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IDENTIFICATION OF NOVEL TARGETS TO TREAT SPINAL MUSCULAR
ATROPHY

by

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ABSTRACT

Identification of novel targets to treat spinal muscular atrophy

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Spinal muscular atrophy (SMA) is a debilitating neuromuscular disease that is one of the leading genetic causes of infant mortality. SMA is caused by insufficient levels of survival motor neuron (SMN) protein and is characterized by the loss of lower motor neurons in the anterior horn of the spinal cord and denervation-dependent muscle atrophy. Patients present with severe muscle weakness and fail to achieve normal motor milestones. Recently approved treatments for SMA, nusinersen and gene therapy, prolong lifespan, but only a small subset of patients have improvements in their motor function. Therefore, there is a critical need to develop additional treatments that can act either alone or in combination with current treatments to improve disease outcomes in all patients. Here, we aimed to identify novel genetic modifiers of SMN protein and molecular pathways in skeletal muscle that could be targeted to improve motor function.

Using a genome-wide RNAi screen, we found that knockdown of *WDR33*, *CPSF1*, and *THOC3*, all components of RNA processing machinery, increase SMN protein levels. Knockdown of *WDR33* and *THOC3*, but not *CPSF1* increases export of

SMN mRNA to the cytoplasm allowing for increased translation. We also found that knockdown of *Neuralized-2* (*Neurl2*), an E3 ligase, increases SMN protein. We show that *Neurl2* ubiquitinates SMN and that reduction of *Neurl2* stabilizes SMN protein levels.

Given that SMA mice have fewer and smaller muscle fibers, and that SMN deficiency impairs myoblast fusion, we sought to identify how loss of SMN impairs myoblast fusion. We found that SMN deficiency reduces expression of myomaker and myomixer, two muscle-specific fusion molecules, in SMA mouse muscle and in SMN-deficient myoblasts. Ectopic expression of myomixer in SMA mice increases myofiber number and improves motor function and further improved muscle histopathology and motor function when administered in combination with an SMN-increasing therapy. These studies provide evidence of novel pathways that can act in combination with current therapies to further improve disease outcomes.

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CHAPTER 1: Introduction

WHAT IS SPINAL MUSCULAR ATROPHY?

Spinal muscular atrophy (SMA) is a neuromuscular disease that is one of the leading genetic causes of infant mortality (92; 115). SMA affects approximately 1 in 10,000 live births annually (170). It is an autosomal recessive disease and has a carrier frequency of 1 in 50 people (170). SMA is characterized by the loss of lower motor neurons in the anterior horn of the spinal cord and denervation-dependent muscle atrophy causing patients to have severe muscle weakness (92).

WHAT CAUSES SMA?

SMA is caused by insufficient levels of the survival motor neuron (SMN) protein (92). SMN protein is produced by two genes located on chromosome 5: *SMN1* and *SMN2*. *SMN1* is the major producer of full-length SMN protein whereas a C-to-T base pair change in *SMN2* leads to alternative splicing of the *SMN2* transcript resulting in the exclusion of exon 7 (98; 116). Exclusion of exon 7 leads to the formation of a less stable, truncated protein known as SMN Δ 7. *SMN2* produces about 85% SMN Δ 7 and about 15% full-length SMN (98; 116). SMA patients have a homozygous loss or deletion of *SMN1*, but retain at least one copy of *SMN2* (92). Because *SMN2* cannot fully compensate for the loss of *SMN1*, patients have reduced levels of SMN protein in all organ systems.

CLINICAL PRESENTATION

Symptoms of SMA

SMA patients present with severe muscle weakness, reduced muscle tone, and reduced or loss of tendon reflexes (50; 120; 133). Many patients have weak movements, a

weakened or absent grasp, and weakened respiratory muscles (120; 133). Patients with severe forms of the disease fail to reach important motor milestones (133). Additionally, many patients experience difficulty eating (133).

A recent retrospective study of insurance claims of SMA patients prior to their first symptoms of a neuromuscular disease revealed many common non-neuromuscular phenotypes including defects in the cardiovascular, gastrointestinal, metabolic, reproductive, and skeletal systems (95). These defects were most prevalent among patients with milder forms of SMA. It remains unknown whether these non-neuromuscular defects are ameliorated with current SMA treatments.

Types of SMA

There are four main types of SMA that are characterized by the age of onset of symptoms (summarized in **Table 1**) (99). Type 1 SMA is the most severe form of SMA and accounts for more than half of all patients (170). SMA type 1 patients present with symptoms before 6 months of age, never achieve the ability to sit unassisted, and die before 2 years of age (40; 99). Type 2 patients are diagnosed between 6 and 18 months of age and are able to sit, but not stand or walk (40; 99). Type 1 and type 2 patients experience weakness of their intercostal muscles, the muscles which are used to cough and clear the lungs, resulting in patients dying from upper respiratory infections such as pneumonia (120). Type 3 patients are diagnosed after 18 months of age, are able to stand and walk, but experience impaired movement and muscle weakness later in life (40; 99). Type 3 patients have a near-normal lifespan. Type 4 is the least severe form of SMA and is adult onset (99). Type 4 patients are able to walk and experience muscle weakness in adulthood.

Table 1. Types of SMA characterized by age of onset, highest motor milestone achieved, and *SMN2* copy number.

Type of SMA	Age of Onset	Highest Motor Milestone Achieved	Age of Death	# of <i>SMN2</i> copies
Type 1	< 6 months	Never sit	< 2 years	1-2
Type 2	6-18 months	Sit; never stand or walk	School age	2-3
Type 3	> 18 months	Stand and walk	Adult	3-4
Type 4	Adult	Walk through adulthood	Adult	4+

Differences in disease severity are caused by variation in *SMN2* copy number. A higher *SMN2* copy number causes a less severe phenotype because patients are able to produce more full-length SMN protein (101; 174). Type 1 patients usually have 1-2 copies of *SMN2*, whereas type 4 patients usually have at least 4 copies of *SMN2* (174). *SMN1* copy number can also be varied between individuals. Majority of Caucasian-Americans have 2 copies of *SMN1* and majority of African Americans have 2-3 *SMN1* copies (67). Majority of sub-Saharan Africans have 2-4 *SMN1* copies and consequently this population has a low SMA carrier frequency (151).

Recently, two additional genetic modifiers of the SMA phenotype were identified: plastin-3 and neurocalcin delta (127; 143). Plastin-3, an actin-binding protein, was shown to be significantly upregulated in unaffected *SMN1*-deleted females (127). Further studies demonstrated that overexpression of plastin-3, using gene delivery, improves the disease phenotype in a mild SMA mouse model (78). Neurocalcin delta, a neuronal calcium sensing protein, was significantly reduced in asymptomatic individuals with 0 *SMN1* copies and 4 *SMN2* copies suggesting that loss of neurocalcin delta is protective (143). Knockdown of neurocalcin delta ameliorated the disease phenotype in zebrafish, *C. elegans*, and mouse models of SMA (143). An extensive screen of the entire genome to identify potential modifiers of the SMA phenotype has not been done.

SMN PROTEIN

SMN is a 38-kDa protein that is ubiquitously expressed in both the nucleus and cytoplasm (38; 96). SMN can dimerize and form a complex with unrip and gemins 2-8 (37). Gemin 2, gemin 3, gemin 5, and gemin 7 bind directly to SMN (56). SMN is ubiquitinated by the E3 ligase mindbomb-1 and is degraded by the proteasome (30; 86). Other E3 ligases have been shown to ubiquitinate SMN including UCHL1 and Itch1 (57; 71). Full-length SMN protein has a half-life of ~4.3 hours whereas SMN Δ 7 has a half-life of 2.2 hours (25).

Although how loss of SMN causes SMA pathology remains unknown, there are several known functions of SMN including a role in pre-mRNA splicing (134). The SMN complex is required for the assembly of the core ring of 7 Sm proteins (135). Together with a small nuclear RNA (snRNA) and proteins specific to the snRNA, the core ring of Sm proteins form a small nuclear ribonucleic protein (snRNP) (181). snRNPs associate with other proteins to form the spliceosome. In SMN-deficient cells and SMA model mice, there is reduced snRNP assembly. Consequently, there are changes in the splicing pattern of many genes in several tissue types, including the spinal cord, in SMA mice (181).

Additionally SMN has a role in the regulation of translation. SMN associates with polyribosomes, which are part of the translation machinery, and is able to repress translation of a generic luciferase reporter *in vitro* (150). SMN has been specifically shown to regulate the translation of CARM1. With the loss of SMN, there is an increase in CARM1 protein expression, but there is no change to CARM1 mRNA or CARM1 protein degradation. Consequently, there is an increase in CARM1 protein in both SMA patient cells and in SMA mice (150).

SMN IN THE MOTOR UNIT

The motor unit is composed of a lower motor neuron and all of the muscle fibers it innervates. SMA is traditionally considered a motor neuron disease and is primarily characterized by the loss of lower motor neurons in the anterior horn of the spinal cord and denervation-dependent muscle atrophy (33; 75; 120; 165). Consequently, lower motor neurons have been extensively studied in SMA. However, all components of the motor unit, including the neuromuscular junction (NMJ), are affected in SMA. Loss of synaptic contact at the NMJ, occurs prior to motor neuron death, and is one of the earliest signs of SMA pathology (15). Loss of SMN-deficient motor neurons is caused by a dying-back mechanism leading to muscle atrophy and weakness (36).

When SMN is insufficient, skeletal muscle is denervated and motor neurons die. In a severe mouse model of SMA, expressing SMN selectively in neurons prevented motor neuron loss and improved motor function, but had a transient increase in body weight and only moderately extended survival suggesting that restoration of SMN in motor neurons alone is not sufficient to fully rescue the SMA phenotype (90).

In another study, severe SMA model mice were co-treated with a subcutaneous injection of an antisense oligonucleotide (ASO) that corrects *SMN2* splicing and a decoy ASO via intracerebroventricular injection that blocks the effects of the splicing modifier in the CNS (72). Systemic injection of the splicing modifier prevented motor neuron loss, rescued AChR clustering patterns in the longissimus capitus, extended survival, improved grip strength, and improved balance and coordination in the rotarod test (72). Co-treatment with the decoy ASO did not block any of the effects of the splicing modifier indicating that peripheral expression of SMN is critical for normal NMJ morphology, motor function, and survival (72).

Additional studies confirm the importance of SMN in the periphery, including its requirement in skeletal muscle. *Drosophila* with a knockdown of SMN selectively in skeletal muscle have a shorter lifespan than *Drosophila* with a knockdown of SMN selectively in motor neurons (29). Furthermore, mice with a deletion of SMN exon 7 and one copy of *hSMN2* in skeletal muscle have reduced body weight, distressed breathing, decreased myofiber diameter, and a shortened lifespan without loss of lower motor neurons (83). Together, these findings suggest that SMN is required in all components of the motor unit.

SMN IN THE SPINAL CORD

SMA Patient Pathology

In SMA patient spinal cord autopsies, there is a loss of lower motor neurons in the anterior horn while the posterior horns and spinal ganglia are normal (33; 75; 120). Some of the remaining motor neurons are chromatolytic, have swollen cell bodies, have their nucleus in the periphery, and show axonal degeneration (32; 33; 120). Swollen neurons often have an accumulation of phosphorylated neurofilaments in the cell body (120). Motor neurons show apoptotic changes including loss of ribosomes on the rough endoplasmic reticulum, nuclear membrane hyperchromatosis, shrinking of the nucleus and cytoplasm, and breakdown of the plasma membrane (153). These motor neurons also have high nuclear expression of p53, an inducer of cell death, and loss of cytoplasmic expression of Bcl-2, an inhibitor of cell death (153). A subset of lower motor neurons have fragmented DNA indicating necrotic cell death (153). Infants with severe forms of SMA had a normal response to ulnar nerve stimulation (118). However, they had slow

conduction velocity in the posterior tibial nerve, which may be explained by the presence of smaller myelinated axons (32; 118).

In addition to lower motor neurons, other cell types have abnormal pathology in the spinal cord. Glial bundles and fibrocytic astrocytes are in the anterior horn of the spinal cord in SMA patients (32; 33). Microglia were found around swollen motor neurons (153).

SMN in Motor Neurons

SMN has several roles in motor neurons including axonal growth, mRNA localization, protein synthesis, and fast axonal transport. SMN is highly expressed in the NMJ and accumulates in the growth cone during neuronal differentiation and during the formation of neurites (48). SMN also co-localizes with GAP43, a major component of the growth cone (48). When grown in culture, motor neurons from SMA mice have shorter axonal processes and smaller growth cones but have normal dendrites (147).

Furthermore, overexpression of SMN in PC12 cells, a neuronal cell line, promotes neurite growth (147). Studies in zebrafish have shown that loss of SMN leads to branching defects and truncation of motor axons leading to poor motor function (59; 89; 108). A lack of positive TUNEL staining in *Smn*-deficient motor neurons of zebrafish suggests that shortened axons are not caused by apoptosis, but are likely due to impaired motor axon development (108).

Studies in mice have not been able to confirm a role for SMN in motor axon development. SMA mouse embryos do not have defects in axonal formation and have no misrouted bundles (107). Additionally, SMA mice do not have unoccupied nicotinic acetylcholine receptor (nAChR) clusters until embryonic day 18.5 (E18.5) suggesting that

motor neurons in SMA mice are able to reach their target muscle during development and that denervation is why there are unoccupied nAChR clusters (107). It is not known whether axonal growth is impaired in SMA patients.

SMA mice have reduced levels of poly(A) mRNA in axons, but not cell bodies of their motor neurons (47). Given that SMN-deficient neurons also have reduced protein synthesis in the growth cone, it is likely that mRNA is not properly transported to the growth cone indicating a role for SMN in the localization of mRNAs (47). Both GAP43 and β -actin mRNA are reduced in SMN-deficient axons (47). GAP43 promotes axon growth which may explain impaired motor axon development and growth with SMN deficiency.

Global protein synthesis efficiency is reduced with SMN deficiency (88). SMN deficiency increases miR-183 in neurites and in SMA type 1 patient fibroblasts. Increased miR-183 alters mTOR activity leading to reduced protein synthesis (88). Knockdown of miR-183 rescued axon growth of SMN-deficient neurons in culture. However, miR-183 inhibition in SMA mice led to minimal improvements in survival, body weight, and grip strength, and had no effect on the number of motor neurons or NMJ morphology (88).

SMN also has a role in axonal transport. Examination of synaptic vesicle proteins in the sciatic nerve of wild-type (WT) and SMA mice revealed reduced expression of synaptic vesicle protein 2 (SV2-c) and synaptotagmin (Syt1) in the distal axon (42). This indicates fewer synaptic vesicles at the distal axon due to reduced anterograde transport with SMN deficiency (42).

SMN in Sensory Neurons

At birth, WT and severe SMA model mice have a similar number of VGluT1⁺ (a marker for proprioceptive inputs) terminals on the soma and dendrites of lower motor neurons (110). By postnatal day 13 (P13), there is a significant loss of VGluT1⁺ terminals on dendrites, but not on the soma of SMA motor neurons. While there are fewer and smaller proprioceptive synapses, there is no change in the number of primary sensory neurons in the dorsal root ganglia in P13 SMA mice (110). Sensory neurons are also functionally impaired with SMN deficiency. Stimulation of L1 dorsal root fibers evoked a reduced monosynaptic reflex in P4 SMA spinal cord with an 85% reduction in peak amplitude (110). Intracellular recordings showed that SMA motor neurons evoked fewer action potentials and respond weakly with a 70% reduction in peak excitatory postsynaptic potential (EPSP) amplitude (110). The decrease in afferent-evoked potentials is likely due, at least in part, to loss of VGluT1⁺ synapses on SMA motor neurons. Sensory neuron defects can be reversed with administration of trichostatin A (TSA), an histone deacetylase (HDAC) inhibitor which was previously shown to increase SMN protein levels, improve motor function, and extend survival of SMA mice (12). TSA treatment increases the size and density of VGluT1⁺ synapses on SMA motor neurons and increases the amplitude of the L1 monosynaptic reflex suggesting that these defects are SMN-dependent (110).

SMN in Glia

When grown in culture, SMA mouse oligodendrocytes proliferate and mature similarly to control oligodendrocytes (126). There is also no difference in morphological maturation. SMA oligodendrocyte progenitor cells (OPCs) display normal migration and SMA oligodendrocytes display normal myelination of axons *in vitro* (126). In SMA mice,

oligodendrocytes mature and myelinate normally suggesting that oligodendrocyte function is not altered with SMN deficiency (126).

There is an increase in GFAP⁺ astrocytes in the grey matter of the anterior horn of SMA patient spinal cords and as well as an increase in IL-6 and IL-1 β expression, which are markers for glial activation (144). Restoration of SMN in astrocytes in SMA mice extends lifespan, increases body weight, improves motor function, and increases the number of fully innervated NMJs in the splenius capitis and longissimus capitis muscles, but has no effect on the number of lower motor neurons in the lumbar region of the spinal cord (144). Additionally, there is a partial reduction of IL-6, IL-1 β , and TNF α in the spinal cord with SMN restoration in astrocytes suggesting that SMN deficiency leads to glial activation (144).

Conditioned media from SMA patient induced pluripotent stem cell- (iPSC-) derived astrocytes induces death of SMA iPSC-derived motor neurons (157). miR-146a, a miRNA involved in inflammatory signaling pathways, is increased in exosomes secreted by SMA iPSC-derived astrocytes (157). SMA iPSC-derived motor neurons co-treated with astrocyte conditioned media and a miR-146a inhibitor were protected from cell death indicating that miR-146a mediates the death of SMN-deficient motor neurons (157).

SMA mice have an increase in Iba1⁺ microglia in the spinal cord, similar to reports in SMA patients (153; 171). Depletion of microglia from SMA mice improves performance in the righting reflex test, but does not rescue motor neuron death suggesting that depletion of microglia alone is not sufficient to prevent motor neuron death (171). Together, these studies suggest that glia may play a role in mediating motor neuron death.

SMN AT THE NMJ

Immature NMJs

Normally during development, the embryonic γ -subunit of the AChR is switched out for the mature ϵ -subunit. Additionally, the NMJ transforms from a plaque-like shape to a pretzel-like shape with many perforations and folds (79). In SMA mice, there is an increase in the γ -subunit and a decrease in the ϵ -subunit indicating immature AChRs (84). SMA mice also have more plaque-like NMJs with a lack of perforations (79). Similarly, NMJs in autopsy tissue from an SMA type 1 patient have increased expression of the γ -subunit and endplates have fewer folds indicative of less complex, immature NMJs (60).

