACAT-3'), and SMN cDNA Taqman probe (FAM-CTTCTGGACCACCAATAATTCCCCCACC-TAMRA). Amplification of ApoB was used as internal duplex PCR control with ApoB forward primer (5'-CACGTGGGCTCCAGCATT-3'), ApoB reverse primer (5'-TCACCAGTCATTTCTGCCTTTG-3'), and ApoB Taqman probe (VIC-CCAATGGTCGGGCACTGCTCA-TAMRA).

2.3: Results

Readthrough Transgene

To develop an intermediate *SMN2*-based model and to examine the in vivo activity of the SMN^{RT} protein, a transgene was generated that would express SMN^{RT} under the control of the native *SMN* promoter. The SMN^{RT} transgene was derived from the original SMNΔ7 cDNA transgene [297] and consists of a 3.4 kb *SMN1* promoter fragment, the 5' untranslated region from the human *SMN1* gene, human SMNΔ7 cDNA with a mutation in the first stop codon of exon 8, and the 3' UTR from the human *SMN1* gene. The SMN^{RT} transgene encodes a protein that would typically be produced from a read-through event and therefore retains a greater degree of stability and functionality compared to SMNΔ7 [302]. The SMN cDNA in the SMN^{RT} transgene is identical to SMNΔ7 cDNA except for a single nucleotide change in the SMNΔ7 stop codon within exon 8. By changing the stop codon sequence from TAG to TAT, a tyrosine residue is encoded instead of a stop codon. Tyrosine was chosen since it is the most likely amino acid that would be incorporated during readthrough due to the wobble position of the stop codon. After the tyrosine, four additional amino acids (Serine, Serine, Threonine, and Lysine), are incorporated prior to recognizing the next stop codon. This SMN^{RT} protein

is the same sequence as previously examined SMN read-through products which demonstrated increased stability and function [241, 301, 302].

Readthrough Lines

13 SMN^{RT} transgenic founder lines were generated by pronuclear injection. To obtain the appropriate genetic background, carriers from the previously described "severe" model (*SMN2*$^{+/+}$;$Smn^{-/-}$) were crossed with the hemizygous SMN^{RT} lines ($SMN^{RT+/RT-}$) to generate unaffected breeder animals (*SMN2*$^{+/+}$;SMN^{RT};$Smn^{+/-}$). The original SMN^{RT} lines were also kept on a non-transgenic background, allowing for a straightforward means to specifically quantify SMN^{RT} transgene expression since only a single nucleotide differentiates *SMN2*- and SMN^{RT}-derived transcripts. Transgene copy numbers were determined for each line by TaqMan real-time PCR using specific primers and probes for human SMN cDNA and ApoB control. Transgene copy number was monitored to ensure a single integration event based upon Mendelian inheritance patterns. Several lines likely contained multiple integration events based upon non-Mendelian segregations patterns and were therefore no longer analyzed. Throughout the following experiments, copy number of the SMN^{RT} transgene was routinely monitored to ensure transgene stability and the strain was maintained on a mixed background (FVB/C57BL/6).

Expression Analysis

SMN^{RT} line 16 (referred to as SMN^{RT} from this point) RNA and protein expression was measured by qPCR and Western blotting, respectively. While RNA levels for SMNΔ7 animals were universally higher, SMN protein levels were much more uniform between SMNΔ7 and SMN^{RT} animals, especially in the brain and spinal cord (data not shown). These results demonstrate that SMN^{RT} animals are expressing SMN in levels that are comparable to SMNΔ7 levels in disease relevant tissues.

Phenotypic Analysis

Assessment of the SMN^{RT} transgenic SMA mouse model revealed that animals were more ambulatory, gained more weight, demonstrated better motor function and lived longer than SMNΔ7 animals. However, at all timepoints these animals weighed less and were weaker than healthy animals (data not shown). At approximately P40-P50, SMN^{RT} mice began showing signs of necrosis on the ears, tail and/or eye (data not shown).

Morphological Analysis

Upon histological analysis, SMN^{RT} muscle fibers were smaller than in wildtype controls, however they were significantly larger than in SMNΔ7 control mice. In addition, the muscle fiber distribution of SMN^{RT} mice indicated the majority of total muscle fibers were in a similar range as wildtype (data not shown).

SMN^{RT} mice showed a similar number of motor neurons compared to hemizygous SMNΔ7 controls. However, SMN^{RT} mice showed a reduction of proprioceptive synapses onto motor neurons compared to hemizygous SMNΔ7 mice (data not shown).

Recently, a discrete subset of muscles had been shown to be particularly vulnerable in SMA mice [275, 288, 307, 308], including the splenius and the longissimus capitis. As the SMA phenotype in SMN^{RT} animals does not present until a relatively later time point, we chose to compare the NMJ pathology to unaffected mice ($SMN2^{+/+}$;$Smn^{+/+}$) since pathology does not present until age-matched SMNΔ7 animals would be dead. To test whether SMN^{RT} SMA mice display synaptic defects, we examined endplate morphology as well as NMJ innervation in the longissimus and splenius capitis muscles of SMN^{RT} mice. Consistent with the intermediate phenotype of this mouse, modest but

significant reduction in NMJ innervation was observed in the longissimus muscle of SMN^{RT} SMA mice.

Further, during development, endplates are initially plaque shaped, and become more complex as perforated, "C"-shaped, and branched morphology, and eventually mature into a pretzel shape [309]. At P19, over 98% of NMJs in unaffected mice have pretzel-shaped (mature) endplates. In contrast, NMJs in SMN^{RT} SMA mice showed a significant increase in the percentage of NMJs with perforated or fragmented endplates, suggesting a defect in synapse maturation in SMN^{RT} SMA mice (data not shown).