Denervation

In SMA zebrafish models, expression of SV2, a marker for presynaptic terminals, was reduced suggesting an NMJ defect with SMN deficiency (58). Similarly, SMA mice have unoccupied AChR clusters in intercostal muscles, partially denervated NMJs in paraspinal muscles, and many multiply-innervated NMJs in the tibialis anterior (TA) muscle (84; 107). An extensive study examining innervation patterns in SMA mice revealed that the majority of axial muscles (those in the head, neck, and trunk) and a few appendicular muscles (those that control limbs) had some form of denervation (94). SMN-deficient lower motor neurons can form synapses on skeletal muscle, but eventually synaptic contact is not maintained and muscles become denervated. For example, at E17.5 the serratus posterior inferior (SPI) muscle is fully innervated in the SMA mouse. However, at birth, only 45% of endplates remain fully innervated with the other endplates either fully or partially denervated (94).

SMN-deficient motor neurons are unable to properly reinnervate muscle. A study in a milder SMA mouse model found high levels of sprouting in the transverse abdominis (TVA), a muscle that is highly denervated in SMA mice (121). Sprouts traversed deep into muscle, but ultimately were not associated with AChRs suggesting impaired NMJ remodeling (121). It is unclear what induces denervation and whether denervation is due to defects in either the motor neuron, the muscle fiber, or both.

Electrophysiology

Electrophysiological recordings reveal reduced endplate current (EPC) amplitude in SMA mice due to fewer vesicles released and reduced probability of neurotransmitter release (84). Additionally, vesicle density is reduced and distribution of vesicles is abnormal with fewer docked vesicles (84).

Schwann cells

Axons of intercostal nerves are both myelinated and unmyelinated in a mouse model of severe SMA (76). SMA mice also have more large axons that are unmyelinated or have thinner myelin sheaths compared to control mice (76). Given that there are no signs of axonal degeneration, but there are myelination defects, it is likely that SMN deficiency affects Schwann cell function. When grown in culture, SMA mouse Schwann cells have upregulated expression of caspase-3, indicating that the cells are apoptotic (76). Additionally, SMA Schwann cells poorly myelinate wild-type neurons in culture (76). Restoration of SMN in Schwann cells in SMA mice rescues myelin sheath pathology, improves motor function, increases the number of innervated NMJs in the levator auris longus (LAL) muscle, and extends survival, but has no effect on myofiber size (77).

SMN IN SKELETAL MUSCLE

SMA Patient Pathology

The limb and trunk muscles of SMA patients show signs of atrophy while some muscles, including the diaphragm, are spared (75). Skeletal muscles show signs of denervation and have groups of atrophied myofibers among groups of normal fibers (75). Patients have fewer myofibrils due to the loss of myofilaments and have a large variation in myofiber diameter (165). Myofibers of SMA patients are small with small central nuclei (50; 75; 165). In atrophied fibers, the sarcolemma is normal, but there are fewer mitochondria and the sarcoplasmic reticulum is either absent or highly disorganized (75). Band structure within the fibers is also disorganized with no clear distinction between the A, I, and Z bands (75). Patient autopsies show the presence of some satellite cells, the skeletal muscle stem cells (165). There is also increased collagen between myofibers indicating formation of fibrous tissue (165). Electromyography (EMG) shows atrophy caused by denervation (32; 120). In one case study, a patient with severe SMA had hypertrophic round type I myofibers which may be indicative of atrophy of myopathic origin whereas atrophy of neurogenic origin would result in angular myofibers (120). This finding further supports the role of muscle in SMA pathogenesis.

SMN in Satellite Cells

SMA mice have smaller myofibers and have a normal number satellite cells compared to unaffected mice (62). Examination of muscle differentiation markers show more Pax7⁺ satellite cells co-express MyoD in SMA mice indicating SMN-deficient satellite cells differentiate more rapidly (62). When grown in culture, SMA mouse satellite cells prematurely differentiate, but are ultimately unable to fuse to form myosin

heavy chain-positive (MyHC⁺) myotubes (62). Restoration of SMN in satellite cells in SMA mice prevents loss of motor function in the first several months of life due to the ability to regenerate myofibers (125). Eventually, the loss of myofibers outcompetes the ability to regenerate new fibers leading to loss of motor function (125). These studies demonstrate the need for SMN in satellite cells in order to properly develop and regenerate myofibers.

SMN in Myoblast Fusion

SMA patients and SMA mice have smaller myofibers than their unaffected counterparts suggesting SMN may have a role in skeletal muscle development (18; 75; 84; 91; 120). Skeletal muscle development is a highly regulated process involving the induction of myogenic programming genes, MyoD and myogenin to produce myosin heavy chain, migration of myoblasts and alignment of their plasma membranes, and finally formation of multinucleated myotubes. These key aspects of skeletal muscle development are well-studied in SMA. Several studies have shown altered myogenic programming with SMN deficiency. MyoD and myogenin levels are reduced in myoblasts from two different SMA mouse models as well as in human embryonic stem cell (hESC)-derived myoblasts from a SMA type 1 patient (18; 21; 66). SMA mice have an increased amount of the perinatal isoform of MyHC and less of the adult isoform suggesting that muscle fibers are immature (84; 91). SMN deficiency also reduces fusion of myoblasts from a SMA type 1 patient, immortalized myoblasts from SMA mice, and Smn-deficient C2C12 myoblast cells (10; 21; 152). Immortalized myoblasts from SMA mice also showed reduced cell motility (21). Myoblasts from a SMA type 1 patient had fewer AChRs and had reduced AChR clustering and hESC-derived SMA myoblasts had

reduced functional Ca^{2+} response to nAChR stimulation (10; 66). Together, these studies show that SMN-deficient myoblasts are not able to properly fuse and mature into functional multinucleated myotubes. The mechanism by which loss of SMN alters myogenic programming to impair myoblast fusion is not known.

Muscle Weakness with SMN Deficiency

SMA mice have muscle weakness prior to loss of lower motor neurons and denervation further supporting a role for SMN in skeletal muscle (20). The tibialis anterior (TA) muscle of SMA mice has reduced twitch force and a lower peak tetanic force (20). How SMN deficiency causes muscle weakness is unknown. Deletion of SMN exon 7 specifically in skeletal muscle in mice causes muscle paralysis by 4 weeks of age as well as decreased spontaneous and induced motor activity (35). However, closer examination of myofibers in this mouse model reveals a dystrophic phenotype rather than atrophic (35).

SMN IN PERIPHERAL TISSUES

Smooth Muscle

One group recently identified a role for SMN in smooth muscle using a mouse model of SMA where SMN is high in neurons, but low in cardiac muscle, skeletal muscle, kidney, and lower GI tract and there is no SMN in the upper GI tract, liver, and spleen (175). SMA mice had increased fecal loading (175). A closer examination of the colon revealed lesions, infiltration of inflammatory cells, and smaller cross-sectional area of smooth muscle fibers in the colon (175). Additionally, SMA mice have a smaller bladder and smaller bladder smooth muscle fibers (175). SMA mice have fewer interstitial cells of Cajal (ICCs) which connect motor neurons to local effector cells such

as smooth muscle cells. This reduction in ICCs may explain abnormal enteric nervous system signaling and poor GI motility (175).

Cardiac Muscle

Hearts from a severe SMA mouse model are significantly smaller and flaccid compared to those of unaffected mice (65). SMA mice had a slow heart rate even prior to loss of motor neurons (65). SMA mice have decreased pumping efficiency and blood flow from the right ventricle to the lungs (65). Additionally, there are fewer sympathetic nerves and the nerves innervating the heart are thinner and have reduced branching (65).

MECHANISMS OF MOTOR NEURON DEATH

Why loss of SMN, despite being a ubiquitously expressed protein, selectively causes lower motor neurons to die is not known (24). Some SMN-deficient motor neurons are more susceptible to cell death (122). For instance, motor neurons that innervate the TVA, rectus abdominis (RA), and external oblique (EO) are vulnerable whereas motor neurons innervating the LAL, auricularis superior (AS), and adductor auris longus (AAL) are less vulnerable to cell death (122). Muscles innervated by vulnerable motor neurons have about one fifth of NMJs denervated while nearly all NMJs were fully occupied in muscles innervated by less vulnerable motor neurons in symptomatic SMA mice (122). When SMA mice are presymptomatic, vulnerable SMA motor neurons have increased expression of cell death factors including parts of the p53 signaling pathway (122). Other studies have confirmed an increase in p53 signaling in motor neurons in different SMA mouse models (39; 154).

In a mild SMA mouse model, p53 expression is upregulated when denervation begins (39). When the mild SMA mouse model is crossed with a mouse with an inducible

p53 knockout background, there is no improvement in body weight, the righting reflex, and motor neuron death, but there is reduced NMJ loss (39).

In a severe SMA mouse model, p53 is highly expressed in vulnerable motor neurons in the L1 and L5 regions in the spinal cord (154). p53 must be phosphorylated to induce motor neuron death. Vulnerable L1 and L5 motor neurons accumulate phospho-p53 whereas resistant L5 SMA motor neurons only accumulate p53 (154). Inhibiting p53 rescues motor neuron death, but has little to no effect on survival, body weight, and motor function (154). Mdm2 and Mdm4 are repressors of p53. In severe SMA model mice, there is an accumulation of alternatively spliced Mdm2 and Mdm4 (168). Overexpression of full-length Mdm2 and full-length Mdm4 reduces p53 in vulnerable SMA mouse motor neurons and decreases the mRNA expression of several downstream targets of p53 (168). Additionally, overexpression of either full-length Mdm2 or full-length Mdm4 reduces motor neuron degeneration in the lumbar region of the spinal cord and improved motor function in the righting reflex test, but does not improve NMJ denervation, the amplitude of the compound muscle action potential (CMAP), body weight, and survival (168). Why NMJs are lost in a severe SMA model mouse even when motor neurons do not degenerate remains unknown.

Genetically delaying Wallerian degeneration in a severe mouse model of SMA does not delay disease onset and has no effect on body weight, motor function, and loss of motor neurons (146). This study suggests that Wallerian degeneration does not cause SMA disease pathogenesis.

MECHANISMS OF MUSCLE ATROPHY

Atrogin-1 and muscle ring finger 1 (MuRF1) are E3 ligases called atrogenes that ubiquitinate muscle proteins to target them for degradation in atrophic myofibers. Atrogin-1 and MuRF1 are upregulated in the iliopsoas muscle, a hip muscle, of type 1 SMA patients and their expression remains unchanged in the diaphragm, a relatively unaffected muscle (22). It remains unknown why some muscles, such as the diaphragm, are relatively spared with SMN deficiency. Severe SMA model mice also have increased expression of atrogin-1 and MuRF1 (22). Atrogene expression is regulated by histone deacetylases (HDACs). Treatment with trichostatin A (TSA), an HDAC inhibitor, inhibits atrogene induction in SMA mice and blocks the interaction of myogenin on the atrogin-1 and MuRF1 promoters (22). How loss of SMN causes myogenin to induce atrogene expression is unclear.

In a different severe SMA model mouse, atrogin-1, but not MuRF1, is increased in hind limb muscle (45). SMA mice also have a subsequent increase in ubiquitinated proteins (45). In a mild SMA model mouse, both atrogin-1 and MuRF1 expression are upregulated and have increased ubiquitinated proteins when mice are symptomatic (45). This model SMA mouse also has increased expression of the autophagosomal markers Gabarapl1, CathepsinL, and Bnip3, and has increased protein levels of LC3-II (45). Treatment of mild SMA mice with TSA decreases expression of atrogin-1 and MuRF1, decreases ubiquitinated proteins, and decreases expression of autophagy markers (45).

TREATMENTS FOR SMA

Restoration of SMN protein levels improves the disease phenotype and prolongs survival of SMA mouse models. Consequently, increasing full-length SMN protein is the goal of approved treatments as well as many of the treatments currently in development

and in clinical trials (summarized in **Table 2**). Increased SMN protein can be achieved by increasing transcription of *SMN2*, modifying *SMN2* transcript splicing, blocking SMN protein degradation, and through SMN gene delivery (4; 12; 74; 131).

Table 2. Current treatments and drugs under consideration for SMA patients.

Drug	Current Phase	Mechanism of Action	Mode of Administration
Nusinersen	FDA Approved	Splicing modifier	Intrathecal injection
AVXS-101	FDA Approved	Gene therapy	Intravenous injection
Risdiplam	Phase 3; FDA decision expected May 2020	Splicing modifier	Oral
Branaplam	Phase 1/2	Splicing modifier	Oral
SRK-015	Phase 2	Myostatin inhibitor	Intravenous injection
CK-2127107	Phase 2	Fast skeletal muscle troponin activator	Oral

Nusinersen

Nusinersen is the first drug approved by the US FDA to treat SMA patients (129). Nusinersen is a 2'-*O*-methoxyethyl-modified antisense oligonucleotide (ASO) that blocks the intronic splicing silencer of intron 7 on *SMN2* (ISS-N1) to promote the inclusion of exon 7 (130). In preclinical studies, intracerebroventricular and systemic injection of the ASO increased total full length SMN protein, improved motor function, and prolonged survival in a mouse model of severe SMA (73; 130).

Nusinersen is administered to patients via intrathecal injection (51). Patients receive 4 loading doses in the first two months of treatment followed by a maintenance dose once every 4 months (51). In a phase 3 sham-controlled study in type 1 SMA

patients, 37/73 (51%) of nusinersen-treated patients saw improvements in their motor function while no patients in the placebo-treated group had improvements in their motor function (51). In the nusinersen-treated group, 22% had full head control, 10% could roll over, 8% could sit unassisted for 30 seconds, and 1% could stand (51). Additionally, significantly fewer patients in the nusinersen treatment group died or required permanent ventilation (51). A separate study found that only 15/47 (32%) SMA type 1 patients were able to sit independently after 14 months of nusinersen treatment (9). In another sham-controlled study, SMA type 2 patients, who were able to sit independently, but not walk at baseline, were treated with nusinersen for 15 months (111). During the course of this study, 13/84 nusinersen-treated and 2/42 control patients achieved at least one new motor milestone (111). Only 1/84 nusinersen-treated patients (vs. 1/42 control patients) could stand alone and only 1/84 nusinersen-treated patients would walk with assistance (vs. 0/42 control patients) after 15 months of treatment (111).

Type 1 and type 2 SMA patients who were treated presymptomatically show the most improvement in motor function with 22/25 patients having the ability to walk independently indicating that early treatment is critical (44). While this was an open-label, non-controlled study, it supports other data showing that the SMN protein is needed most during early development (140). Human spinal cord and skeletal muscle autopsy tissue have high levels of SMN protein during fetal development followed by a decrease in expression in the perinatal period (140). SMN protein levels remain low postnatally (140).

In patients treated with nusinersen, there is uptake of ASO in the anterior horn neurons with the highest uptake of in the lumbar, sacral, and thoracic regions and less

uptake in the cervical spinal cord, consistent with drug administration (140).

Interestingly, there is no increase in SMN protein levels in whole spinal cord and no change in full-length *SMN2* in the liver and skeletal muscle suggesting that the effects of nusinersen are limited to the site of injection and the surrounding area (140).

Gene Therapy

AVXS-101 is a self-complementary adeno-associated virus 9 (scAAV9) used to deliver SMN complementary DNA through an intravenous injection (52; 109).

Intravenous injection of scAAV9-SMN increased SMN protein in the brain, spinal cord, and skeletal muscle of SMA mice (52). Restoration of SMN increased weight of SMA mice compared to scAAV9-GFP treated SMA mice, but remained smaller than control mice (52). scAAV9-SMN improved motor function in the righting reflex test, increased endplate current amplitude, and extended survival (52). Additionally, scAAV9-SMN-treated SMA mice have no signs of denervation and no neurofilament accumulation in motor neurons at P9-10 (52).

In clinical trials, type 1 SMA patients received a single intravenous dose of AVXS-101 (5; 109). Historically, by 20 months of age, only 8% of patients do not require permanent ventilation (109). In an open-label study, no patients (0/15) required permanent ventilation and all reached at least 20 months of age (109). Out of the 12 patients that received the proposed therapeutic dose, 11 gained full head control, 9 could sit unassisted for at least 30 seconds, 9 could roll over, and 2 were able to crawl, stand, and walk independently (5; 109).

Why increasing SMN levels using nusinersen or gene therapy prolongs lifespan in many SMA patients, but only improves motor function in a small subset is not well

understood. How to enhance these treatments to improve motor function in patients remains a major focus of SMA research.

Ongoing Clinical Trials and Research

Splicing Modifiers

Two splicing modifiers are currently being tested in clinical trials. Risdiplam, an orally administered small molecule, increases *SMN2* exon 7 inclusion in SMA patient fibroblasts and in motor neurons differentiated from SMA patient iPSCs (142). In SMA mice, risdiplam increases SMN protein levels in the brain and skeletal muscle, extends survival, prevents denervation and loss of motor neurons, and increases muscle size (137; 142). In ongoing clinical trials (NCT02908685, NCT02913482, NCT03779334), SMA patients receive a daily liquid oral dose of risdiplam. The FDA is expected to make a decision on the approval of risdiplam in May 2020 (2).

Branaplam is a small molecule that promotes *SMN2* exon 7 inclusion by stabilizing the interaction between the spliceosome and *SMN2* pre-mRNA (31). Branaplam increases SMN protein and extends survival of SMA mice (31). In a current phase 1/2 clinical trial (NCT02268552), SMA patients receive a weekly oral dose of the splicing modifier.

Increasing *SMN2* Transcription

SMN-AS1 is a long non-coding RNA that blocks PRC2, a repressor complex, from interacting with the *SMN2* promoter (41). Blocking SMN-AS1 increased *SMN2* gene transcription and SMN protein levels in cell culture and improved motor function and extended lifespan in SMA mice (41). Blocking SMN-AS1 in combination with a splicing modifier further improved the SMA disease phenotype (41). This suggests that

combining drugs with different mechanisms of action (i.e. increasing transcription and modifying splicing) can act synergistically to improve the SMA phenotype.

Blocking SMN Protein Degradation

SMN protein is degraded by the ubiquitin-proteasome pathway (30). Targeting the ubiquitin-proteasome system using the proteasome inhibitor, Bortezomib, can increase SMN protein levels and improve motor function of SMA mice (30). Specifically blocking mindbomb-1, the E3 ligase that ubiquitinates SMN, using the small molecule ML372, increased SMN protein levels, improved motor function, and moderately improved survival of SMA mice (4).

Myostatin Inhibition

The myostatin pathway regulates muscle growth with high expression of myostatin inhibiting muscle growth (97). SRK-015 is a monoclonal antibody that binds to latent myostatin to block its activation leading to increased muscle mass and strength in SMA mice when co-administered with a splicing modifier (97). An ongoing clinical trial (NCT03921528) is examining the safety and efficacy of SRK-015 administered by intravenous injection once every 4 weeks in SMA type 2 and type 3 patients.

Recently, SMA mice were treated with an AAV-myostatin propeptide (AAV-MPRO), which inhibits myostatin by preventing myostatin from binding its receptor (182). When administered in combination with a splicing modifier, AAV-MPRO increases myofiber diameter and improves motor function (182).