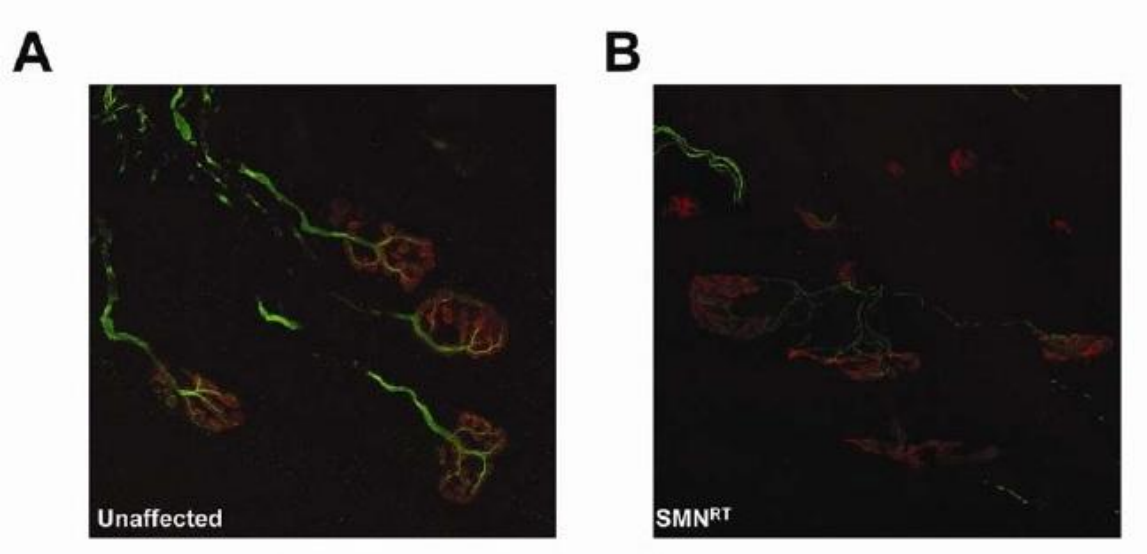

Figure 28: Endplate morphology in SMNRT muscles at P19.

Longissimus capitis muscle was dissected from SMNRT and control mice at P19 and processed for immunohistochemistry with the following antibodies: anti-neurofilament (1:2000; Chemicon) and anti-synaptophysin (1:200, Invitrogen). AChRs were labeled with Alexa Fluor 594-conjugated a-bungarotoxin (Invitrogen). Fluorescently labeled NMJs were observed by epifluorescence or confocal microscopy. For imaging of NMJs, Z-stack images of immunostained whole-mount muscles were obtained at sequential focal planes 1 mm apart using the Zeiss LSM 510 META confocal microscope. Illustrated images are flattened projections of Z-stack images.

2.4: Discussion

SMA research has made tremendous progress moving from the bench to the bedside. In the past year, SMA-specific compounds have entered clinical trials with more on the way. SMA animal models have been exceptionally valuable from the standpoint of understanding the biology of the disease and they have served as a valuable means to evaluate potential therapeutic candidates in vivo [310]. One caveat common to many of the SMA mouse models is that they are incredibly severe, with the two most widely used models having life spans of 5 or 14 days [198]. From a biological as well as translational perspective, the development of a *SMN2*-based intermediate model would be a valuable addition to the SMA field. To this end, the SMN^{RT} model fills an important void based upon several important parameters: 1) the native human *SMN2* gene is present and can therefore be targeted by *SMN2*-specific therapeutics; 2) the life span is longer than the SMNΔ7 model, but is still severe enough to allow for a relatively rapid analysis of therapeutics; 3) a clearly identified, yet intermediate level of NMJ pathology is present, and 4) restoration of SMN via a viral vector fully rescues the SMA phenotype.

Another intermediate model of SMA has been useful in the field for identifying SMN-independent therapeutics. This model, $Smn^{2B/-}$ is based on a mutation in mouse *Smn* exon 7, resulting in decreased inclusion of *M.m.Smn* exon 7. However, the Smn^{2B} mutation is not equivalent to the *SMN2* sequence, nor are the flanking intron sequences homologous. The $Smn^{2B/-}$ model cannot be used to examine *SMN2*-targeting compounds such as splice-site switching antisense oligonucleotides. The SMN^{RT} model complements the $Smn^{2B/-}$ model in that it validates the intermediate lifespan, muscle and neuromuscular junction phenotypes seen with modest levels of SMN in mice, however the SMN^{RT} has the advantage of testing *SMN2*-based therapeutics.

Additional models have been developed such as temporal, tissue-specific models as well as a titrating allelic series [113, 311], however, even the "humanized" transgenic model does not present an overt neuronal phenotype or significantly shortened life span [312], both of which are important advantages regarding the use of the present model as an in vivo model for therapeutic analysis. Importantly, all of the components of the SMA phenotype that were examined in this report were significantly different than wildtype mice, demonstrating the potential utility for SMA therapeutics in a longer-lived model.

The SMN^{RT} mice display an important intermediate phenotype with regard to NMJ development. While the splenius muscle has a mild reduction in the number of fully innervated NMJs, the longissimus has a significant reduction in fully innervated NMJs and a significant increase in partially innervated NMJs. Interestingly, while nearly 80% of the longissimus NMJs are fully innervated, the relative state of maturation suggests that NMJ development is delayed. SMN^{RT} muscles display NMJ morphologies that are consistent with developing NMJ structures, such as the perforated and branched morphologies, compared to the fully developed "pretzel-like" structures observed in unaffected animals. Interestingly, total motor neuron numbers are not significantly reduced in SMN^{RT} animals. In contrast, proprioceptive synapses are reduced nearly 75% compared to unaffected tissue.

One of the additional objectives in generating SMN^{RT} animals was to examine the in vivo activity of the purported SMN^{RT} protein. Several studies have shown that the C-terminus of SMN appears to possess flexible amino acid sequence requirements and that the exon 7 region is involved in conferring stability [239, 241, 301, 302]. We and others have shown that the SMNΔ7 protein encoded by a translational read-through event (SMN^{RT})

exhibits increased functionality in vitro and in cell based assays for protein stability, axonal extension, intracellular localization, and snRNP assembly [302]. Additionally, small molecules that induce translational read-through events such as aminoglycosides can reduce disease severity, including a modest extension in survival [240, 301, 303-305]. However, none of these experiments addressed whether SMN^{RT} would function in vivo, in particular, within tissues that are relevant to SMA development. In the SMN^{RT} animals, RNA expression is below that of the SMNΔ7 model, while SMN protein levels are nearly equivalent. These results are consistent with the in vitro and cell-based analysis that led to our initial hypothesis that a SMNΔ7 read-through would confer a greater degree of functionality compared to the SMNΔ7 protein. By no means is the SMN^{RT} protein equivalent to full-length SMN, but much like the SMN^{RT} phenotype as a whole, the functionality of the SMN^{RT} protein retains an intermediate level of activity: not as severe as SMNΔ7, but not quite as functional as full-length SMN. The development of the SMN^{RT} model demonstrates that SMN^{RT} protein confers a significant level of SMN-related activity to SMN-deficient tissues, as well as providing evidence that the SMN^{RT} product is not toxic. Collectively, these experiments detail the development of an *SMN2*-based intermediate model of SMA that can aid in the development of therapeutic candidates for SMA and can provide insight into the biology behind a less severe form of this devastating disease.

3: Neuromuscular Junction Analysis of a Novel SMN-2 Targeting Splicing Enhancer

Parts of this work are published as "Osman, EY, **Miller MR**, Robbins KL, Lombardi A, Atkinson AK, Brehm AJ, Lorson CL. Morpholino Antisense Oligonucleotides Targeting Intronic Repressor Element1 Improve Phenotype in SMA Mouse Models. Hum Mol Genet. 2014 Sep 15;23(18):4832-45"

3.1: Introduction

All SMA patients rely on the nearly identical copy gene, *SMN2*, which produces low levels of functional SMN protein. SMN is ubiquitously expressed and is a critical factor in a variety of RNA pathways. Even though the *SMN2* gene is 99% identical in nucleotide sequence and is completely identical in amino acid sequence, approximately 90% of *SMN2*-derived transcripts are alternatively spliced and encode a truncated protein lacking the final coding exon (exon 7). This aberrant splicing event is the result of a silent, non-polymorphic C to T nucleotide transition 6 nucleotides within exon 7 [34, 313]. *SMN2*, however, is an excellent target for therapeutic intervention. Previously, cis-acting negative regulatory regions that surround *SMN2* exon 7 have been identified and described[35, 98, 314-320]. In particular, ISS-N1 has been a hotspot for experimental therapeutics, especially antisense oligonucleotides (ASOs). ASO molecules of various lengths and backbone chemistries have been used to inhibit the repressor activity of ISS-N1, leading to an increase in SMN protein and significant extensions in survival in animal models of SMA. Presently, the 2'-MOE chemistry used by Ionis Pharmaceuticals in the development of their ASO, SMN-Rx, is in Phase II clinical trials [321, 322]. Similar

Morpholino-based ASOs have shown excellent pre-clinical promise in severe SMA mice and are under further development.

Currently, no effective treatment exists for SMA, and the complexity and expansive clinical spectrum strongly argue that a single compound will be insufficient to address the needs of all SMA patients. Going forward, it is essential to broaden the range of tractable genetic targets for SMA. To this end, we have focused upon a genetic region upstream of *SMN2* exon 7 called Element1 (E1). Using a mini-gene system, E1 was shown to function as a potent repressor of *SMN2* exon 7 splicing[320, 323] and was bound by splicing repressor proteins[323]. Native RNA antisense molecules expressed from a viral backbone modulated *SMN2* exon 7 splicing and elevated SMN protein levels, although the inherent instability of the native RNA molecules likely accounted for the poor impact upon disease severity and poor extensions in survival[323].

In this report, Morpholino-based ASOs targeting the E1 region have been developed and examined in two important animal models of disease: the "gold standard" SMNΔ7 mouse, which is a very severe model living only ~14 days; and a recently developed model called SMN^{RT}, in which animals live ~35 days and represent a less severe population. Using a relatively low dose of the Element1 ASO ($E1^{MO}$-ASO), the SMA phenotype at the molecular, cellular, and organismal levels were largely rescued, including a 300-700% extension in survival for the two mouse models. From a pre-clinical perspective, there is excellent target engagement (*SMN2* splicing), molecular efficacy (SMN protein production), functional improvement (NMJ maturity) and robust phenotypic rescue in two complementary models of disease. Collectively, this work identifies a lead ASO candidate that targets a distinct region of the *SMN2* pre-mRNA.

3.2: Methods

Element1 antisense oligonucleotides ($E1^{MO}$-ASO)

The following oligos were modified at every base with Morpholino chemistry groups (GeneTools L.L.C., Philomath, OR 97370 USA); $E1^{MO}$-ASO (26-mer) 5'-CUA UAU AUA GAU AGU UAU UCA ACA AA -3', and negative scrambled control provided and tested by GeneTools L.L.C. (25-mer), 5'- CCU CUU ACC UCA GUU ACA AUU UAU A-3'.

Standard methods

All protocols for mouse handling, ICV injection, and NMJ imaging were performed as described previously within this document.