Fast skeletal muscle troponin activator

CK-2127107 is a fast skeletal muscle troponin activator that binds to troponin and slows the off rate of calcium ions from troponin which sensitizes the complex to calcium ions (8). This lowers the firing threshold to increase muscle contractility (8). SMA type 2, 3, and 4 patients receive an oral dose of CK-2127107 in an ongoing phase 2 clinical trial (NCT02644668). This trial aims to examine the effects of CK-2127107 on skeletal muscle function and fatigue.

RATIONALE FOR STUDIES

Combinatorial approaches to increase SMN protein levels improve disease outcomes in SMA animal models (41; 87). For example, co-treatment of severe SMA model mice with TSA, which increases *SMN2* transcription, and bortezomib, which inhibits SMN degradation, synergistically increase SMN protein levels (87). Together, TSA and bortezomib increase myofiber number, increase body weight, and extend survival compared to either treatment administered alone (87). Another study co-treated SMA mice with an ASO targeting SMN-AS1, a long non-coding RNA that suppresses *SMN2* expression, and an ASO that corrects *SMN2* splicing (41). This combination treatment increases SMN protein levels, improves survival, increases body weight, and improves motor function in the rearing test (41).

More recent approaches to treat SMA combine an SMN-increasing therapy with an SMN-independent treatment (167; 182). In one study, SMA mice were treated with an ASO that modifies *SMN2* splicing and an ASO that reduces expression of neurocalcin delta, a protein that when reduced is a protective modifier of SMA (143; 167). Simultaneous correction of *SMN2* splicing and neurocalcin delta reduction in SMA mice increased CMAP amplitude, rescued NMJ maturity, increased muscle fiber diameter, and

increased grip strength (167). Another study examined the effects of treating SMA mice with a splicing modifier in combination with a myostatin inhibitor (182). This combination treatment increased body weight, myofiber diameter, and grip strength in the hanging wire grip test, but did not extend survival compared to SMA mice treated with the splicing modifier alone (182).

These studies suggest that combinatorial mechanisms that increase SMN protein levels may provide therapeutic benefit and that SMN-independent treatments may further ameliorate the SMA disease phenotype. SMN protein is required early in development which may explain why current treatments are very effective when administered to pre-symptomatic SMA patients (44; 140). However, treating patients pre-symptomatically is not always feasible. Therefore, some SMN-irreversible changes (i.e. those that are not fixed by increasing SMN) may need to be addressed through SMN-independent treatments (68). Given this, there is a need to identify novel SMN-increasing mechanisms and SMN-independent mechanisms to treat SMA.

One potential peripheral tissue that can be targeted in SMA patients is skeletal muscle. There is evidence that current therapies do not increase SMN protein levels in skeletal muscle or increase muscle mass (52; 140). Additionally, given the direct function of skeletal muscle in motor function, targeting skeletal muscle may allow patients to achieve new motor milestones. Lastly, muscle weakness occurs prior to motor neuron loss and denervation suggesting that targeting skeletal muscle is equally important as targeting motor neurons (20).

HYPOTHESIS AND SPECIFIC AIMS

Current treatments extend survival for many SMA patients, but only a small subset see major improvements in their motor function. The safety and long term effects of nusinersen and AVXS-101 are still not known. Additionally, some patients have spondylodesis (fusing of the spine) and advanced scoliosis which can make it difficult to administer nusinersen through intrathecal injection (159). Our long term goal is to identify novel mechanisms to treat SMA patients. We hypothesize that the SMA phenotype can be ameliorated through genetically modifying SMN protein levels and by targeting SMN-independent pathways in peripheral tissues. We will test our hypothesis with the following specific aims:

Aim 1: To identify genetic modifiers of SMN protein.

Aim 2: To determine how SMN deficiency impairs myoblast fusion.

CHAPTER 2: A High-Throughput Genome-wide RNAi Screen Identifies Novel Modifiers of Survival Motor Neuron

This chapter is a modified version of a manuscript that has been submitted for publication in a peer-reviewed journal.

ABSTRACT

Spinal muscular atrophy (SMA) is the one of the leading genetic causes of infant death. SMA results from mutations of the survival motor neuron 1 gene (*SMN1*), leading to deficiency of the survival motor neuron (SMN) protein. While current therapies increase SMN protein levels and improve patient survival and motor function, none are cures, making it necessary to identify complementary strategies to further improve the disease phenotype. In this study, we sought to identify genes or pathways that regulate SMN expression. We performed a genome-wide RNAi screen using a luciferase-based activity reporter to identify genes involved in regulating SMN gene expression, RNA processing, and protein stability. Functional clustering analysis revealed several candidate genes linked to protein quality control and RNA processing that when knocked down dramatically altered SMN levels. Here, we report the identification of pathways that regulate SMN RNA processing and protein stability. First, we show that SMN levels are regulated by components of the TRanscription EXport (TREX) complex through the regulation of nuclear/cytoplasmic RNA transport, independent of splicing. Also, we show that the E3 ligase, Neurl2, works cooperatively with Mib1 to ubiquitinate and promote SMN degradation. Together, our screen uncovered novel pathways through which SMN expression is regulated, potentially revealing novel therapeutic strategies to treat SMA.

INTRODUCTION

Spinal muscular atrophy (SMA) is a neuromuscular disease that is one of the leading genetic causes of infant mortality (92). SMA is characterized by the loss of lower motor neurons in the anterior horn of the spinal cord and muscle atrophy leading to muscle weakness (92). SMA is caused by the homozygous loss of *SMN1*, the predominant producer of SMN protein (92; 98). Humans have a second copy of this gene, *SMN2*, which has a cytosine-to-thymine base pair change leading exclusion of exon 7 in the transcript to primarily produce a truncated form of SMN known as SMN Δ 7 (98; 116). *SMN2* is unable to fully compensate for the loss of *SMN1* in SMA patients leading to a deficiency of SMN protein. SMA patients have variable copy numbers of *SMN2* which correlates with disease severity (101; 174). Although the function of SMN is not completely known, SMN has been shown to be required for the assembly of small nuclear ribonucleoproteins (snRNPs) (135). It remains unclear how loss of SMN contributes to SMA pathology.

Restoration of SMN protein has been shown to ameliorate the disease phenotype. Strategies to increase SMN in SMA animal models and patients include increasing *SMN2* transcription, promoting exon 7 inclusion, blocking SMN protein degradation, and gene delivery (4; 11; 74; 131). Currently, there are two recently approved treatments for SMA patients. Nusinersen, an antisense oligonucleotide, increases SMN protein levels by promoting the inclusion of exon 7 in the *SMN2* transcript whereas gene therapy uses an adeno-associated virus (AAV)-9 to deliver *SMN1* complementary DNA (cDNA) to patients (52; 74; 109). While some patients have significant improvements in their ability to sit, walk, and run, other patients see little to no improvement in their motor function (9; 51; 109; 111). For example in one study, only 32% of SMA type 1 patients were able

to sit unassisted for 30 seconds following 14 months of nusinersen treatment (9).

Therefore, ongoing clinical trials and research focus on identifying alternative ways to further increase SMN protein levels or complementary strategies to improve disease outcomes by targeting non-SMN modifying genes and pathways.

In addition to *SMN2*, only two disease modifiers have been identified: plastin-3 and neurocalcin delta (127; 143). Plastin-3 is upregulated in unaffected *SMN1*-deleted females (127). Expression of neurocalcin delta is reduced in asymptomatic, *SMN1*-deleted individuals with 4 copies of *SMN2* (143). Upregulation of plastin-3, an actin-binding protein, lessens disease severity potentially by stabilizing the growth cone, while upregulation of neurocalcin delta improves the disease phenotype by restoring endocytosis in SMA mice (78; 127; 143). To date, very few regulators of SMN transcript have been reported. HDAC inhibitors have been used in cell and animal models of the disease to increase SMN transcript, but have been shown to have limited efficacy in clinical trials (11; 162). Moreover, HDAC inhibitors have been reported to activate expression of ~2% of the genome which reduces enthusiasm to further develop these drugs for SMA treatment (82; 132; 169).

In this study, we sought to identify, using an unbiased approach, modifiers of SMN protein levels utilizing a genome-wide cell-based RNAi screen against >20,000 individual genes. Human embryonic kidney (HEK) 293 cells stably expressing an *SMN2*-luciferase minigene cassette were used as the cellular model system for the screen, and subsequent validation analyses were carried out using SMA patient-derived fibroblasts. We have identified and validated novel regulators of SMN expression which were grouped into transcriptional regulation, RNA processing, and protein stability.

MATERIALS AND METHODS

siRNA screening

SMN2-HEK-luciferase reporter cells were reverse transfected with siRNAs in 384-well optical plates (2 µl per well, 0.8 pmol). Each well contained, 20 µl of DMEM medium (Life Technologies) and 4 µl/ml RNAiMAX (Life Technologies). After 30 min incubation, 20 µl of a 37,500 cells per ml suspension was added to each well for reverse transfection. Plates with cells were incubated at 37 °C/5% CO₂ for a further 72 h. Media was DMEM supplemented with luciferin (1 µM) (Promega) and B-27 supplements (Invitrogen), and the plates were sealed with an optically clear film (USA Scientific). Plates were incubated at 37°C incubator and bioluminescence recorded with a PE ViewLux (Perkin Elmer). Secondary screens were similarly performed with a unique set of siRNAs performed. All screens were performed in temperature-controlled enclosures with automated liquid and plate handling.

Cell culture

SMA patient 3813 fibroblasts (Coriell) were maintained in DMEM (Thermo Fisher) supplemented with 10% fetal bovine serum (Millipore Sigma) and 1% Pencicillin-Streptomycin Glutamine (Thermo Fisher). Cells were incubated at 37°C with 5% CO₂.

String analysis

Gene Ontology (GO) enrichment analysis of the top genes that increased or decreased SMN expression was performed with the “cellular components enrichment” tool of the STRING database (<http://string-db.org/>). Statistical significance for

enrichment by genome-wide false discovery rate (FDR) was calculated using the STRING database (164).

RNAi screen validation

250,000 fibroblasts were plated and transfected with 30 pmol siRNA (Thermo Fisher) using Lipofectamine RNAiMAX (Thermo Fisher) in Opti-MEM. Cells were collected 48 hours after transfection for further analysis. SiRNAs were all purchased from Thermo Fisher.

Protein expression analysis

Cells were lysed in 1% NP-40, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and protease inhibitor cocktail (Roche) on ice for 10 minutes. Protein concentrations were determined using a DC assay (Bio-Rad) according to the manufacturer's protocol. Protein lysates were resolved by 4-12% SDS-PAGE and transferred to PVDF membranes (Bio-Rad). Membranes were blocked in 5% milk in TBST for 1 hour at room temperature. Membranes were incubated with primary antibody (mouse anti-SMN, 1:1000, BD Biosciences) followed by incubation in secondary antibody (goat anti-mouse IgG HRP conjugate, Enzo Life Sciences). Mouse anti- β -actin peroxidase (Millipore Sigma) was used as a loading control. Western blots were quantified using ImageJ.

RNA isolation and gene expression analysis

Total RNA was extracted using the QIAzol-chloroform method as previously described. RNA was purified using the RNeasy kit (Qiagen) using the manufacturer's instructions. cDNA was synthesized from one microgram of RNA using the High Capacity cDNA Reverse Transcriptase Kit (Thermo Fisher). qRT-PCR was performed

using Taqman reagents (Bio-Rad) and TaqMan primers were all purchased from Thermo Fisher). The SurePrep Nuclear or Cytoplasmic RNA Purification Kit (Thermo Fisher) was used for mRNA fractionation.

SMN pre-mRNA

SMN pre-mRNA levels were determined as previously described (41). Briefly, SMN pre-mRNA was amplified using reverse transcriptase (Thermo Fisher) and a strand specific primer (5'-CGA CTG GAG CAC GAG GAC ACT GAG GAA GAA GGG TGC TGA GA-3'). qRT-PCR was used to determine relative SMN pre-mRNA levels using Taqman reagents (Bio-Rad) and a custom Taqman primer (F: 5'-CAG GAG GAT TCC GTG CTG TT-3'; R: 5'- CGA CTG GAG CAC GAG GAC ACT GA-3'; Probe: 5'-AAT AGG GAG ACT GCA CTG-3'; Thermo Fisher).

SMN2 mRNA splicing

SMN2 transcripts were amplified from cDNA using PCR SuperMix (Thermo Fisher). The PCR primers were F: 5'-CCC ACC ACC TCC CAT ATG TCC-3' and R: 5'-AAC TGC CTC ACC ACC GTG CTG-3'. PCR products were run on a 2% agarose gel at 150 V for 30 minutes. The full-length SMN (421 bp) and the SMN Δ 7 (367 bp) products were quantified using ImageJ. The ratio of full-length SMN to SMN Δ 7 was calculated.

mRNA stability

Actinomycin D (5 μ g/mL, Millipore Sigma) was added to cells 48 hours after transfection with siRNA. Cells were collected 0, 1, 3, 5, and 8 hours after the addition of

actinomycin D. RNA was extracted and purified from cells and then transcribed into cDNA. Relative *SMN* mRNA levels over time was determined using qRT-PCR.

Immunoprecipitation

Fibroblasts were transfected with either GFP-Neurl2 or Neurl2-GFP constructs. *SMN* was immunoprecipitated using an anti-*SMN* antibody and samples were resolved by 4-12% SDS-PAGE. Western blots were probed with an anti-GFP antibody.

***In vitro* ubiquitination**

In vitro ubiquitination reactions were performed as previously described (86). Briefly, recombinant *SMN* protein (Enzo Life Sciences) was incubated with ubiquitin, an E1 ubiquitin-activating enzyme (Boston Biochem), an E2 ubiquitin-conjugating enzyme, and recombinant Mib1 or Neurl2 (OriGene Technologies) in reaction buffer (50 mM Tris-HCl, pH 7.5, 2 mM ATP, 4 mM MgCl₂, 2 mM dithiothreitol (DTT)) for 1 hour at 37°C. Reactions were quenching with 5X Laemmli sample buffer, boiled for 5 minutes, and separated via SDS-PAGE. Western blots were probed with an antibody against polyubiquitin (FKI; Enzo Life Sciences).

Pulse-chase protein labeling

Pulse-chase analysis was performed as previously described (25). Briefly, SMA patient fibroblasts were incubated in cysteine-methionine-free medium (Sigma) for 2 h followed by incubation in methionine-free medium containing 100 µCi of ³⁵S-labeled cysteine-methionine (GE Healthcare) for 1 h. After labeling, cells were washed with culture medium containing an excess of unlabeled cysteine and methionine and then further incubated with the same medium. Cells for collected at the indicated time points.

SMN was immunoprecipitated using an anti-SMN antibody (BD Transduction Laboratories). Samples were separated on SDS-10% PAGE which were then dried and exposed to a phosphorimager screen. The signal was visualized using a Storm phosphorimager system (Molecular Dynamics). Densitometry analysis of SMN protein bands was carried out using ImageQuant PhosphorImager software (Molecular Dynamics).

Statistics

All experiments were repeated at least three times, and quantitative data are presented as the mean $\pm$ SEM. All statistical analyses were performed using GraphPad Prism v7.05 (GraphPad Software, Inc.). Statistical significance was determined by Student's *t* test or one-way ANOVA. Significant differences were identified by post hoc analysis using the Tukey–Kramer post hoc method for multiple comparisons. Assessments were considered significant with $p < 0.05$ and nonsignificant with $p > 0.05$.

RESULTS

Genome-wide RNAi screen identifies genes that regulate SMN expression

To identify novel modifiers of SMN expression, we conducted a genome-wide RNAi screen using HEK-293 cells that stably express a *SMN2*-luciferase minigene (179). The luciferase reporter gene assay combines the promoter and splicing based cassettes in tandem with the major portion of the native *SMN2* cDNA with luciferase (**Figure 1A**). Luciferase is expressed when full-length SMN protein is generated with luminescence signal proportional to SMN protein levels. The screen was conducted in three stages (**Figure 1A**). To determine baseline signals, standard deviation values, and optimal incubation times post-transfection, we conducted a pilot screen, targeting 100 genes that

are predicted to be druggable with three independent siRNAs per gene. The pilot screen was completed successfully with robust *SMN2*-luciferase expression that could be detected following siRNA transfection. Many genes when knocked down altered luciferase expression, including genes that were previously shown to modulate SMN protein expression (shown in red) (**Table 3**).

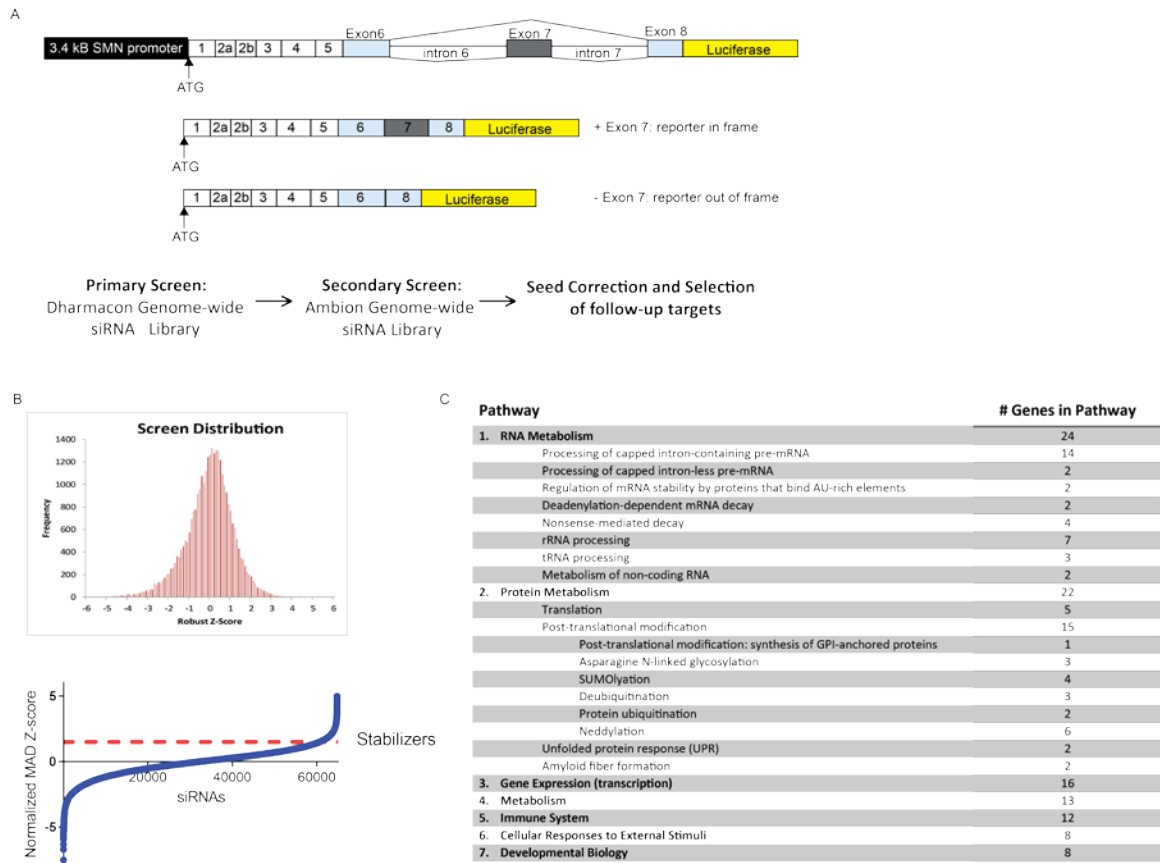


Figure 1. Genome-wide RNAi screen identifies genetic modifiers of SMN expression. (A) Schematic of *SMN2* luciferase reporter cassette stably expressed in 293 cells used in an RNAi screen to identify modifiers of SMN. Two independent screens using Dharmacon and Ambion genome-wide siRNA libraries were performed followed by seed correction to eliminate off-target effects. (B) Distribution of z-scores and distribution of z-scores normalized to median absolute deviation (MAD). (C) Reactome pathway analysis was used to categorize top hits by predicted molecular function.