3.3: Results

To determine whether delivery of the $E1^{MO}$-ASO to SMA mice on P2 improved the phenotype; survival, weight gain, righting reflexes and strength measurements were collected. Untreated SMNΔ7 animals as well as those injected with scrambled ASO lived less than two weeks. Similarly, delivery of the $E1^{MO}$-ASO via a single intraperitoneal (IP) injection failed to extend survival beyond 1-2 days. However, in this severe model, a single ICV injection of $E1^{MO}$-ASO led to nearly a 400% improvement in life span, with more than one third of the treated animals living beyond 50 days. Similarly, this therapeutic introduced into the intermediate *SMN2*-based model, SMN^{RT}, provided a robust extension in life span. Whereas most knockout animals from this line die around day 32, all $E1^{MO}$-ASO treated animals were still alive at P175, at which point the experiment was stopped and animals were euthanized. $E1^{MO}$-ASO was seen to improve

fidelity in the splicing of SMN exon 7 and accordingly increase FLSMN protein levels. Together these results suggest that on a molecular level as well as an organismal level, $E1^{MO}$-ASO could be a viable therapeutic for SMA.

We further sought to understand the effect of $E1^{MO}$-ASO at a physiologic level. To this end, we analyzed the neuromuscular junctions in both untreated and treated mice. An important hallmark of the SMA phenotype that directly relates to disease pathogenesis is the integrity of the neuromuscular junctions (NMJs). As expected, NMJs from untreated SMA mice appear immature, poorly developed and there was little overlap between the pre- and post-synaptic endplate. In contrast, the wild type and $E1^{MO}$-ASO-treated tissues exhibit well developed NMJs with a high degree of connectivity between the axons and the post-synaptic endplate. These results are consistent with the significant correction of the SMA phenotype at the organismal level and provide evidence that a molecular correction of *SMN2* splicing using an $E1^{MO}$-ASO can profoundly reverse the severe SMA phenotype observed in SMNΔ7 mice.

Figure 5

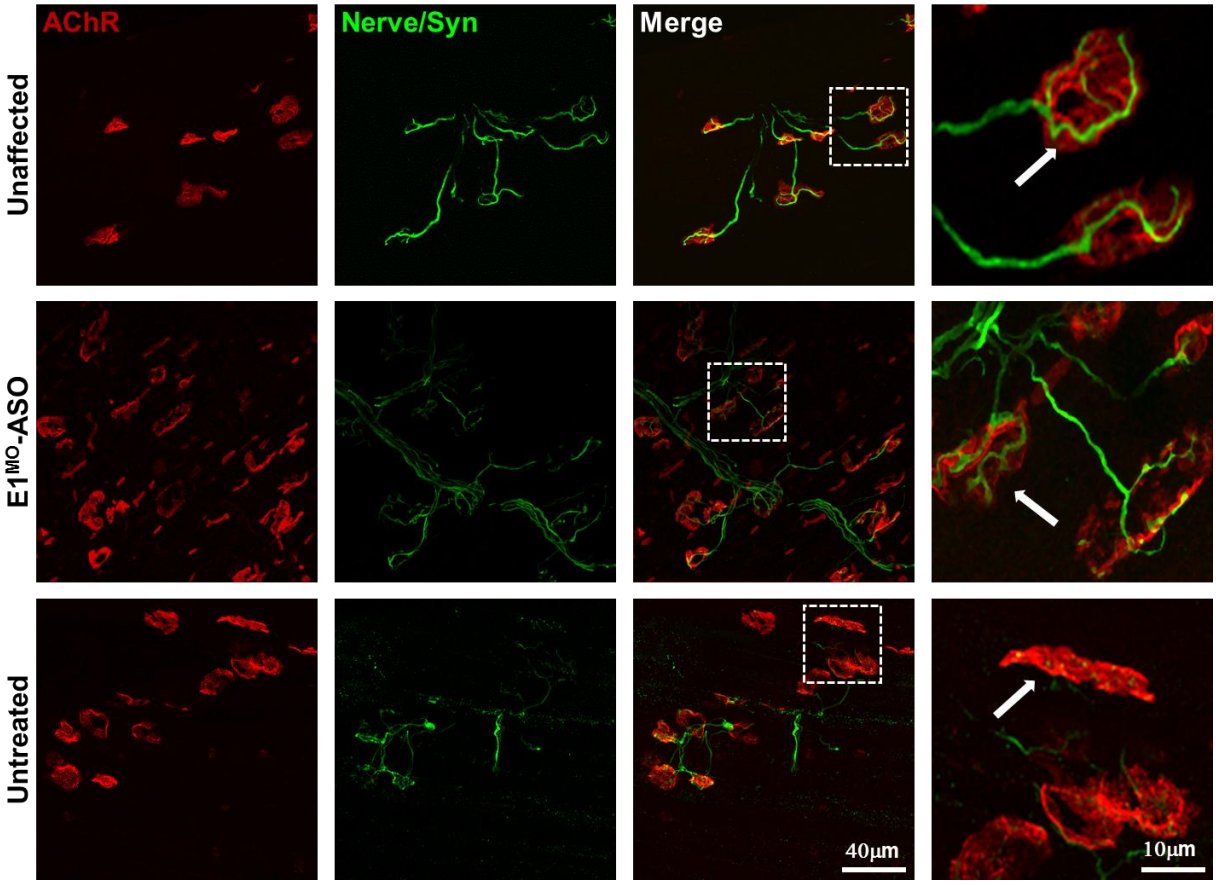

Figure 29: Improvement in neuromuscular junction (NMJ) pathology.

The *longissimus capitis* (LC) muscles from ICV injected and control animals at P12 were immunostained for nerve terminals with anti-neurofilament/anti-synaptophysin [Nerve/Syn] (in green) and motor endplates with α-bungarotoxin (in red). While the untreated SMNΔ7 mice displayed typical severe denervation, $E1^{MO}$-ASO treatment substantially restored NMJ's pretzel-like structures. Selected high-magnification images of NMJs in LC muscles of control and SMNΔ7 mice are shown (last column). Arrows indicate occupied endplates in both treated and unaffected animals contrasting the empty endplate seen in untreated controls.