We next performed primary and secondary genome-wide RNAi screens. The primary screen was performed using the siGenome from the Dharmacon genome-wide library, comprising a single pool of three siRNAs for 18,120 genes. Independently, we performed a secondary screen using the Ambion Silencer Select Version 4, a non-pooled library of three independent siRNAs for 21,566 genes. Luciferase signals were normalized to non-targeted control. Together, we completed three independent screens with at least three siRNA targeting almost all genes in the genome.

Table 3. Table of genes in the druggable genome and their median absolute deviation (MAD) z-score for each of the three siRNAs tested and the mean MAD z-score for each gene. Positive MAD z-scores indicate an increase in SMN2-luciferase expression while negative MAD z-scores indicate a decrease in SMN2-luciferase expression.

Symbol	RNAi 1	RNAi 2	RNAi 3	Mean	Gene Name
CPSF1	2.33	5.42	-3.64	3.29	cleavage and polyadenylation specific factor 1, 160kDa
WDR33	3.60	2.15	2.82	3.27	WD repeat domain 33
SFRS3	1.01	3.33	1.61	2.74	serine/arginine-rich splicing factor 3
UBA7	2.46	-0.64	1.36	2.41	ubiquitin-like modifier activating enzyme 7
CAD	2.03	3.28	2.62	2.37	carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase
TRYX3	3.47	-0.55	1.74	2.30	Trypsin X3
PUF60	-0.69	1.95	2.40	2.26	poly-U binding splicing factor 60KDa
SAP18	2.05	-0.09	1.66	2.14	Sin3A-associated protein, 18kDa
U2AF2	0.18	0.72	1.99	2.14	U2 small nuclear RNA auxiliary factor 2
U2AF1	4.18	1.43	2.75	2.08	U2 small nuclear RNA auxiliary factor 1
EGLN1	0.99	0.88	0.50	2.05	egl nine homolog 1 (C. elegans)

TRO	2.44	0.40	2.38	2.04	trophinin
XPA	2.00	2.11	-0.49	2.03	xeroderma pigmentosum, complementation group A
ISX	0.24	0.65	1.45	1.99	intestine-specific homeobox
YWHAG	3.12	-1.80	1.52	1.98	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide
YY1	2.28	1.03	2.92	1.94	YY1 transcription factor
EP400	1.00	2.48	1.61	1.83	E1A binding protein p400
GTF2E1	0.68	0.05	-1.00	1.76	general transcription factor IIE, polypeptide 1, alpha 56kDa
TTF1	3.03	-1.19	1.42	1.76	transcription termination factor, RNA polymerase I
ADAM32	2.08	1.07	-0.87	1.75	ADAM metallopeptidase domain 32
PTPRZ1	0.63	1.37	-0.90	1.73	protein tyrosine phosphatase, receptor-type, Z polypeptide 1
MAFK	2.52	-0.42	1.49	1.73	v-maf musculoaponeurotic fibrosarcoma oncogene homolog K (avian)
KIR2DL5B	-1.88	1.58	2.50	1.72	killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 5B
PTK2B	-1.44	2.33	1.82	1.71	PTK2B protein tyrosine kinase 2 beta
CRIP3	2.15	0.62	2.29	1.70	cysteine-rich protein 3
EIF5B	1.36	1.32	1.14	1.68	eukaryotic translation initiation factor 5B
MAP4	-0.65	2.23	1.18	1.67	microtubule-associated protein 4
APBA2	0.25	0.38	-1.33	1.66	amyloid beta (A4) precursor protein-binding, family A, member 2
BARX1	-0.75	-0.08	-0.45	1.63	BARX homeobox 1
UBE2D3	2.75	1.56	1.31	1.62	ubiquitin-conjugating enzyme E2D 3
NACC1	-1.35	0.01	0.45	1.59	nucleus accumbens associated 1, BEN and BTB (POZ) domain containing
RAB1B	-0.45	0.89	0.47	1.55	RAB1B, member RAS oncogene family
RANBP2	-0.39	3.23	1.77	1.53	RAN binding protein 2

TCFL5	0.75	1.41	-0.39	1.52	transcription factor-like 5 (basic helix-loop-helix)
LONP2	1.97	0.36	2.82	1.52	lon peptidase 2, peroxisomal
VIP	1.64	-0.79	1.16	1.52	vasoactive intestinal peptide
SIAH1	-0.48	0.80	1.11	1.50	seven in absentia homolog 1 (Drosophila)
TSG101	2.65	0.76	1.74	1.48	tumor susceptibility gene 101
HCFC1	1.61	1.33	-0.08	1.47	host cell factor C1 (VP16-accessory protein)
PSMD4	0.77	0.58	1.46	1.46	proteasome (prosome, macropain) 26S subunit, non-ATPase, 4
POU4F2	0.14	-0.22	0.76	1.46	POU class 4 homeobox 2
MEP1B	0.92	2.12	0.56	1.46	meprin A, beta
ADAMTS20	0.04	0.50	-1.36	1.44	ADAM metallopeptidase with thrombospondin type 1 motif, 20
SLC6A4	0.52	2.25	2.24	1.43	solute carrier family 6 (neurotransmitter transporter, serotonin), member 4
GABPA	1.79	1.33	1.63	1.43	GA binding protein transcription factor, alpha subunit 60kDa
PPP1R10	1.94	1.28	2.31	1.41	protein phosphatase 1, regulatory subunit 10
RPS6KB1	1.09	1.38	-0.58	1.40	ribosomal protein S6 kinase, 70kDa, polypeptide 1
NEURL2	1.42	-0.78	2.06	1.39	neuralized homolog 2 (Drosophila)
YWHAE	1.88	1.84	2.84	1.38	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide
MRS2	0.13	2.65	2.26	1.38	MRS2 magnesium homeostasis factor homolog (S. cerevisiae)
ZNF213	-0.01	-1.40	4.23	1.37	zinc finger protein 213
ANGPTL4	-1.06	1.19	2.34	1.37	angiopoietin-like 4
ATP2B2	1.21	1.88	2.05	1.37	ATPase, Ca ⁺⁺ transporting, plasma membrane 2
PGS1	2.98	1.88	-0.34	1.36	phosphatidylglycerophosphate synthase 1
MEOX2	0.85	2.23	0.56	1.36	mesenchyme homeobox 2
DHX15	2.54	0.15	2.27	1.36	DEAH (Asp-Glu-Ala-His) box polypeptide 15

PIGM	-0.13	1.22	0.44	1.36	phosphatidylinositol glycan anchor biosynthesis, class M
CAPRIN1	1.43	-0.21	2.82	1.36	cell cycle associated protein 1
UBA1	3.77	0.48	1.21	1.34	ubiquitin-like modifier activating enzyme 1
HNRNPA2B1	1.34	0.94	1.08	1.33	heterogeneous nuclear ribonucleoprotein A2/B1
NCL	1.89	1.46	-0.98	1.33	nucleolin
LSG1	1.13	2.59	1.07	1.31	large subunit GTPase 1 homolog (S. cerevisiae)
TEAD1	0.41	0.69	0.43	1.31	TEA domain family member 1 (SV40 transcriptional enhancer factor)
KCTD9	1.94	0.88	-0.62	1.31	potassium channel tetramerisation domain containing 9
HMX1	0.66	1.13	-1.42	1.30	H6 family homeobox 1
SLC4A5	1.92	-0.49	2.20	1.30	solute carrier family 4, sodium bicarbonate cotransporter, member 5
TMPRSS6	1.27	-1.16	2.32	1.29	transmembrane protease, serine 6
PNN	1.11	1.33	0.70	1.29	pinin, desmosome associated protein
TCEB2	1.96	1.92	1.06	1.28	transcription elongation factor B (SIII), polypeptide 2 (18kDa, elongin B)
ACTR1A	0.71	1.37	1.67	1.28	ARP1 actin-related protein 1 homolog A, centractin alpha (yeast)
TFAP2C	0.71	0.29	1.68	1.28	transcription factor AP-2 gamma (activating enhancer binding protein 2 gamma)
PHPT1	1.30	-0.11	0.17	1.27	phosphohistidine phosphatase 1
DDI2	3.77	-0.44	-0.58	1.27	DNA-damage inducible 1 homolog 2 (S. cerevisiae)
ANKRA2	0.87	0.81	0.30	1.26	ankyrin repeat, family A (RFXANK-like), 2
ACAT2	-0.83	1.09	1.77	1.25	acetyl-CoA acetyltransferase 2
TMPRSS5	1.63	-0.16	2.63	1.25	transmembrane protease, serine 5
PDE6A	1.00	-2.20	1.32	1.25	phosphodiesterase 6A, cGMP-specific, rod, alpha

UCP3	2.16	-0.30	1.65	1.23	uncoupling protein 3 (mitochondrial, proton carrier)
KCNB2	2.57	-0.21	1.68	1.22	potassium voltage-gated channel, Shab-related subfamily, member 2
GLT8D1	2.21	-1.50	1.74	1.22	glycosyltransferase 8 domain containing 1
HS3ST1	0.30	1.05	0.71	1.21	heparan sulfate (glucosamine) 3-O-sulfotransferase 1
PATE1	0.79	1.27	1.98	1.21	prostate and testis expressed 1
LEO1	0.99	-3.18	0.93	1.20	Leo1, Paf1/RNA polymerase II complex component, homolog (<i>S. cerevisiae</i>)
CNOT1	1.39	0.90	2.45	1.20	CCR4-NOT transcription complex, subunit 1
SUPT16H	1.58	1.46	1.49	1.20	suppressor of Ty 16 homolog (<i>S. cerevisiae</i>)
ZNF281	-0.63	0.40	1.04	1.19	zinc finger protein 281
WHSC1	0.82	0.48	1.22	1.17	Wolf-Hirschhorn syndrome candidate 1
A2BP1	0.80	1.32	0.47	1.17	
ITGAV	-0.27	0.24	1.70	1.17	integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)
ZNF74	-0.28	0.48	0.42	1.17	zinc finger protein 74
SR140	0.44	0.53	1.33	1.16	
DLST	1.03	2.27	1.36	1.16	dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex)
HGS	1.42	0.97	-0.63	1.16	hepatocyte growth factor-regulated tyrosine kinase substrate
RELB	1.00	0.44	0.45	1.16	v-rel reticuloendotheliosis viral oncogene homolog B
SAPS1	0.56	-1.04	0.67	1.16	protein phosphatase 6, regulatory subunit 1
PCDHB8	0.99	-0.25	0.80	1.15	protocadherin beta 8
NOP2	1.29	0.54	2.31	1.15	NOP2 nucleolar protein homolog (yeast)
KRT83	0.95	-0.38	0.99	1.15	keratin 83
PPP4C	0.35	2.17	1.28	1.15	protein phosphatase 4, catalytic subunit
PPP2R5C	2.03	0.53	0.88	1.14	protein phosphatase 2, regulatory subunit B', gamma

Candidate gene selection used a robust statistical measure of median absolute deviation (MAD) to standardize siRNA activities from each screen (34). Common Seed Analysis was used to help prioritize clean hits as opposed to those appearing to arise from seed based off-target effects (23; 102). Outcomes of screened siRNAs with positive z-score reflect enhancement in full-length SMN expression and a negative z-score showing a reduction in SMN levels (**Figure 1B**). To identify strong modifiers, we set a z-score cutoff of greater than ± 1.0 (2-fold change). Z-score analyses indicated a continuous distribution of hits (**Figure 1B**); 112 genes gave Z-scores smaller than -1.0 (reduced expression) and 89 larger than $+1.0$ (increased expression) ranging from -5.5 to $+3.3$ (**Table 4, 5**). Importantly, several previously described regulators of SMN expression were re-identified in the screen (**Figure 2**), thus validating the effectiveness of our assay. We divided hits into groups based on predicted molecular functions to gain better insight into molecular pathways that would lead to enhancement of SMN protein levels. These categories were enriched for genes involved in RNA processing, protein metabolism, and transcription regulation (**Figure 1C**).

Table 4. List of genes that when knocked down increase SMN protein levels in primary and secondary RNAi screens.

Gene Symbol	MAD Z-Score
CPSF1	3.2869
WDR33	3.26524
SFRS3	2.74404
UBA7	2.40669
CAD	2.36826
TRYX3	2.30498
PUF60	2.26025
SAP18	2.14174

U2AF2	2.14055
U2AF1	2.07882
EGLN1	2.04871
TRO	2.04461
XPA	2.03244
ISX	1.99354
YWHAG	1.97794
YY1	1.94377
EP400	1.83392
GTF2E1	1.76068
TTF1	1.7596
ADAM32	1.751
PTPRZ1	1.73326
MAFK	1.7332
KIR2DL5B	1.7219
PTK2B	1.70745
CRIP3	1.70405
EIF5B	1.67578
MAP4	1.66953
APBA2	1.66215
BARX1	1.63319
UBE2D3	1.62228
NACC1	1.59103
RAB1B	1.55105
RANBP2	1.52544
TCFL5	1.52411
LONP2	1.52328
VIP	1.5201
SIAH1	1.50039
TSG101	1.47981
HCFC1	1.46689
PSMD4	1.46195
POU4F2	1.45887
MEP1B	1.45535
ADAMTS20	1.43637
SLC6A4	1.42748
GABPA	1.42562
PPP1R10	1.40706
RPS6KB1	1.40267
NEURL2	1.38708

YWHAE	1.38074
MRS2	1.37727
ZNF213	1.37399
ANGPTL4	1.3698
ATP2B2	1.36706
PGS1	1.36032
MEOX2	1.35924
DHX15	1.35805
PIGM	1.35789
CAPRIN1	1.35746
UBA1	1.3427
HNRNPA2B1	1.33207
NCL	1.32633
LSG1	1.31376
TEAD1	1.31208
KCTD9	1.30825
HMX1	1.30136
SLC4A5	1.29897
TMPRSS6	1.2912
PNN	1.28887
TCEB2	1.28305
ACTR1A	1.28146
TFAP2C	1.27853
PHPT1	1.26988
DDI2	1.2671
ANKRA2	1.2594
ACAT2	1.25448
TMPRSS5	1.25288
PDE6A	1.24748
UCP3	1.22571
KCNB2	1.22336
GLT8D1	1.22172
HS3ST1	1.21298
PATE1	1.21143
LEO1	1.20353
CNOT1	1.19902
SUPT16H	1.19643
ZNF281	1.18831
WHSC1	1.17263
A2BP1	1.17203

ITGAV	1.17102
ZNF74	1.16902
SR140	1.16428
DLST	1.16202

Table 5. List of genes that when knocked down decrease SMN protein levels in primary and secondary RNAi screens.

Gene Symbol	MAD Z-Score
RRM1	-5.512
POLR2J3	-5.4248
CHEK1	-5.1021
POLR2B	-4.8099
NXF1	-4.4643
POLR2G	-4.192
WEE1	-4.0512
SUPT5H	-4.0213
RRM2	-3.844
POLR2A	-3.8139
POLR2D	-3.7949
POLR2K	-3.7221
SF3B1	-3.6489
POLR2F	-3.5197
POLR2I	-3.2739
MED14	-3.2174
SNRPB	-3.1686
ZNF207	-3.1044
SNRPG	-3.089
RAN	-3.0882
THOC1	-3.0407
SNRPD1	-2.9657
POLR2C	-2.9547
SNRPE	-2.7716
POLR2H	-2.7406
SNRPA1	-2.7072
SNRPD3	-2.6912
SNRPF	-2.6601

PLK1	-2.5737
SF3B4	-2.5582
SUPT6H	-2.5368
SF3B3	-2.5296
AP3B2	-2.4487
RCN3	-2.4429
TXNDC14	-2.3915
CPSF6	-2.3817
CREM	-2.3667
MED24	-2.3048
PRPF4B	-2.2859
CTDP1	-2.2856
PSMC1	-2.2328
MED6	-2.1546
HNRNPC	-2.1521
DDX42	-2.1295
MLL2	-2.1092
UNC13A	-2.1046
SF3A3	-2.0982
SNRPA	-2.0867
MED17	-2.0637
MED30	-2.0598
PSKH1	-2.0279
A2M	-1.9595
PSMC2	-1.958
MAT2A	-1.9013
MFSD4	-1.8859
PIK3R3	-1.8769
CKAP5	-1.8705
USPL1	-1.8672
MED26	-1.862
RBM39	-1.8207
CHMP6	-1.8095
MYH4	-1.8029
SCNN1D	-1.7993
IRF6	-1.7945
UBE2E2	-1.7684
FCER2	-1.7452
SNAPC3	-1.717
RETSAT	-1.7041

SF3A2	-1.6956
SPEN	-1.6949
SFRS1	-1.6645
ABCC4	-1.6625
MED20	-1.6597
SUZ12	-1.646
ODC1	-1.6391
SSU72	-1.6388
MED27	-1.6229
DNAJC8	-1.6181
CSNK2B	-1.6158
PSMD1	-1.6108
MED8	-1.6059
KCNJ6	-1.6018
GFRA4	-1.5852
SRMS	-1.5829
DTD1	-1.5822
NMNAT2	-1.5785
ACO2	-1.5654
ITGAM	-1.5629
ABCC10	-1.5564
AQR	-1.5551
PGD	-1.5514
RNGTT	-1.55
VPS26A	-1.5488
POLE	-1.5486
POLM	-1.5474
SMNDC1	-1.5439
NANS	-1.5425
THOC4	-1.5411
KPNA6	-1.5402
PRPF8	-1.538
POU2F3	-1.5288
FUBP1	-1.5221
SETD8	-1.5218
PPAT	-1.5082
UCK2	-1.5073
XPNPEP1	-1.5029
MX1	-1.4871
BAT1	-1.485

LSM2	-1.4849
TBRG1	-1.4695
CDK8	-1.4679

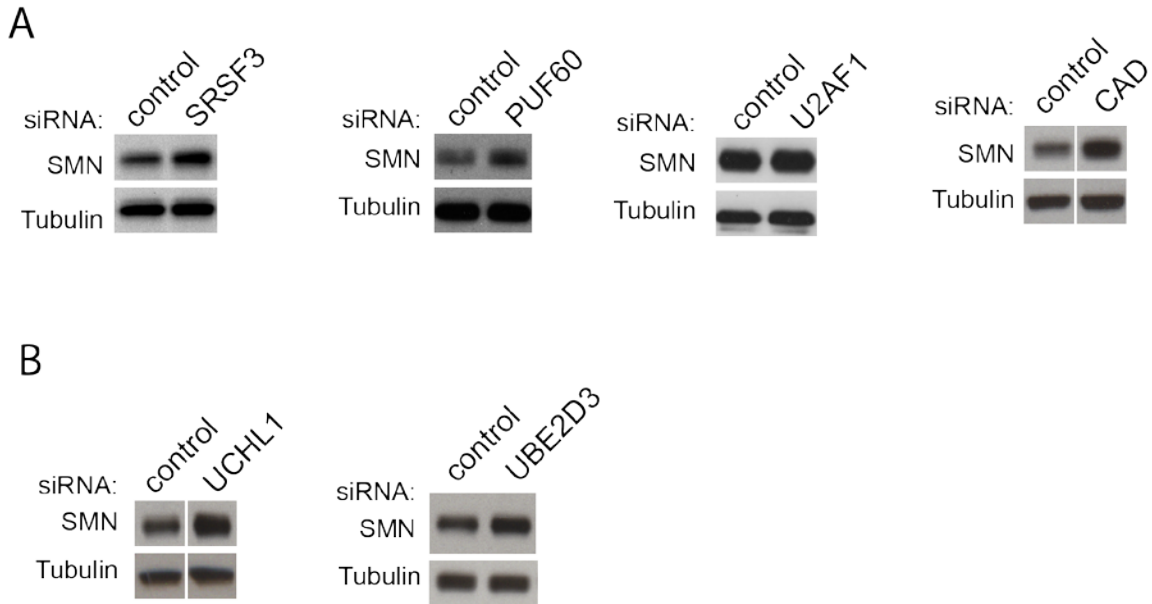


Figure 2. RNAi screen re-identifies modifiers of SMN. (A) SMA patient fibroblasts were transfected with siRNAs targeting SRSF3, PUF60, U2AF1, and CAD for 48 hours. Western blot analysis shows that reduction of each of these genes increases SMN protein levels. (B) SMA patient fibroblasts were transfected with siRNAs targeting UCHL1 and UBE2D3 for 48 hours. Western blot analysis confirms that reduction of each of these genes increases SMN protein levels.

Identification of SMN exon 7 splicing regulators

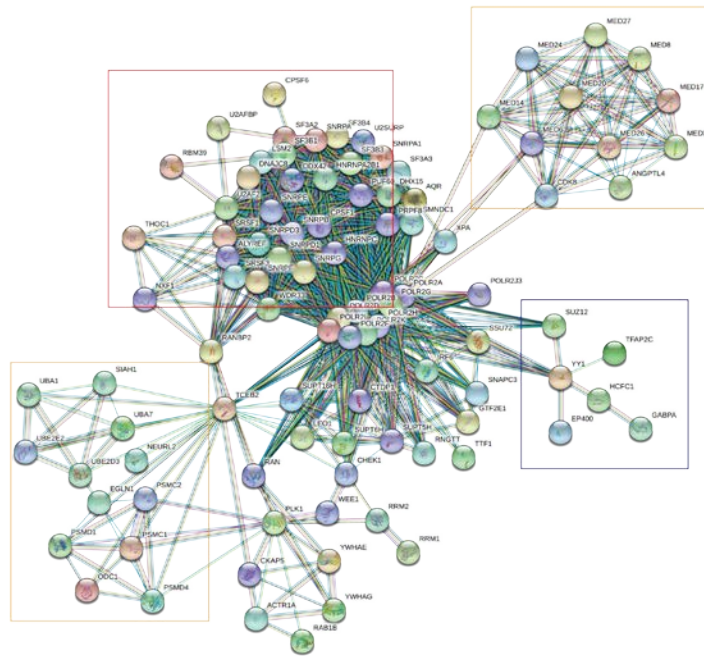
An overwhelming number of genes (75 out of 201 hits) had well characterized roles in mRNA splicing, indicating that splicing alterations might be the most robust mechanism to increase SMN expression. Of the SMN-modifying genes we identified several genes that have previously been shown to enhance SMN exon 7 inclusion. The previously described *SMN2* splicing modifiers hnRNP A1, hnRNP A2, U2AF1, U2AF2, PUF60 and SRSF3 were all top scored hits in the screen (61; 80; 105; 172). All of these genes were previously shown to promote SMN exon 7 exclusion consistent with why knocking them down increased full-length SMN expression. Interestingly, genes

previously described to promote SMN inclusion (ASF/SF2, SRp30c, Tra2- β , TIA1) were not picked up in the screen (27; 70; 156; 176). This may point to the limitations of this assay to detect genes that further suppress SMN expression or, more interestingly, genes that regulate SMN exon 7 inclusion are redundant with overlapping functions.

Gene Cluster and Interactome Analyses

We next sought to determine if specific cellular pathways altered SMN expression by grouping hits into functional clusters using STRING analysis (164). The genes that met our selection criteria (increased or decreased SMN expression) were selected for further analysis (**Figure 3A**). We looked for over-representation of biological functions among the candidate genes by gene set enrichment analysis using the KEGG, REACTOME, GO, and PANTHER databases. Each of these analyses groups genes by biological function or predicted function. Key components of the RNA polymerase II transcription machinery including the mediator complex were highly enriched in the genes that when knocked down reduced SMN expression, consistent with their role as transcriptional activators. Indeed, knockdown of CDK8, Med6, Med8, Med14, Med17, Med20, Med24, Med26, Med27, Med30 or Med31 all resulted in decreased SMN expression (**Figure 3B**). We could distinguish two networks visualized with STRING analysis that are enriched in genes whose reduction increased SMN expression: the TRanscription-EXport (TREX) complex and the ubiquitin proteasome system (**Figure 3B**).

A



B

Mediator Complex

Gene	Symbol	MAD Z-Score
Cyclin Dependent Kinase 8	CDK8	-1.5
Mediator Complex Subunit 20	MED20	-1.7
Mediator Complex Subunit 26	MED26	-1.9
Mediator Complex Subunit 17	MED17	-2.0
Mediator Complex Subunit 30	MED30	-2.1
Mediator Complex Subunit 6	MED6	-2.2
Mediator Complex Subunit 24	MED24	-2.3
Mediator Complex Subunit 14	MED14	-3.2

Splicing and Nucleocytoplasmic Trafficking

Gene	Symbol	MAD Z-Score
WD Repeat Domain 33	WDR33	3.3
Cleavage And Polyadenylation Specific Factor 1	CPSF1	3.3
Serine And Arginine Rich Splicing Factor 3	SFRS3	2.7
U2 Small Nuclear RNA Auxiliary Factor 1	U2AF1	2.1
U2 Small Nuclear RNA Auxiliary Factor 2	U2AF2	2.0
THO Complex 3	THOC3	1.7
Aly/REF Export Factor	THOC4 (ALYREF)	-1.5
Serine And Arginine Rich Splicing Factor 1	SFRS1	-1.7
RNA Binding Motif Protein 39	RBM39	-1.8
THO Complex 1	THOC1	-3.0
Nuclear RNA Export Factor 1	NXF1	-4.5

Ubiquitin-Proteasome Degradation System

Gene	Symbol	MAD Z-Score
Ubiquitin Like Modifier Activating Enzyme 7	UBA7	2.4
Ubiquitin Conjugating Enzyme E2 E6	UBE2E2	1.8
Ubiquitin Conjugating Enzyme E2 D3	UBE2D3	1.6
Proteasome 26S Subunit, Non-ATPase 4	PSMD4	1.5
Siah E3 Ubiquitin Protein Ligase 1	SIAM1	1.5
Neutralized E3 Ubiquitin Protein Ligase 2	NEURL2	1.4
Ubiquitin-Activating Enzyme E1	UBA1	1.3
Transcription Elongation Factor B (SII), Polypeptide 2	TCEB2	1.3
Proteasome 26S Subunit, Non-ATPase 1	PSMD1	-1.6
Proteasome 26S Subunit, ATPase 2	PSMC2	-2.0
Proteasome 26S Subunit, ATPase 1	PSMC1	-2.2

Transcription Factors

Gene	Symbol	MAD Z-Score
Zinc Finger Protein 143	ZNF143	2.4
YY1 Transcription Factor	YY1	1.9
Host Cell Factor C1	HCFC1	1.5
GA Binding Protein Transcription Factor Subunit Alpha	GABPA	1.4
SUZ12 Polycomb Repressive Complex 2 Subunit	SUZ12	-1.6

Figure 3. Interactome analysis of top hits. (A) STRING analysis of top hits showed four gene clusters: mediator complex, splicing and nucleocytoplasmic trafficking, ubiquitin-proteasome degradation system, and transcription factors. (B) List of genes and their MAD Z-score in each cluster.

The TREX complex regulates SMN levels

Little is known about the factors that regulate SMN mRNA trafficking. The TREX complex couples transcription elongation by RNA polymerase II to mRNA export (81). TREX coordinates with the elongating RNA polymerase II and facilitates RNP

assembly and recruitment of RNA processing factors. Depletion of TREX components reduces expression of some, but not all genes (106). We found from our screen that RNAi targeting *THOC1*, *THOC4* (*ALYREF*), *NFX1*, and *CPSF6* reduced SMN expression suggesting they may be indispensable to SMN mRNA nucleocytoplasmic translocation.

Interestingly, we found that knocking down *THOC3*, another member of the TREX complex, resulted in increased SMN expression. This result conflicted with its predicted role in nuclear export of mRNA (81). It has been proposed that the TREX and CPSF complexes play sequential roles in mRNA maturation and trafficking (28; 81). Unlike other TREX complex subunits, *THOC3* contains a WD40 repeat domain like the CPSF complex protein *WDR33*, suggesting that both proteins may have redundant or complementary roles in mRNA processing (28; 63). We selected *CPSF1*, *THOC3*, and *WDR33* to further characterize their roles in SMN RNA processing.

We transfected cells with siRNA targeting *CPSF1*, *WDR33*, and *THOC3* and isolated protein and mRNA 48 hours after transfection. We confirmed that knocking down each gene individually increased SMN protein levels (**Figure 4A, B**). Additionally, simultaneously transfecting SMA patient-derived fibroblasts with all three siRNAs significantly increased SMN protein levels (**Figure 4C, D**). We are unable to directly compare the individual knockdowns from the triple knockdown to determine whether the knockdown of all three genes produces a greater increase in SMN protein compared to knockdown of each gene individually. We next examined if knocking down each gene induced SMN transcription, altered mRNA stability, or nuclear export. To determine if knocking down each gene affected SMN transcription we used qRT-PCR primers spanning SMN intron-exon boundaries to quantify SMN pre-mRNA.

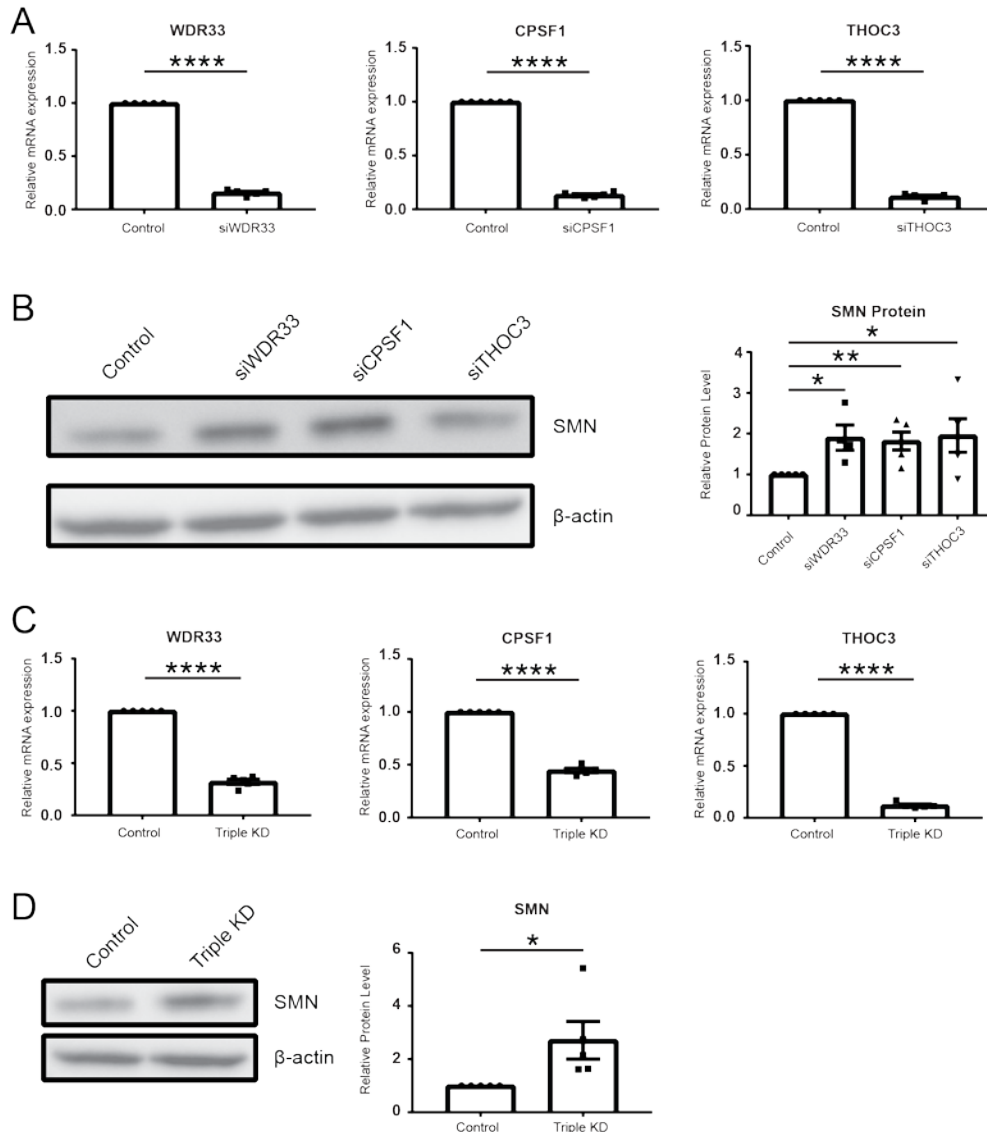


Figure 4. Knockdown of RNA processing machinery components increases SMN protein levels. SMA patient-derived fibroblasts were transfected with siRNA for 48 hours. (A) Gene expression validates knockdown of *WDR33*, *CPSF1*, and *THOC3*. N = 5. (B) Western blot and quantification of SMN protein levels 48 hours after siRNA transfection. SMN protein levels are increased with knockdown of each gene. N = 5. (C) SMA patient-derived fibroblasts were simultaneously transfected with siRNAs targeting *WDR33*, *CPSF1*, and *THOC3* (Triple KD) for 48 hours. Gene expression validates knockdown of *WDR33*, *CPSF1*, and *THOC3*. N = 5. (D) Western blot and quantification of SMN protein levels 48 hours after transfection with all 3 siRNAs. SMN protein levels are increased in fibroblasts with all three genes knocked down. N = 5. All data represented as mean $\pm$ SEM. *P < 0.05, **P < 0.01, ****P < 0.0001.

We found that *SMN2* pre-mRNA levels were not changed by knocking down each gene (**Figure 5A**). In addition, total *SMN2* mRNA was unchanged by knocking down each gene (**Figure 5B**). We next examined *SMN2* mRNA stability after stopping gene transcription with actinomycin D. Knocking down *CPSF1*, *WDR33*, and *THOC3* did not alter *SMN2* mRNA stability or the *SMN2* splicing through exon 7 inclusion (**Figure 5C**, **D**).

Lastly, we looked at the nucleocytoplasmic distribution of *SMN2* mRNA after knocking down each gene. We found increased cytoplasmic localization of the *SMN2* mRNA 48 hours after knocking down *WDR33* and *THOC3*, but not *CPSF1*, offering one explanation for the increased SMN expression (**Figure 5E**). Together, we show that components of the mRNA trafficking complexes are enriched in our screen and confirm in patient cells that knocking down some of these genes increase SMN expression possibly through regulating *SMN2* mRNA nucleocytoplasmic shuttling.

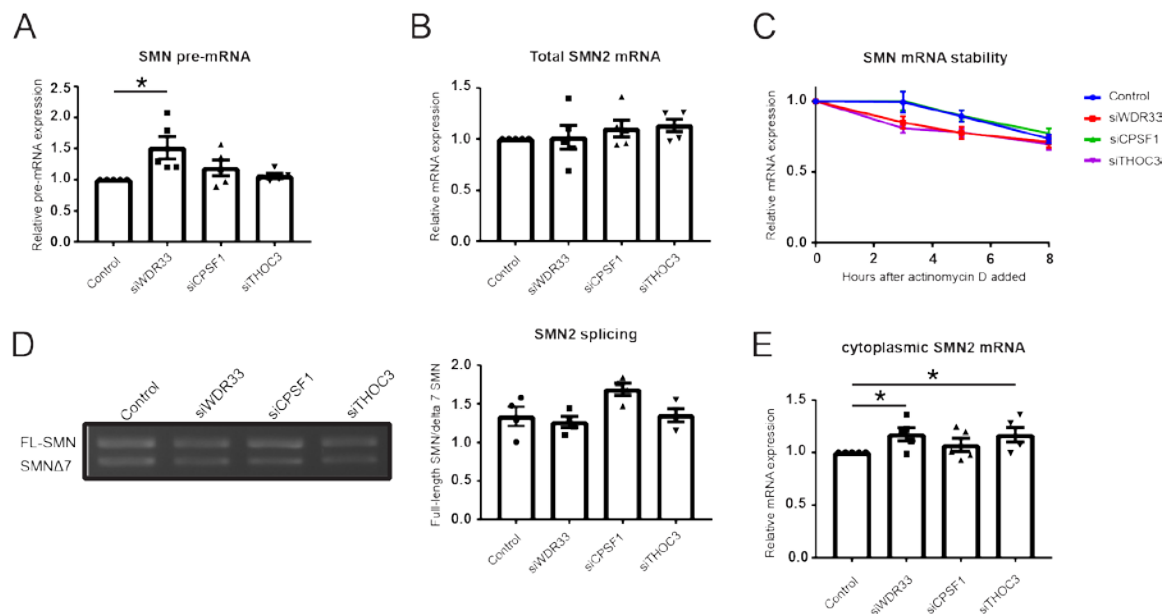


Figure 5. Knockdown of RNA processing machinery components increases cytoplasmic SMN mRNA. SMA patient-derived fibroblasts were transfected with siRNA targeting WDR33, CPSF1 or THOC3 for 48 hours. SMN pre-mRNA (A), total SMN mRNA (B) were examined using qRT-PCR. Total SMN mRNA was unchanged with knockdown of each gene. N = 5. (C) SMA patient-derived fibroblasts were transfected with siRNA for 48 hours and were then treated with actinomycin D to inhibit transcription. SMN mRNA expression was examined for 8 hours after the addition of actinomycin D. SMN2 mRNA stability was unchanged with reduction of each gene. N = 3-4. (D) Splicing of SMN2 transcript and quantification of the ratio of full-length SMN to SMN Δ 7. SMN2 splicing was unchanged with reduction of each gene. N = 5. (E) Cytoplasmic SMN mRNA expression were examined using qRT-PCR. Cytoplasmic SMN mRNA expression was increased with reduction of WDR33 and THOC3, but not CPSF1. N = 5. All data represented as mean $\pm$ SEM. *P < 0.05.