3.4: Discussion:

ASOs have become important tools to modulate splicing in SMA with the overall goal of moving these compounds from pre-clinical models to the clinic. To date, the ASOs have exhibited a number of important properties that are common to successful candidate compounds, including compound/ASO stability, target specificity, and a well-defined molecular pathway (*SMN2* exon 7 splicing). To this end, in this report we have developed a Morpholino-based ASO that targets the E1 regulatory region which is upstream of *SMN2* exon 7 and is distinct from the target of other ASO strategies, ISS-N1. Using the nuclease-resistant Morpholino backbone, the $E1^{MO}$-ASO was not only able to modulate *SMN2* exon 7 splicing and increase SMN in disease relevant tissues such as the spinal cord, $E1^{MO}$-ASO was also able to largely correct the SMA pathology in two important models of disease: the severe SMNΔ7 and the "intermediate" SMN^{RT} mouse models. Improvements were consistently demonstrated at molecular, physiological and organismal levels.

The overwhelming majority of ASO strategies have focused upon the intronic repressor downstream of *SMN2* exon 7, ISS-N1. The exceptions include antisense molecules targeting the intron 7/exon 8 junction[324-326] and our previous report developing bifunctional RNAs that target E1 [323].Currently, the ASO developed by Ionis Pharmaceuticals and colleagues has entered Phase II/III clinical trials. Additional Morpholino-based ASOs targeting the same genetic region have shown considerable promise in the SMNΔ7 model. Morpholino-based ASOs that induce exon-skipping in other disease contexts have already entered clinical trials, such as for Duchene Muscular Dystrophy[327], demonstrating the practicality of the Morpholino backbone and the relatively low toxicity associated with systemic administration of Morpholino-based

ASOs. The current work targeting E1 is by no means designed to diminish these efforts that focus upon ISS-N1, rather, for such a complex and devastating disease like SMA, it is imperative to have as many well-validated pre-clinical successes as possible considering at this juncture there are no proven or approved SMA treatments. It will be particularly interesting going forward to determine whether combinations of ASOs elicit great responses, perhaps even lowering the doses for total ASO per individual. Alternatively, combining ASOs with small molecules may prove to be a potent combination. For example, ASOs have an exceptionally well-defined mode-of-action: *SMN2* exon 7 splicing. By combining this activity with a small molecule that increases promoter activity or enhances *SMN2* pre-mRNA stability, a synergistic activity could be observed. While it is clear that additional work needs to be done, ongoing efforts to successfully translate the exceptionally detailed and mechanistic understanding of *SMN2* pre-mRNA splicing has reached the point at which bona fide candidate compounds are entering clinic and providing hope for SMA patients.

4: Quantifying the Diminishing Effect of Delayed Delivery of SMN on Neuromuscular Junction Integrity

Parts of this work have been published as "Robbins KL, Glascock JJ, Osman EY, **Miller MR**, Lorson CL. Defining the Therapeutic Window in a Severe Animal Model of Spinal Muscular Atrophy. Hum Mol Genet. 2014 Sep 1;23(17):4559-68"

4.1: Introduction

As new therapeutics advance, understanding the temporal requirement for SMN will be important, particularly as neonatal screening for SMA is not routinely performed. By genetic means, restoration of SMN at early developmental periods is shown to have robust effect, but this effect is diminished by delayed induction[195, 203]. These results suggest that there is a very narrow window to deliver SMN and significantly impact disease severity. Further, a small study in which SMN was provided throughout early development and later deprived suggested that high levels of SMN could be required early during neonatal development, but that a lower maintenance threshold exists that is sufficient for survival. The genetic approach has clear biological implications, but it is more difficult to extrapolate these results to a therapeutic window. To address this, we have initiated time point studies with a gene therapy vector shown to provide a dramatic rescue of the SMA phenotype.

SMN gene therapy with a self-complementary adeno-associated virus serotype 9 (scAAV9) has resulted in the greatest extension in survival whether the vector was delivered systemically or directly into the CNS [101, 176, 180, 182, 184, 205, 328]. Therefore, using this highly effective paradigm we have performed a systematic analysis, utilizing the well characterized SMNΔ7 model, to determine the effects of delaying scAAV9-SMN administration. We treated each mouse with a single injection of scAAV9-SMN (1×10^{11}

viral genomes [v.g.]) at a single time point, PND2 through PND8. Early SMN induction provided the most benefit whereas injection at later time points decreased the efficacy of the vector. All treated mice lived significantly longer, but SMN induction at the earliest time point (PND2) provided the most robust rescue with the fewest early deaths. While SMN induction in the CNS and periphery was observed, NMJ pathology was largely uncorrected at later time points, indicating that even high levels of SMN protein could not correct the phenotype once the disease has progressed to this advanced stage. Collectively, the earliest administrations provided the greatest degree of phenotypic rescue. Importantly, however, even at late time points, a significant extension in survival was observed, suggesting that even symptomatic delivery of an effective SMN-inducing therapeutic could impact the course of disease.

4.2: Methods

All protocols including viral preparation, mouse handling, ICV injection and phenotypic assessment were performed as described previously within this document.

4.3: Results

To provide a continuous view of the temporal requirements of SMA, SMNΔ7 mice were injected with a single administration of scAAV9-CBA-SMN at a dose of 1×10^{11} viral genomes on either PND 2, 3, 4, 5, 6, 7 or 8. Animals showed a dose-dependent extension in survival, weight gain, and motor function (data not shown). According to these measurements, animals treated on similar days could be grouped together: those injected on PND2, PND3 or PND4 displayed similar weight gain from birth to peak, and there was no significance when compared with each other and animals injected on PND5, PND6 or PND7 had comparable percent weight gain and also did not show significance when compared to one another.