Reducing expression of core components of the ubiquitin proteasome system increases SMN levels

We and others have shown that SMN protein stability is regulated by the ubiquitin proteasome pathway (25; 30; 86). Functional clustering analysis reveal enrichment of genes involved protein clearance that increase SMN expression when knocked down. From the screen, we identified core components of ubiquitin proteasome degradation system that when knocked down increase SMN levels (**Figure 3B**). These genes include *UBA1*, the enzyme that catalyzes the first step in ubiquitin conjugation cascade, which is responsible for tagging proteins for trafficking and degradation (7). Interestingly, mutations in *UBA1* have been previously associated with X-linked infantile spinal muscular atrophy (141). We have shown that the E3 ubiquitin ligase Mindbomb-1 (Mib1) directly ubiquitinates and promotes the degradation of SMN (86). We found siRNAs that target the E2 conjugating enzyme *UBE2D3*, which works in combination with Mib1 to ubiquitinate target proteins, also increase SMN protein levels (**Figure 3B**). We confirm in patient-derived cells that knocking down *UBE2D3* increases SMN levels (**Figure 2B**). The E1 and E2 conjugating enzymes confirm that the ubiquitin proteasome degradation system is a key regulator of SMN protein levels, but because E2 enzymes have multiple

targets they are not viable therapeutic targets for human diseases. The E3 ligase is responsible for the substrate specificity for protein ubiquitination. In addition to Mib1, the screen re-identified the E3 ligase UCHL1 (**Figure 2B**) which has previously been confirmed to regulate SMN protein levels (71). We identified two additional E3 ligases, SIAH1 and Neurl2, as potential regulators of SMN levels. We confirmed in SMA patient-derived fibroblasts that knocking down *Neurl2* increased SMN levels (**Figure 6A**). Together, we show here that the ubiquitin proteasome system is a major regulator of SMN protein levels and provide additional therapeutic targets to help stabilize the SMN protein.

Neurl2 interacts with SMN and regulates its turnover

From our RNAi screen, we identified three additional E3 ligases that when knocked down increased SMN levels in SMA patient-derived fibroblasts. However, only Neurl2 depletion resulted in increased SMN protein levels in every cell type tested (**Figure 6A**). We next assessed if Neurl2 associated with endogenous SMN. SMA patient fibroblasts were transfected with GFP-Neurl2 or Neurl2-GFP, and endogenous SMN was immunoprecipitated followed by western blot analysis with an anti-GFP antibody. We found robust association of Neurl2 and SMN, confirming interaction (**Figure 6B**). Neurl2 has been reported to, in cooperation with Mib1, ubiquitinate and decrease expression of X-Delta (158). Therefore, we next asked if Mib1 and Neurl2 cooperatively modulated SMN levels. Knocking down Mib1 and Neurl2 separately increases SMN levels in patient cells. Knocking down Mib1 and Neurl2 together resulted in a similar increase in SMN levels as knocking down each ligase individually suggesting both enzymes function

in redundant pathways (**Figure 6C**). We next examined the potential mechanism through which *Neur12* affected SMN protein stability. We looked to see if, like *Mib1*, *Neur12*

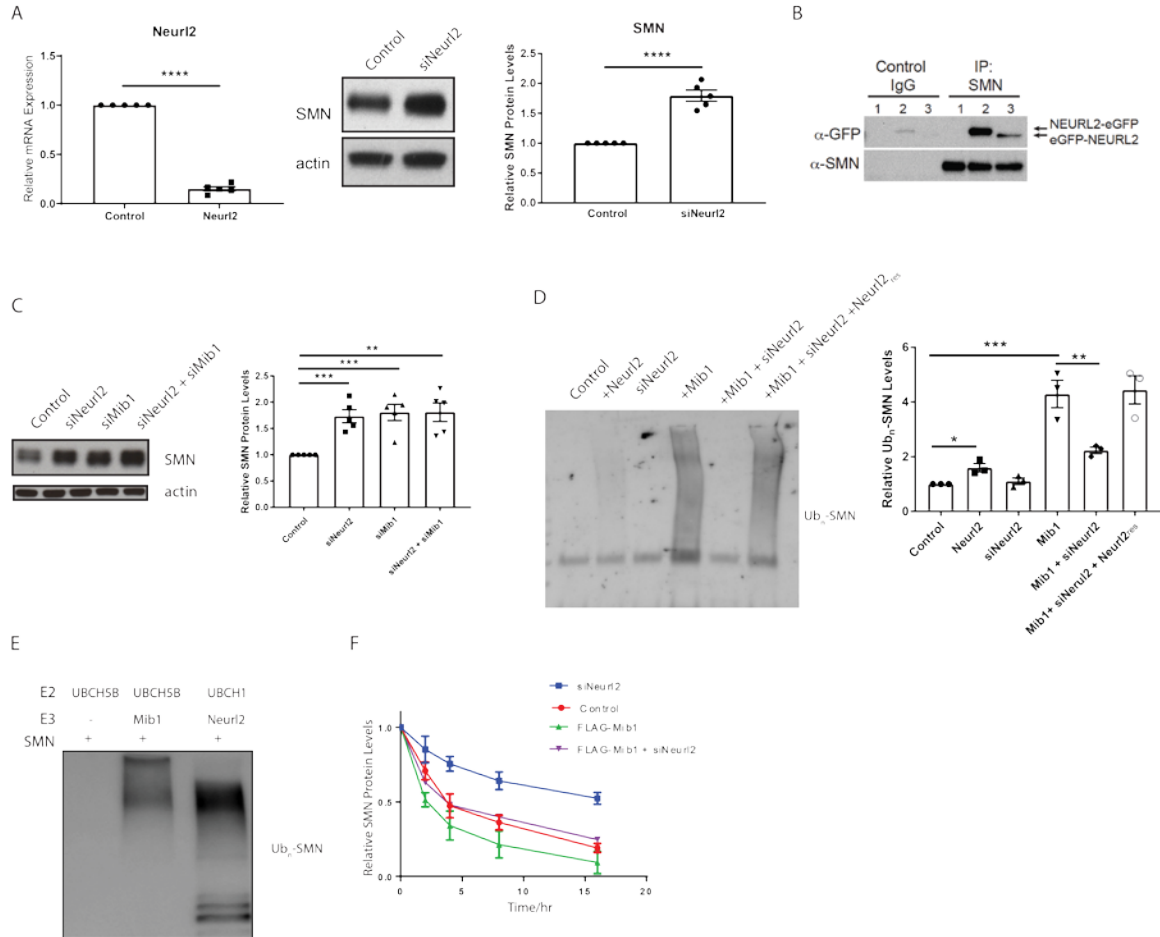


Figure 6. Knockdown of *Neur12* blocks SMN degradation to increase SMN protein levels. (A) SMA patient-derived fibroblasts were transfected with siRNA targeting *Neur12*. Gene expression and western blot analysis shows that *Neur12* knockdown resulted in increased SMN levels. N = 5. (B) SMA patient fibroblasts were transfected with GFP-*Neur12* or *Neur12*-GFP. SMN was immunoprecipitated and *Neur12* was associated confirming their interaction. (C) Patient fibroblasts were transfected with siRNA targeting *Neur12*, *Mib1* or both. Western blot analysis of SMN levels 48 hours following transfection. Knockdown of *Neur12*, *Mib1*, and the combination of both E3 ligases increases SMN protein levels. N = 5. (D) Patient cells were transiently transfected with 1 μ g of HA-ubiquitin and FLAG-*Mib1*, siRNA targeting *Neur12* or siRNA-resistant *Neur12* (*Neur12_{res}*). Ubiquitinated SMN was immunoprecipitated and

ubiquitination was determined by western blot using FK1 antibody. N = 3. (E) Cell-free SMN ubiquitination assay. Recombinant SMN was incubated with E1 and E2 (UBCH5B or UBCH1) enzymes with or without Mib1 or Neurl2 and ubiquitin for 1 hour at 37°C. Western blots were probed with an anti-polyubiquitin antibody (FK1). (F) SMN protein stability following siRNA knock down of Neurl2, overexpressing FLAG-Mib1 or both. Overexpression of FLAG-Mib1 accelerates degradation of SMN protein. Knockdown of Neurl2 stabilizes SMN protein levels. The data represent mean $\pm$ the SEM of four independent experiments. All data represented as mean $\pm$ SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

ubiquitinates SMN. We transfected SMA patient-derived fibroblasts with HA-tagged Neurl2 and collected lysates 48 hours after transfection. We immunoprecipitated SMN from these lysates and quantified SMN ubiquitination by western blot analysis. We found that overexpressing Neurl2 increased SMN ubiquitination (**Figure 6D**). To determine if Neurl2 directly ubiquitinates SMN we performed an *in vitro* ubiquitination assay. Since we did not know which E2 conjugating enzyme would facilitate Neurl2-dependent SMN ubiquitination we performed the assay in the presence of each known E2 conjugating enzyme. We found that Neurl2 directly ubiquitinates SMN only in the presence of the E2 enzyme Ubch1 (**Figure 6E**). Finally, we looked to determine if Neurl2 cooperates with Mib1 to regulate the rate of SMN turnover. We compared the rate of SMN turnover in cells transiently overexpressing Mib1, alone or when Neurl2 is knocked down. Consistent with our previous studies, SMN is more rapidly degraded in cells overexpressing Mib1 (**Figure 6F**). However, the rate of SMN turnover is slowed when Neurl2 levels are reduced suggesting that both genes cooperate to regulate SMN turnover.

DISCUSSION

The recent approval of two therapies to treat SMA has resulted in a dramatic enhancement of lifespan and reduced motor deficits of the most severe type of SMA.

However, SMA patients remain severely debilitated, suggesting additional treatments to improve or complement the current therapies are sorely needed. Based on the new SMA landscape, several SMN-dependent and -independent therapeutic strategies are under clinical consideration including efforts to provide systemic boosting of SMN levels or enhance skeletal muscle size and function. In this study, we opted to utilize a human genome-wide RNAi screen, robust seed correction, and bioinformatics analysis, to help identify new genes that potentially regulate SMN expression. Based on the rigorous nature of our screening studies, we were able to generate a list of genes and pathways that are highly likely to alter SMN gene expression through transcription, RNA processing, and post-translational modifications.

Transcriptional regulation of SMN expression

Not much is known about the regulatory mechanisms at the *SMN1* and *SMN2* promoters even though changes to SMN expression have been reported in several tissues throughout development (46; 117; 148). Putative consensus binding sites for the transcriptional repressor YY1 were previously described in the *SMN2* promoter (46). YY1 was a highly ranked hit from the screen and the highest ranked transcription factor. YY1 is thought to induce transcriptional repression by directing the activity of histone deacetylases at gene promoters. Intriguingly, histone deacetylase inhibitors have been shown to increase SMN expression and were previously under clinical consideration for treating SMA (11; 162). While numerous off-target effects precluded further clinical consideration of these class of drugs, it is possible specific interactions with transcription factors could provide another avenue for therapeutic intervention in this pathway.

STRING analysis showed several transcription factors clustered around YY1 including HCFI, GABP and SUZ12. *HCFC1* gain- and loss-of-function mutations have been described in neurodevelopmental disorders, suggest it plays critical roles in CNS development (55; 85; 177). *HCFC1* associates with DNA-binding transcription factors and chromatin modifiers to either activate or repress transcription. One report estimates that close to 90% of promoters that *HCFC1* binds to also bind YY1 or GABP (112). Association of GABP with *HCFC1* is thought to be essential for *HCFC1* to regulate the transcription of several genes (112). Knocking down YY1, GABP, or *HCF1* individually increase SMN expression.

Nuclear transport of SMN RNA mediates SMN expression

Prior research on regulating SMN gene expression through the *SMN2*-derived mRNA has focused on increasing mRNA synthesis, correcting the splicing to promote exon 7 inclusion, or slow metabolism (4; 11; 74; 162). Increasing evidence suggests that the export of mRNA is a key regulator of gene expression, and selective mRNA export (54; 173). Here, we provide initial evidence that increased export of *SMN2* mRNA into the cytoplasm leads to increased SMN protein levels suggesting a novel mechanism to increase SMN protein and potentially treat SMA.

Regulators of SMN protein turnover

We and others have shown that SMN protein is targeted for degradation by the ubiquitin proteasome system (30; 86). In this study, we show that siRNAs targeting enzymes that catalyze protein ubiquitination or the substrate recognition domains of the proteasome increase SMN levels, consistent with our previous findings. We identified an

additional E3 ligase, Neurl2, which promotes SMN ubiquitination and its subsequent degradation.

CONCLUSION

Many human diseases are caused by toxic accumulation of deleterious proteins or the inappropriate clearance of proteins resulting in functional deficits. The genes and pathways that regulate the delicate balance between gene transcription and protein degradation have been difficult to identify due to the number of molecular steps involved and the inherent redundancy involved in maintaining protein homeostasis. High throughput genome-wide gene suppression assays are ideal to begin to identify critical proteins involved in regulating steady-state levels of a protein. Using this strategy, we have identified many genes that have not previously been implicated in regulating SMN protein levels. We validated several of the hits in SMA-patient derived cells and provided initial molecular characterization of their modes of action.

Chapter 3: SMN Deficiency Slows Myoblast Fusion through Reduced Myomaker and Myomixer Expression

This chapter is a modified version of a manuscript that has been submitted for publication in a peer-reviewed journal.

ABSTRACT

Spinal muscular atrophy is an inherited neurodegenerative disease caused by insufficient levels of the survival motor neuron (SMN) protein. Recently approved treatments aimed at increasing SMN protein levels have dramatically improved patient survival and has altered the disease landscape. While restoring SMN levels slows motor neuron loss, many patients do not achieve normal motor milestones. It remains unclear why SMN restoration is not sufficient to fully restore muscle size and function. We and others have shown that SMN-deficient muscle precursor cells fail to efficiently fuse into myotubes, and restoring SMN only partially rescues myoblast fusion. In this study, we show that SMN-deficient myoblasts readily fuse with wild-type myoblasts, demonstrating fusion competency. Conditioned media from wild-type differentiating myoblasts does not rescue the fusion deficit of SMN-deficient cells, suggesting that compromised fusion may primarily be a result of altered membrane dynamics at the cell surface. Transcriptome profiling of skeletal muscle from SMN-deficient mice reveal marked reduction in expression of the muscle fusion proteins myomaker and myomixer. Suppressing SMN expression reduces expression of myomaker and myomixer in C2C12 myoblast cells and restoring SMN levels only partially restores myomaker and myomixer expression. Restoring myomixer improves muscle size and motor function in SMA model mice and

enhances motor function when administered in combination with an antisense oligonucleotide that increases SMN protein levels.

SIGNIFICANCE STATEMENT

Spinal muscular atrophy (SMA), one of the leading genetic causes of infant mortality, results from reduced expression of the survival motor neuron (SMN) protein. Recently approved therapies slow the disease progression, but many patients still fail to achieve motor milestones. Current therapeutic strategies to further improve motor function include increasing muscle size or improving muscle function. However, little is known about the role of SMN in muscle development and if replacing SMN is sufficient to restore muscle size and function in treated patients. Here, we show that SMN deficiency alters expression of critical membrane fusion proteins. We demonstrate that strategies to enhance the fusion capacity of SMN-deficient myoblasts, in combination with a current therapy, improve muscle pathology and motor function.

INTRODUCTION

Spinal muscular atrophy (SMA) is one of the leading genetic causes of infant mortality affecting 1 in 10,000 live births annually (92; 115). SMA is caused by insufficient levels of survival motor neuron (SMN) protein (92). SMN protein is encoded by two genes—*SMN1*, the major producer of full-length SMN protein, and *SMN2*, which primarily produces truncated SMN protein. A C-to-T base pair change in *SMN2* results in the exclusion of exon 7 in the *SMN2* transcript and the production of a less stable, truncated protein referred to as SMN Δ 7 (98; 116). SMA patients have a homozygous loss or deletion of *SMN1* and retention of at least one copy of *SMN2*. While *SMN2* produces some full-length SMN protein, it is unable to compensate for the loss of *SMN1* (92).

SMA is characterized by the loss of lower motor neurons and muscle atrophy causing patients to have severe muscle weakness (92). SMN is a ubiquitously expressed protein and has pleiotropic cellular roles (38; 134). How loss of SMN contributes to SMA pathology remains unclear (24). Although SMA is traditionally considered a motor neuron disease, there is increasing evidence showing intrinsic muscle deficits in SMA including reduced clustering of nicotinic acetylcholine receptors in differentiated myoblasts from SMA patients, reduced quantal content, altered myogenic programming in SMN-deficient myoblasts, and smaller myofiber size in SMN Δ 7 mice (10; 17-22; 66; 84; 123). Additionally, our lab and others previously found reduced fusion of SMN-deficient myoblasts into myotubes (21; 152). Why SMN deficiency impairs myoblast fusion, and ultimately muscle fiber size, is unknown.

Current treatments for SMA patients are Nusinersen, an antisense oligonucleotide (ASO) that corrects the splicing of the *SMN2* transcript to include exon 7, and gene therapy, an intravenous dose of AAV9-SMN1 (52; 74; 155). While some patients see improvements in their motor function and lifespan, others still fail to achieve normal motor milestones (43; 51; 109; 111). Therefore, there is a need to identify SMN-independent mechanisms, such as those in skeletal muscle, which can be targeted in combination with current therapies to treat SMA (78). Here, we performed transcriptome analysis of skeletal muscle from wild-type and SMA mice and found reduced expression of myomaker, a protein required for myoblast fusion and muscle formation (93; 113). Expression of myomixer, a second protein required for myoblast fusion, was also reduced in SMA mouse skeletal muscle (14; 93; 138; 180). We found that both myomaker and myomixer, as well as MyoD and myogenin expression, are reduced in SMN-deficient

myoblasts. Restoring MyoD or myogenin expression increased myomaker and myomixer and partially rescued fusion of SMN-deficient myoblasts. Importantly, AAV9-mediated delivery of myomixer to SMA mice increased myofiber size and number and improved motor function.

MATERIALS AND METHODS

Cell Culture

C2C12 cells were maintained in DMEM (Thermofisher) with 10% fetal bovine serum (Sigma) and 1% penicillin-streptomycin glutamine (Thermofisher). To initiate differentiation, C2C12 cells were cultured in DMEM supplemented with 2% horse serum (Thermofisher) and 1% penicillin-streptomycin glutamine.

Generation of SMN knockdown myoblast line

SMN shRNA (Genecopoeia, Rockville, MD, USA) was packaged into a lentivirus using a Lentivirus packaging kit (Takara Bio Inc., Japan). C2C12 cells (ATCC, Manassas, VA, USA) were infected with a lentivirus containing SMN shRNA. Puromycin-resistant, GFP-positive clones were selected and expanded. Knockdown of SMN mRNA and protein levels was confirmed by RT-PCR and western blot analyses, respectively.

An antisense oligonucleotide (ASO) (Integrated DNA Technologies, Coralville, IA, USA) was designed to bind and repress the SMN shRNA. Cells were plated and 24 hours later transfected with 1 μ g of SMN shRNA ASO using Lipofectamine 3000 (Thermo Fisher, Waltham, MA, USA). Cells were collected 48 hours after transfection and used for either mRNA or protein expression analysis.

Protein expression analysis

Cell and tissues were lysed in 1% NP-40, 50 mM Tris-HCl (pH 8), 150 mM NaCl and protease inhibitor cocktail (Roche, Indianapolis, IN, USA) on ice for 10 min. The lysates were centrifuged at 4°C for 10 min and the supernatants collected. Protein concentration was determined using the DC assay (Bio-Rad, Laboratories, Hercules, CA, USA) according to the manufacturer's protocol. Protein lysates were resolved by SDS-PAGE (4-12%) and transferred to PVDF membranes. The membranes were blocked in 5% milk and probed with mouse anti-SMN (1:1000; clone 8, BD Biosciences, San Diego, CA, USA), mouse anti- β -actin-peroxidase (Millipore Sigma, Burlington, MA, USA), and goat anti-mouse IgG HRP conjugate (Enzo Life Sciences, Farmingdale, NY, USA).

Myoblast fusion assay

Wild-type and SMN-deficient C2C12 cells were plated separately and differentiated for 2 days. Cells were then loaded with either QTracker 585 (Thermo Fisher) or QTracker 655 (Thermo Fisher) according to the manufacturer's instructions. Briefly, 10^6 cells were incubated with QTracker at 37°C and mixed every 15 minutes for 1 hour. Cells were washed twice with differentiation media. Cells were mixed and re-plated on coverslips and differentiated for an additional 5 days. Cells on coverslips were fixed with 4% paraformaldehyde and stained with DAPI (Cell Signaling Technology, Danvers, MA, USA). Five images per condition were quantified for each N. Cell mixing images were quantified by dividing the number of double-labeled cells by the total number of cells in the field of view.

Myoblast fusion with conditioned media

Wild-type C2C12 cells were plated on coverslips and then differentiated for 24 hours. Conditioned differentiation media from wild-type myoblasts was added to either wild-type or SMN-deficient C2C12 cells. New conditioned differentiation media was added every 24 hours for a total of 7 days. Cells were fixed with 4% formaldehyde and immunolabeled with an anti-myosin antibody (1:200, Developmental Studies Hybridoma Bank, Iowa City, IA, USA) and DAPI. Five images per condition were quantified for each N. Myoblast fusion was quantified by calculating the fusion index (the number of nuclei in myosin-positive myotubes divided by the total number of nuclei).

Satellite cell isolation

We isolated satellite cells from SMA mice and their wild type littermates as previously described (21). Briefly, hind limb muscles were isolated from P10 mice and put into plain Dulbecco's Modified Eagle Medium (DMEM) in 6-well plate. The muscles were minced incubated at 37°C in 2 ml of 0.2% type II collagenase and displaced for 1 h 30 min into the incubation. Samples were passed through a 40-µm cell strainer into a 50-ml conical tube, and 3 ml of media containing DMEM, 5% l-glutamine, 10% FBS (Life Technologies, Grand Island, NY, USA), 20% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA) and 5% 1× penicillin and streptomycin mixture (Life Technologies). Samples were centrifuged for 5 min at 1500 rpm, supernatant removed, and pellet resuspended and plated onto one well of a 6-well plate. Every 24 h for the next 3 days, the samples were pre-plated on a new 6-well plate. Twenty-four hours after last pre-plate, a new 6-well plate was coated with Matrigel (BD Bioscience, 1:5 dilution in plain DMEM). The samples were then plated onto coated 6-well plate for growth.

Satellite cell differentiation: satellite cells were removed from 6-well plate using 0.05% trypsin for 3 min at 37°C.

Transcriptome profiling by RNA sequencing

Total RNA was quantified via a fluorescence dye-based methodology (RiboGreen) on a Spectramax Gemini XPS plate reader (Molecular Devices, Mountain View, CA) as previously described (119). RNA integrity was assessed using automated capillary electrophoresis on a Fragment Analyser (Advanced Analytical Technologies, Inc, Santa Clara, CA). Total RNA input of 200 ng was used for library preparation using the TruSeq Stranded mRNA Library Preparation Kit (Illumina, San Diego, CA). Sequencing libraries were quantified by PCR using KAPA Library Quantification Kit for NGS (Kapa, Wilmington, MA) and assessed for size distribution on a Fragment Analyser. Sequencing libraries were pooled and sequenced on a NextSeq. 500 Desktop Sequencer (Illumina) using a NextSeq. 500 High Output Kit v2 with paired-end reads at 75 bp length. Raw sequencing data was demuxed using bcl2fastq2 Conversion Software 2.17 before alignment using TopHat Alignment v1.0 and differential expression analysis using Cufflinks Assembly & DE v1.1.0 on BaseSpace Onsite (Illumina). Functional enrichment analysis was performed using the PANTHER Classification System⁷¹. Significantly overrepresented gene ontology biological processes at $p < 0.01$ were adjusted using a Bonferroni correction for multiple testing.

RNA isolation and gene expression analysis

Total RNA was isolated from muscle cells using QIAzol (Qiagen, Hilden, Germany), and the RNeasy kit (Qiagen) was used to purify the RNA according to the manufacturer's instructions (45). One microgram of RNA was converted to cDNA using

the High Capacity cDNA Reverse Transcription kit (Thermo Fisher), following the manufacturer's instructions. Gene expression was determined by qRT-PCR using either TaqMan or SYBR Green reagents (Bio-Rad, Hercules, CA, USA). TaqMan primers (all Thermo Fisher) were MyoD (TaqMan assay Mm00440387_m1), Myogenin (TaqMan assay Mm00446194_m1), Pax7 (TaqMan assay Mm00834079_m1), Pax3 (TaqMan assay Mm00435493_m1), SMN1 (TaqMan assay Mm00488313_m1), myomaker (TaqMan assay Mm00481256_m1), and gusb (TaqMan assay Mm01197698_m1). SYBR primers (all Integrated DNA Technologies) were myomixer (F: 5'-GTT AGA ACT GGT GAC CAG GAG-3' R: 5'-CCA TCG GGA GCA ATG GAA-3') and GAPDH (F: 5'-GCA TGG CCT TCC GTG TTC-3' R: 5'-ATG TCA TCA TAC TTG GCA GGT TTC-3'). Transcript levels were quantified by threshold cycle values using gusb or GAPDH as a control. For cell culture experiments, values were normalized to unaffected cells for each N. For animal experiments, values were normalized to the average ΔC_T of all wild-type animals.

Myomaker and myomixer surface expression analysis

Wild-type and SMN-deficient C2C12 cells were plated on coverslips and differentiated for 7 days using DMEM with 2% horse serum. Cells were fixed using 4% paraformaldehyde. Cells were stained for rabbit anti-myomaker (Abcam, Cambridge, UK) or sheep anti-myomixer (R&D Systems, Minneapolis, MN, USA) and DAPI. Secondary antibodies rabbit 546 (Thermo Fisher) or donkey anti-sheep 557 (R&D Systems) were used. Five images per condition were quantified for each N. Pixel intensity was quantified using ImageJ. Pixel intensity was normalized to wild-type levels for each N.

MyoD and myogenin overexpression

Wild-type and SMN-deficient C2C12 cells were plated and after 24 hours, transfected with 1 µg MyoD (Addgene, Watertown, MA, USA) or myogenin (Origene Technologies Inc., Rockville, MD, USA) using Lipofectamine 3000 (Thermo Fisher). Cells were collected 48 hours after transfection. For differentiation experiments, wild-type and SMN knockdown C2C12 cells were plated. 24 hours after plating, cells were simultaneously differentiated and transfected with 1 µg of either MyoD or myogenin. Cells were transfected again 48 hours after the first transfection to maintain expression of MyoD or myogenin. Cells were fixed after five days of differentiation. Five images per condition were quantified for each N. Images were quantified by calculating the fusion index. Values were normalized to wild-type cells for each N.

mRNA stability

Wild-type and SMN knockdown C2C12 cells were plated on a six-well plate and 24 hours later were differentiated. After 48 hours of differentiation, 5 µg/mL actinomycin D (Sigma Aldrich) was added to cells. Cells were collected 0, 1, 3, 5, and 8 hours after the addition of actinomycin D. RNA was isolated and purified. RNA was reverse transcribed into cDNA. qRT-PCR was used to determine relative mRNA expression over time. ΔC_T values were normalized to the 0 hour time point for wild-type and SMN knockdown cells for each N.

Chromatin immunoprecipitation

Wild-type C2C12 cells were differentiated for 48 hours and then fixed with 1% paraformaldehyde. Chromatin was isolated and purified using the EZ Magna ChIP /G Kit (Millipore) according to the manufacturer's instructions. Anti-SMN antibody (BD

Biosciences) was used to immunoprecipitate chromatin. Anti-polymerase II, clone CTD4H8 antibody and Normal Mouse IgG were used as a positive and negative control, respectively (Millipore). qRT-PCR was used to quantify chromatin. C_T values were normalized to Normal Mouse IgG for each N. Primers used to detect chromatin (Integrated DNA Technologies) were MyoD promoter (F: 5'-ACA CTC CTA TTG GCT TGA GGC-3' R: 5'-GGC GCC CTG GGC TAT TTA TC-3') and myogenin promoter (F: 5'-GAA TCA CAT GTA ATC CAC TGG A-3' R: 5'-ACA CCA ACT GCT GGG TGC CA-3').

MO^{E1v11} ASO therapeutic

MO^{E1v11} was synthesized as a phosphorodiamidate morpholino oligomer (GeneTools L.L.C.). MO^{E1v11} is a 20-nucleotide oligomer (5'-CUAUAUAUAGUUAUUCAACA-3').

Production and purification of AAV9- myomixer vector

Viral production was performed as previously described (78). Briefly, 293T HEK cells (ATCC CRL 3216) were cultured in four 10-floor cell factories until approximately 85% confluent. Cells were triple transfected with Rep2Cap9 (serotype 2 Rep proteins, serotype 9 capsid proteins), pHelper (adenovirus helper constructs; Stratagene), and scAAV-CBA-PLS3 using 25-kDa polyethyleneimine at a molar ratio of 1:1:1. Cells were harvested at 48 hours after transfection and suspended in 10 mmol Tris, pH = 8.0, lysed by 5 freeze-thaw cycles in liquid nitrogen, DNase treated, and protease treated. CsCl crystals were added to the lysate (0.631 g of CsCl per ml of the lysate) to generate a solution with a density of approximately 1.4 mg/ml. After incubation at 37°C for 45 minutes, the solution was centrifuged at 3,184 g in an Eppendorf 5810 R at 4°C. Virus

was purified from lysate by 3 rounds of density gradient centrifugation at an average RCF of 158,000. Vector titers were determined by quantitative real-time PCR. The final fractions were dialyzed against PBS and stored at 4°C until use.

SMA mice

The original breeding pairs for the SMA mice (*Smn*^{+/-}*SMN2*^{+/+}*SMNΔ7*^{+/+}) on the FVB background were purchased from Jackson Laboratories. The breeding colony was maintained by interbreeding *Smn*^{+/-}*SMN2*^{+/+}*SMNΔ7*^{+/+} mice, and offspring were genotyped using PCR assays on tail DNA as previously described (4).

ASO and AAV9- myomixer treatment

Animals were treated with 2 nmol MO^{E1v11} via a single intracerebroventricular injection (ICV) injection on postnatal day 2. Viral delivery of AAV9-myomixer was performed by intravenous injection (1×10^{11} vector genomes) into the superficial vein on postnatal day 2. On day postnatal day 11 animals were anesthetized using isoflurane and sacrificed via decapitation. Hind limb muscle were harvested and flash frozen in liquid nitrogen. Tissues were stored at -80 °C until processed.

Histological analysis of hind limb muscle fiber

Mice were transcardially perfused with 4% paraformaldehyde. Distal hind limbs were dissected and postfixed overnight. Hind limb tissues were decalcified, embedded in paraffin, and cross-sectioned at the midpoint of the muscle. All analyses were done blinded. Sections (10 μm) were mounted on slides and stained with H&E. Digital images were captured using a Zeiss Axiovert 100M microscope and analyzed with NIH ImageJ software for total myofiber number (original magnification, ×20), and myofiber diameter

(original magnification, $\times 20$). Myofiber diameter was determined by measuring the largest diameter of at least 300 neighboring myofibers per animal.

Statistics

All statistical analyses were done using GraphPad Prism v7.05 (GraphPad Software, Inc.). Data were analyzed using either a Student's *t* test or a 2-way ANOVA. A *P* value less than 0.05 was considered statistically significant.

Study approval

Animals were housed and treated in accordance with the Animal Care and Use Committee guidelines of the Uniformed Services University of the Health Sciences and the University of Missouri. The Animal Care and Use Committee of the Uniformed Services University and the University of Missouri approved these studies.

RESULTS

SMN deficiency reduces expression of membrane fusion proteins

We previously showed that immortalized myoblasts from SMA model mice poorly fuse into myotubes, possibly due to altered myogenic gene expression, compromised cell migration, and membrane fusion (21). How SMN deficiency alters these pathways remains unclear. In this study, we sought to determine the role of SMN in myoblast membrane fusion, the terminal stage of myotube formation. We generated SMN knockdown C2C12 myoblast cell lines that stably express an SMN shRNA and showed, using an antisense oligonucleotide designed against the shRNA, that we could restore SMN expression 48 hours after antisense exposure (**Figure 7A**). We next investigated the fusion capacity of SMN-deficient cells using a cell mixing assay. We co-plated and

differentiated wild-type and SMN-deficient myoblasts labeled with fluorescent trackers and found that SMN-deficient myoblasts fuse with wild-type myoblasts, but not with each other, suggesting that SMN-deficient myoblasts are not fusion-incompetent (**Figure 7B**).

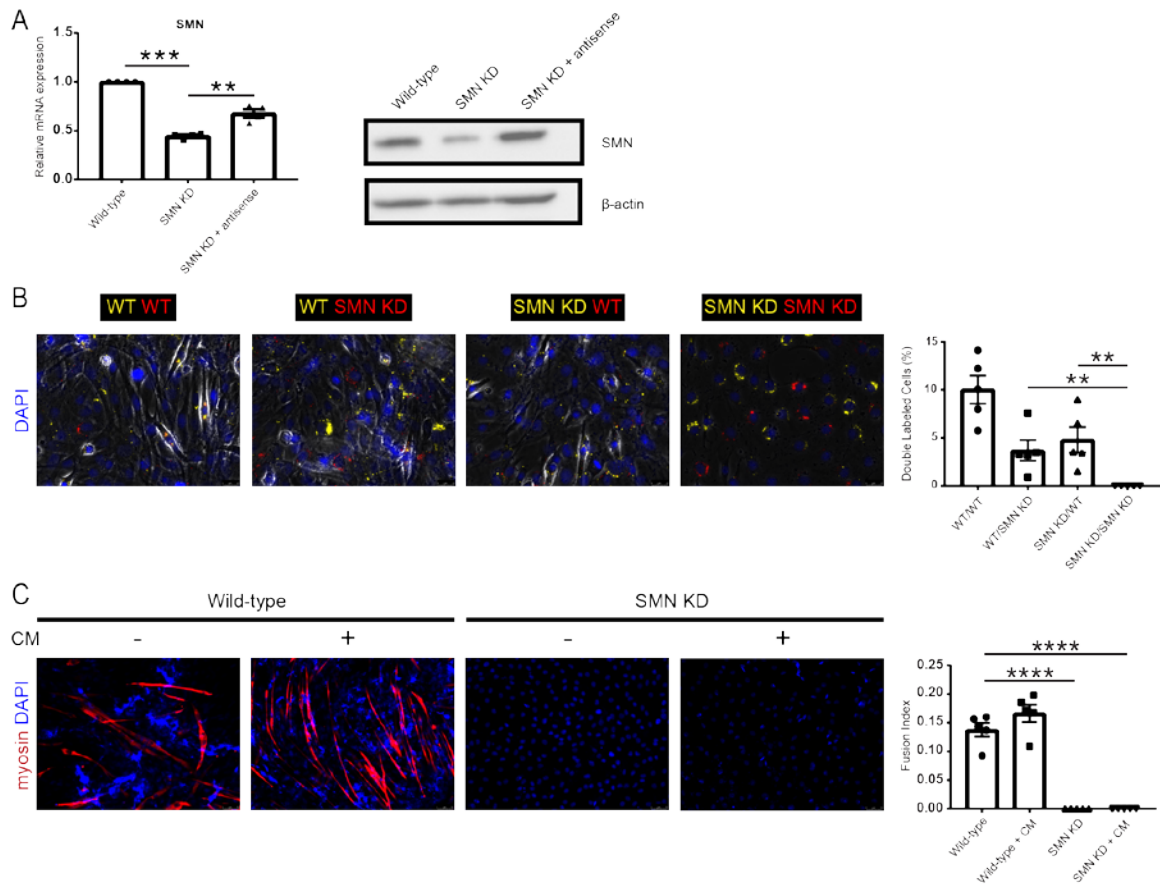


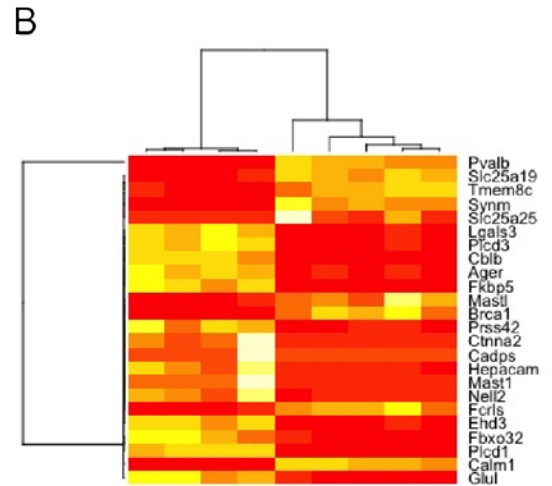
Figure 7. SMN deficiency impairs myoblast fusion. (A) SMN mRNA and protein levels in wild-type and SMN knockdown C2C12 cells and SMN knockdown cells (SMN KD) transfected with an antisense oligonucleotide designed to suppress the SMN shRNA. (B) Wild-type and SMN-deficient C2C12 cells were differentiated for 2 days and then labeled with QTracker 585 (yellow) and 655 (red). After labelling, cells were mixed and differentiated for an additional 5 days. Cells were stained with DAPI (40X; scale bar: 25 μ m). The number of double-labeled cells was counted. N = 5. (C) Wild-type and SMN-deficient C2C12 cells were treated with conditioned media (CM) from differentiating wild-type C2C12 cells (20X; scale bar: 50 μ m) and quantification of fusion index. N = 5. All data represented as mean $\pm$ SEM. **P < 0.01, ***P < 0.001, ****P < 0.0001.