From gross phenotypic analysis, we can discern a remarkable difference between early and late delivery of scAAV. In order to further characterize the differences in phenotype associated with early vs. late delivery, animals injected on PND2 were compared to animals injected on PND7 for in-depth analysis.

First, we posited that that these differences in phenotypic improvement could be due to differential levels of SMN protein in the mouse. To determine if this was the case, mice were injected on PND2 or PND7 and harvested at PND11. SMN levels in the brain, spinal cord, muscle and heart were compared to non-injected. This indicated that mice injected on PND2 exhibited significantly higher SMN levels compared to PND7 injected mice and both PND2 and PND7 injected animals had significantly more SMN protein than age-matched, non-injected SMA control mice (data not shown). This could partially be explained by the fact that at PND11 harvest, animals injected at PND2 have been expressing the viral transgene for a longer period of time. However, when these animals were harvested at PND6, similar high levels of expression were seen, indicating that this is an SMA-related effect rather than a technical effect due to viral expression (data not shown).

In order to determine viral spread, a vector containing GFP was delivered on PND2 or PND7 into healthy animals. Both cohorts demonstrated robust GFP expression in heart, liver, spleen and kidney tissues, indicating that the temporal differences seen are not likely due to large changes in viral penetration (data not shown).

Histological comparison between these different cohorts indicated that animals injected on PND7 had larger muscle fiber diameter than uninjected animal, and those injected on PND2 had still larger muscle fiber diameter although still not as large as those

from healthy animals (data not shown). This intermediate level of improvement corresponds to the rescue effects previously described.

We further wanted to characterize the effects of different delivery timepoints at a physiological level. To this end we analyzed improvement at the level of the neuromuscular junction (NMJ). Several NMJ defects have been well documented in SMA models, including the demonstration that a subset of muscles is particularly vulnerable to SMN deficiency, such as the longissimus capitis. Therefore, we investigated the effects of early and late SMN restoration in the longissimus capitis; a proximal muscle required for head stability and movement. SMNΔ7 mice were injected on PND2 or PND7 and harvested on PND14, along with SMA and wild type mice. Neurofilament accumulation and endplate structure was analyzed by immunofluorescence. While similar numbers of NMJs and axonal projections were observed, the endplate morphology in the PND7-treated tissues strongly indicated a developmental delay or inability to appropriately innervate as the majority of endplates lacked the well-defined "pretzel-configuration" consistent with well-developed mature end plates. As anticipated, tissue from the untreated SMA cohort was poorly innervated, consistent with previously published observations. The PND2 injected NMJs closely resembled the unaffected samples at PND14 where endplates were well innervated and largely free of neurofilament accumulations. While PND7-treated mice exhibited all stages of NMJ development, these treated NMJs displayed a greater number of partially innervated endplates and reduced number of fully innervated endplates compared with PND2-treated tissues. Our results strongly suggest that there is a time limit in which

SMN replacement is most beneficial to NMJs; however, reinstatement after the onset of symptoms (PND7) still provides a modest improvement to NMJ pathology.

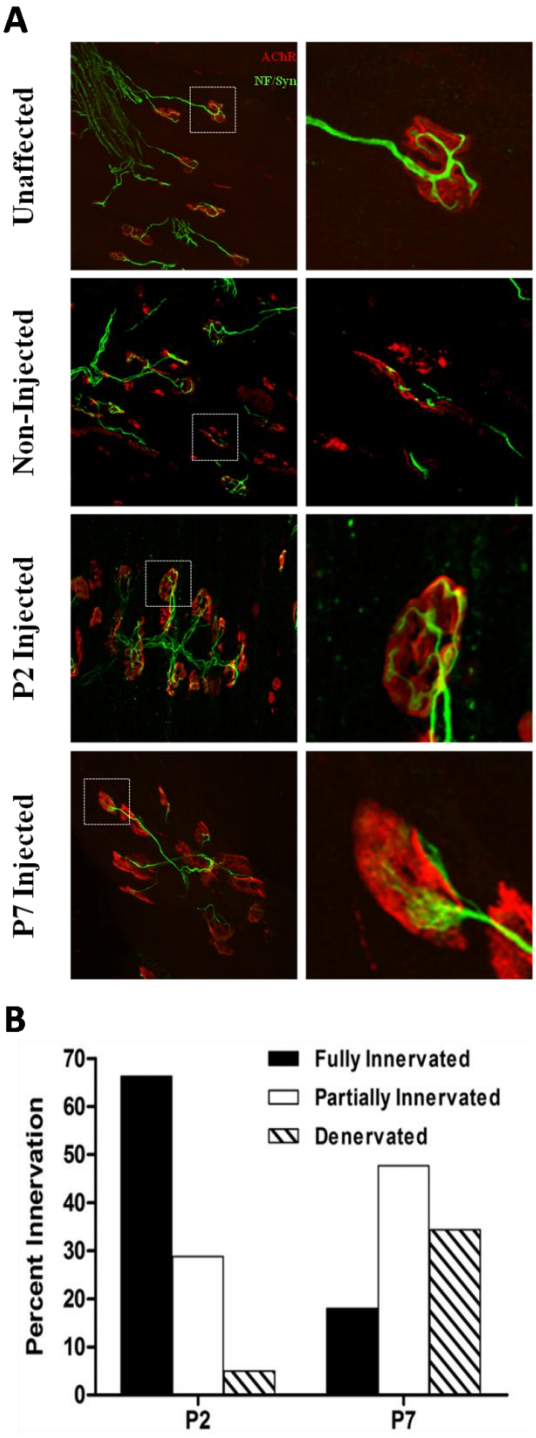

Figure 30: Timing of SMN restoration correlates with improved NMJ pathology.

(A) Representative confocal images of NMJ pathology on day 12 in the vulnerable muscle, longissimus capitis, of unaffected, non-injected and P2 or P7 treated mice. *Left*, 40X magnification of respective tissues; *Right*, enlargement of inset. Presynaptic components (green) were immunostained with anti-synaptophysin (Syn) and anti-neurofilament (NF). Acetylcholine receptors (AChRs) are labeled with alpha-bungarotoxin (red). Unaffected mice exhibited well-innervated NMJs with mature pretzel-shape endplates. Non-injected SMA mice exhibit a high degree of denervation and an increased number of immature endplates. scAAV9-SMN administration on P2 restores NMJ defects as observed by the fully-innervated, mature endplates. NMJ pathology is only partially restored following a delayed delivery (P7). (**B**) Bar graph representing fully innervated (black), partially innervated (open) and denervated (hatched) for P2- or P7-

injected SMA mice harvested on P12. Student's *t*-test: P2/P7 fully innervated: $P < 0.01$; partially innervated: $P < 0.01$; denervated: $P < 0.05$.

4.4: Discussion

Therapeutic approaches for the treatment of SMN can be broadly categorized into two groups: those that modulate *SMN2* splicing to generate more full-length transcripts and direct SMN replacement. Designing an effective therapeutic using either strategy requires knowledge of the tissue-specific and temporal requirements for SMN.

In an effort to elucidate the latter, we performed a thorough analysis of SMN replacement via scAAV9 at sequential postnatal time points in the SMNΔ7 mouse model of SMA. We observed that as treatment is delayed, the resulting phenotypic benefit achieved is diminished. However, even when treatment was administered when overt SMA-like symptoms were present (PND8) a significant, albeit lesser, phenotypic improvement was seen.

It is encouraging to see that our investigation into the therapeutic window produced similar results to transgenic approaches previously described. Two different inducible models have compared early and late SMN induction. One model demonstrated dramatic rescue of SMA mice with embryonic or postnatal induction while induction at PND6 in a significantly reduced rescue[203]. In another model, early induction (PND4) of SMN resulted in the most profound rescue whereas induction at later time points (PND6, PND8, PND10) resulted in a decreasingly substantial phenotypic rescue; mice receiving SMN induction at PND10 did not live longer than untreated controls[195].

Other investigations utilized a scAAV9 approach to answer this question. In the SMNΔ7 model, IV delivery at PND1 and PND2 produced a full lifespan rescue. Delivery at PND5 resulted in a much less pronounced rescue. By P10 delivery of scAAV9-SMN

no longer produces any phenotypic benefit [101]. While the same viral dose (1e11 vg) was used in both studies, ICV injection at PND5 resulted in ~30% of mice surviving past 100 days, while IV injection produced a maximum lifespan of 30 days. This difference highlights the importance of route of delivery [184, 205].

Finally, our finding that even late delivery can be beneficial is corroborated by other research. In the severe mouse model, in which symptoms are evident at birth, delivery of 2e11 vg at PND1 produces a significant extension of lifespan (7.5 to 17 days) [205]. Thus, delivery of scAAV9-SMN after the onset of symptoms in both the SMNΔ7 and severe models is able to modestly improve survival; however, not to the same extent as delivery prior to symptom onset.

Understanding why scAAV9-SMN efficiency diminishes when treatment is delayed would shed light on disease pathogenesis and aid in the development of AAV vectors as therapeutics. One possibility is the inability of the virus to escape from the CNS following an ICV injection at later time points. As many peripheral organs and tissues have been reported to be affected in both SMA patients and animal models (reviewed in [188]), the ability scAAV9 to cross the BBB is likely necessary to achieve a full life span rescue. It is known that scAAV9 crosses the BBB in P2 neonatal mice [175]. However, the ability of scAAV9 to permeate the BBB at later time points is not known. To investigate this, we injected scAAV9-GFP into unaffected animals and observed GFP expression in peripheral organs. Our findings demonstrate the even when scAAV9-GFP is delivered on PND7, robust GFP expression in all peripheral organs examined is

observed. Thus, inability of the virus to cross the BBB is not the reason for the lack of rescue observed in mice treated at later time points.

As low levels of SMN result in the specific loss of lower motor neurons, SMN replacement in those cells is critical for AAV-mediated gene therapy. Previous studies have shown that following a P2 IV injection of scAAV9-GFP, ~42% of lumbar spinal motor neurons were transduced [175]. Here we utilized an ICV delivery because our prior work in severe SMA mouse models demonstrated that direct injection into the central nervous system provided an enhanced phenotypic rescue compared with IV delivery [184, 190]. SMN expression throughout the spinal column in these P2-treated mice was greatly enhanced in the ICV-treated cohort, suggesting elevated transduction compared with IV-treated samples. However, it is expected that transduction levels would be equal to or more than that observed following an IV injection. It is not known if or how transduction levels change as treatment is delayed. Growing evidence suggests that additional cell types in the CNS beyond motor neurons contribute to the overall pathology of SMA, including astrocytes, and Schwann cells [132, 199]. Therefore, it is possible that the astrogliosis observed in SMNΔ7 mice has progressed to a point that leads to irreparable damage, consistent with the loss of >50% of the total motor neuron population[132]. Alternatively, all though not to the exclusion of the astrogliosis, the reduced motor neuron numbers observed by others [132]may simply indicate that sufficient motor neuron targets no longer exist since even a PND7 injection would likely not result in robust transgene expression until PND9, at which time a percentage of SMNΔ7 mice have already died. Even if motor neurons are present, they may be functionally compromised.

Therefore, it remains a possibility that an insufficient population of functional motor neurons is available at later time points, rendering SMN replacement less beneficial.

Another possibility for the lack of rescue may be the accumulation of irreversible neuromuscular junction (NMJ) defects. It has been previously reported that at PND2, NMJs in SMA and unaffected littermates are indistinguishable [290, 292]. However, by PND5 ~25% of all nerve terminals in SMA mice appear thick and swollen with abnormal NF aggregates. As the animals age, there is a progressive increase in the number of such defective terminal axons, reaching ~90% by PND14. Additionally, SMA axons fail to form the fine terminal arbors characteristically found as early as PND8 at normal NMJs. There have been varying reports as to the appearance of unoccupied endplates in SMA animals, with some groups reporting significant denervation and other reporting a lack thereof [290, 292]. However, the absence of anatomically denervated endplates in SMA mice does not suggest that these endplates have maintained their functionality. Further investigation will be needed to determine if these NMJ defects contribute to the inability of sAAV9-SMN to rescue when administration is delayed.

Understanding the underlying reason as to the insufficiency of later therapeutic treatment to fully rescue may aid in the development of new therapeutics and the betterment of existing ones. Nonetheless, early therapeutic intervention may be critical for therapeutic success.

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VITA

Madeline Miller was raised on a farm outside of Holcomb, Kansas to Tim and Rebecca Miller, and an older brother named Bryce. Madeline performed well in the Holcomb School District, finishing high school as Salutatorian. During this time she participated in many sports and musical organizations and learned an appreciation for informal public speaking. Holcomb provided her with many nurturing and inspiring teachers, including a particularly impactful biology teacher. Mr. Wilson is well known in Holcomb for challenging students to learn a solid background of understanding complex subjects as well as for modeling a general curiosity and an appreciation for lifelong learning. Madeline was no exception.

With a long family history at Kansas State University, Madeline chose this to be her alma mater without hesitation. She pursued a degree in biology, a minor in communications and chose to be involved in the Alpha Chi Omega sorority. After taking a genetics course from Dr. Susan Brown, Madeline requested to work in her lab, and Dr. Brown served as a valuable mentor for the rest of her undergraduate career. Dr. Brown encouraged Madeline to apply for awards such as research funding through the Kansas Idea Network of Biomedical Research Excellence and ultimately to pursue a Ph. D. Dr. Brown further introduced Madeline to an exciting research opportunity with Dr. Tonatiuh Melgarejo. Here Madeline received her own project in identifying whether a panel of cat tissues express a novel antimicrobial peptide. Dr. Melgarejo then used this work to build his search for antimicrobial peptides in captive and wild hyenas. He and Dr. Annika Linde brought Madeline on their trips to acquire samples from hyenas. This lead to the

Oklahoma City zoo, and ultimately to a safari trip to South Africa and Botswana in order to obtain biological samples from hyenas, as well as biometric readings from wild lions and leopards. Both Dr. Brown and Dr. Melgarejo helped Madeline apply at the University of Missouri to pursue a Ph.D.

At the University of Missouri, Madeline was granted admission into the Genetics Area Program, and was awarded a Life Sciences Fellowship. Knowing that she wanted to pursue translational research, Madeline met with Dr. Christian Lorson to discuss a possible rotation and appreciated his work in the field of Spinal Muscular Atrophy(SMA). After rotating in other labs, Madeline joined the Lorson lab and began learning viral gene therapy techniques. These techniques served as the basis of her doctoral research, investigating which regions within SMN are required to prevent SMA. Early in her career Dr. Lorson also advised Madeline to travel to California to learn dissection and staining techniques to study neuromuscular junctions (NMJs). This unique opportunity allowed Madeline to contribute to the work of her colleagues, amounting to three co-author publications. Later, when Madeline expressed interest in writing a review article, Dr. Lorson helped her author a text book chapter on the topic of gene therapy in Spinal Muscular Atrophy. Dr. Lorson helped Madeline pursue research interests that involved breaking into the field of fruit fly research and a genial collaboration with Dr. Bing Zhang. This new field of research was foreign, but very rewarding as a geneticist, and Dr. Zhang has said that he has never had a student so quickly grasp the complex genetic crossing schematics involved in fly research; genetic fly research seemed to suit Madeline's strengths.

Due to Madeline's interest in teaching, she appreciated that the Genetics Area Program encourages students to teach as part of their graduate training. For this, Madeline chose to serve as a teaching assistant for Dr. Elizabeth Bryda's writing intensive Human Inherited Diseases course. This was an insightful experience as Dr. Bryda shared the thoughtful ways in which she prepares for class and handles the unexpected situations that inevitably arise with students. Madeline enjoyed her time teaching with, and learning from Dr. Bryda. Together with her work in the Lorson lab, this course instilled in Madeline an appreciation for the challenges associated with rare diseases.

At MU, Madeline has found a number of ways in which to incorporate her newfound appreciation of rare diseases with her general interest in communication. Together with a close friend, Linh Ngo, Madeline helped organize Rare Disease Day events at the University of Missouri. These two have spoken about rare diseases in a public Saturday Morning Science forum and have started a blog called Sciwalk Cafe along with another friend, Adam Johnson. The blog serves as a creative outlet for scientists to present stories that bridge science and humanity. Adam and Madeline both also became involved with a local science radio show called The Big Electron. Here they discuss primary literature, news, and the science behind various cultural events directed toward a lay audience. Through these opportunities Madeline has merged her aptitude for communication with a cause that is worth sharing.

Columbia has provided Madeline with many other fulfilling ventures. She has enjoyed participating in various dancing classes including ballroom dance and swing. She found a bright and charming friend with which to share Columbia when she signed up for

Big Brothers and Big Sisters and met her little, Alaina Kimmons. Along with all of these meaningful interactions, the Unitarian Universalist Church of Columbia served Madeline as a regular reminder of the things that are bigger and more important than bench work. Madeline shared every aspect of graduate school and life with Simon Bolding. Though he lived afar, Simon encouraged Madeline every day through the struggles and joys she found in Columbia. The two will be married October 1st, 2016.

Upon leaving Columbia, Madeline plans to pursue a career in science communication and education. In the short term, she plans to continue developing science writing skills through Sciwalk Cafe and developing science educational tools for the Columbia Afterschools Program.