Impaired myoblast fusion could be caused by loss of soluble factors that triggers signaling cascades to augment muscle fusion or disruption of dynamic membrane processes mediated by integral membrane proteins (69; 149). To determine if impaired fusion is due to loss of released soluble myogenic factors, we treated wild-type and SMN-deficient myoblasts with conditioned media from differentiating wild-type myoblasts and found that conditioned media initiated fusion of wild-type, but not SMN-deficient, myoblasts suggesting that compromised fusion is likely due to altered expression or activity of membrane proteins and not the release of soluble myogenic factors (**Figure 7C**).

We next sought to determine whether genes involved in the regulation of myoblast fusion are altered when SMN is deficient. We isolated and performed transcriptome profiling of skeletal muscle from postnatal day 10 (PND10) wild-type and SMA model mice (**Figure 8A-B**). Protein ANalysis THrough Evolutionary Relationships (PANTHER) revealed altered expression of four cell adhesion molecules including myomaker, a protein required for myoblast fusion and muscle formation (**Figure 8A, C**) (113). Using qRT-PCR, we confirmed that myomaker is reduced in skeletal muscle of SMA mice at PND10 as well as earlier during development at PND4/5 (**Figure 8D-E**). Given that myomaker is required by both cells for fusion to occur, and we found that SMN-deficient myoblasts readily fuse with wild-type myoblasts, myomaker may not be the only fusion molecule deficient in SMA skeletal muscle (113). Therefore, we examined expression of a second fusion protein, myomixer, also known as Gm7325, myomerger, and minion (14; 138; 180). Unlike myomaker, myomixer is only required by one fusing cell (14; 138; 180). We confirmed by qRT-PCR that myomixer expression is

A

Protein Class	% of genes altered
calcium binding protein	3.1
cell adhesion molecule	1
chaperone	0.7
cytoskeletal protein	7.8
defense/immunity protein	2.7
enzyme modulator	12.9
extracellular matrix protein	1.4
hydrolase	13.9
isomerase	1.4
ligase	2.7
lyase	1.7
membrane traffic protein	2.4
nucleic acid binding	8.1
oxidoreductase	6.4
receptor	3.4
signaling molecule	7.1
structural protein	0.7
transcription factor	5.1
transfer/carrier protein	3.1
transferase	8.1
transmembrane receptor/regulatory/adaptor protein	0.3
transporter	6.1



C

Gene		Log2 fold change
Lgals3	Galectin-3	2.11
Ager	Advanced glycosylation end product-specific receptor	1.6
Tmem8c	Myomaker	-1.61
Fcrls	Fc receptor-like 5, scavenger receptor	-1.95

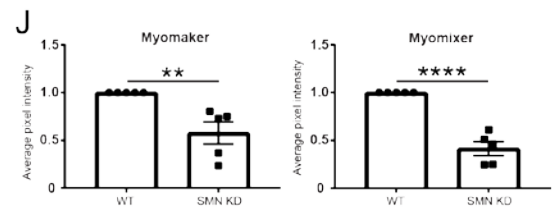
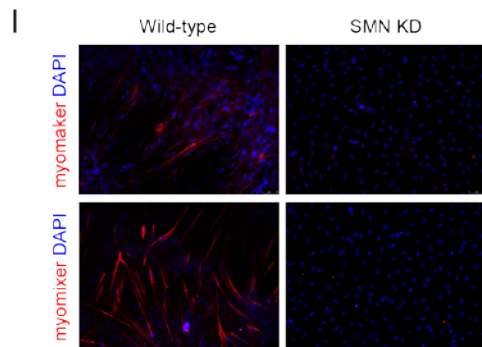
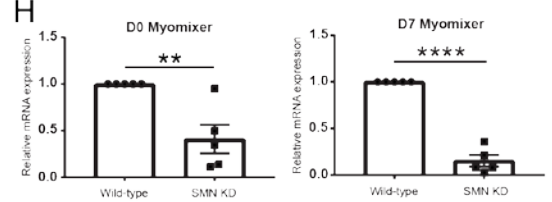
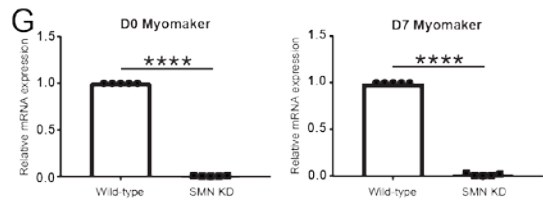
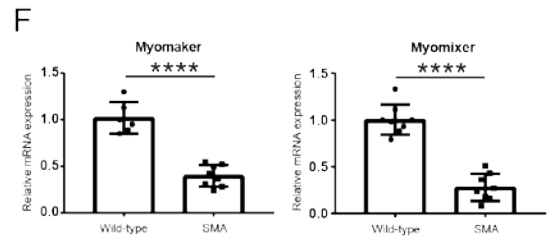
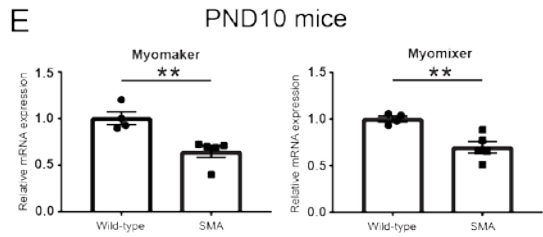
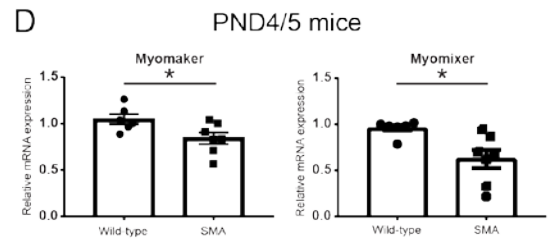


Figure 8. Myomaker and myomixer expression is reduced with SMN deficiency. (A) PATHER analysis of altered genes sorted by protein class. (B) Heat map of select genes (calcium binding proteins, cell adhesion molecules, and ligases) from transcriptome analysis of skeletal muscle from PND10 wild-type and SMN Δ 7 mice. Red indicates high gene expression. Yellow indicates low gene expression. (C) Cell adhesion molecules significantly altered in skeletal muscle of SMN Δ 7 mice. Myomaker and myomixer mRNA expression in PND 4/5 (D) and PND 10 (E) skeletal muscle of wild-type and SMN Δ 7 mice. N = 5-7. (F) Myomaker and myomixer mRNA expression in satellite cells isolated from gastrocnemius muscle of wild-type and SMN Δ 7 mice. N = 8. Myomaker (G) and myomixer (H) mRNA expression in proliferating (D0) and differentiated (D7) wild-type and SMN-deficient C2C12 cells. N = 5. (I) Differentiated C2C12 cells were immuno-labeled with anti-myomaker or anti-myomixer antibodies and DAPI (20X; scale bar: 50 μ m) and (J) quantification of pixel intensity. N = 5. All data represented as mean $\pm$ SEM. *P < 0.05, **P < 0.01, ***P < 0.0001.

significantly reduced in skeletal muscle from both PND4/5 and PND10 SMA mice (Figure 8D-E). Additionally, we examined the expression of myomaker and myomixer in satellite cells, skeletal muscle stem cells, isolated from gastrocnemius muscle of SMA mice and their wild-type littermates. We found that myomaker and myomixer expression were reduced in satellite cells isolated from SMA mice compared to wild-type mice (Figure 8F).

Myomaker and myomixer expression are usually low in satellite cells and upregulated during myoblast fusion (14; 113). We next compared the induction of myomaker and myomixer during differentiation. We found reduced expression of myomaker and myomixer in proliferating SMN-deficient myoblasts consistent with SMA mouse myofibers (Figure 8G-H). After 7 days of differentiation, myomaker and myomixer expression remain reduced in SMN-deficient cells compared to wild-type cells (Figure 8G-H). Given that SMN deficiency did not completely ablate myomaker and myomixer mRNA expression, we looked at the protein expression of each protein in

wild-type and SMN-deficient myoblasts. Immunohistological analysis revealed almost undetectable expression of myomaker and myomixer in SMN-deficient C2C12 cells (**Figure 8I-J**). To determine if restoring SMN rescues myomaker and myomixer expression we treated SMN-deficient myoblasts with an ASO to the SMN-targeting shRNA to relieve SMN gene repression. We found that while SMN expression was restored 48 hours after treatment with the ASO, myomaker and myomixer expression were not fully rescued to wild-type levels (**Figure 9A-B**).

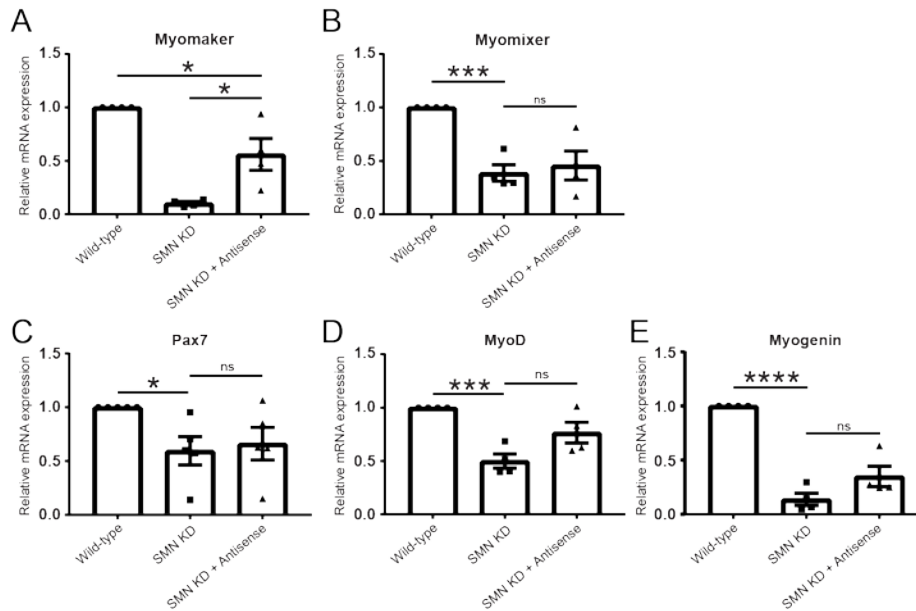


Figure 9. Restoration of SMN does not fully rescue myogenic programming. SMN knockdown C2C12 cells were transfected with an antisense oligonucleotide designed to suppress the SMN shRNA for 48 hours. mRNA expression of myomaker (A), myomixer (B), Pax7 (C), MyoD (D), and myogenin (E) in wild-type, SMN knockdown cells, and SMN knockdown cells transfected with the antisense oligonucleotide. N = 4. *P < 0.05, ***P < 0.001, ****P < 0.0001.

Overexpressing MyoD and myogenin increases myomaker and myomixer expression and rescues fusion deficit of SMN-deficient myoblasts

Expression of myomaker and myomixer is regulated, in part, by the transcription factors MyoD and myogenin (**Figure 10A**) (100; 114; 166). We previously showed that

MyoD and myogenin expression are altered in SMN-deficient myoblasts (21). We first showed that MyoD and myogenin are reduced in SMN-deficient C2C12 cells after 7 days of differentiation, consistent with our previous findings (**Figure 10B-C**) (21). Restoration of SMN did not fully rescue expression of MyoD, myogenin, or Pax7, a marker for muscle stem cells (**Figure 9C-E**). We next examined if restoring MyoD or myogenin is

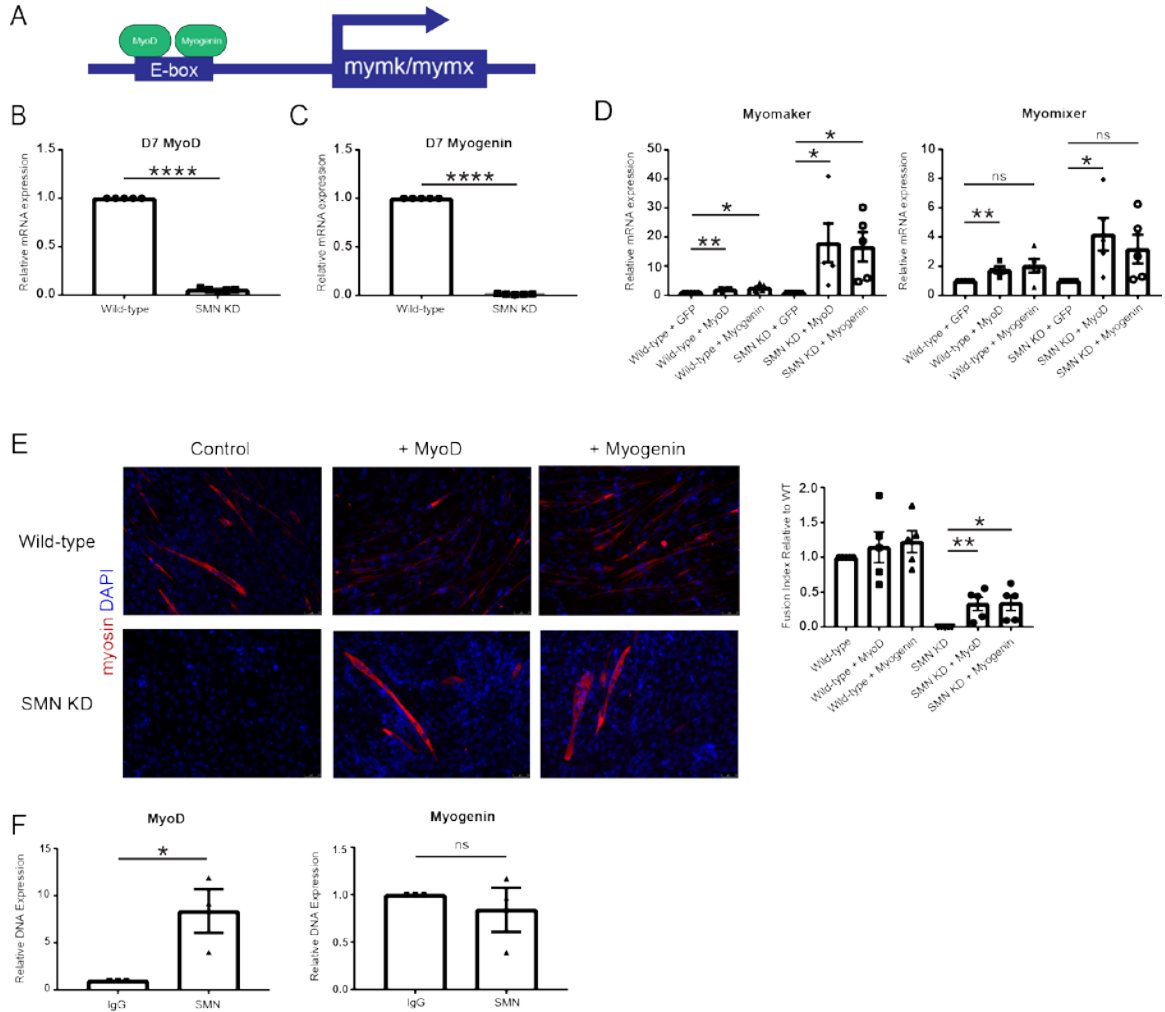


Figure 10. SMN deficiency alters the expression of MyoD and myogenin. (A) Schematic showing binding of MyoD and myogenin to E-box elements upstream of the myomaker and myomixer promoters. MyoD (B) and myogenin (C) mRNA expression in wild-type and SMN-deficient C2C12 cells after 7 days of differentiation. N = 5. (D) Myomaker and myomixer mRNA expression 48 hours after MyoD or myogenin overexpression. N = 5. (E) MyoD or myogenin were overexpressed in wild-type and SMN-deficient C2C12 cells and differentiated for 5 days. Cells were fixed and stained for myosin and DAPI

(20X; scale bar: 50 μ M) and quantification of the fusion index. N = 5. (F) Wild-type C2C12 cells were differentiated for 48 hours and fixed. Chromatin co-immunoprecipitated with SMN protein was probed with primers to the MyoD and myogenin promoters. N = 3. All data represented as mean $\pm$ SEM. *P < 0.05, **P < 0.01, ****P < 0.0001.

sufficient to increase myomaker and myomixer levels when SMN levels are reduced. Overexpressing MyoD or myogenin for 48 hours stimulated myomaker and myomixer expression in SMN-deficient C2C12 cells (**Figure 10D**). Moreover, overexpressing MyoD or myogenin partially restored fusion of SMN-deficient myoblasts (**Figure 10E**). Together, these data indicate that insufficient expression of MyoD and myogenin is at least partially responsible for the reduced expression of myomaker and myomixer in SMN-deficient myoblasts.

We next sought to determine how SMN deficiency alters MyoD and myogenin mRNA expression. We first examined whether SMN deficiency alters MyoD and myogenin mRNA stability. After 48 hours of differentiation, we treated wild-type and SMN-deficient C2C12 cells with actinomycin D to inhibit the synthesis of new mRNA. We found no difference in MyoD or myogenin mRNA stability with SMN deficiency (**Figure 11A-B**). To determine if SMN alters MyoD and myogenin transcription, we immunoprecipitated SMN from wild-type C2C12 cells 48 hours after differentiation and probed the bound chromatin for the MyoD and myogenin promoters. We found that SMN interacted with the MyoD, but not the myogenin, promoter (**Figure 10F**). Together, these data suggest that SMN modulates the myogenic program through regulating MyoD transcription.

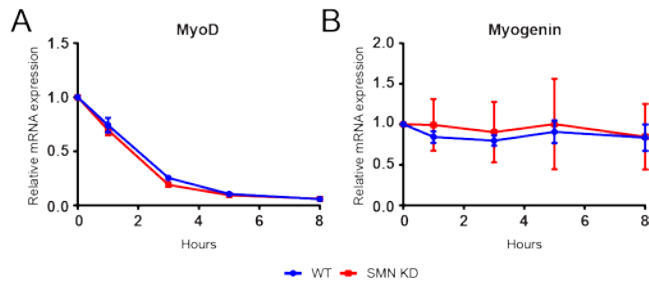


Figure 11. MyoD and myogenin mRNA stability does not change with SMN deficiency. Wild-type and SMN knockdown cells were differentiated for 48 hours and then treated with actinomycin D. MyoD (A) and myogenin (B) mRNA expression following the addition of actinomycin D. N = 5.

Restoring SMN levels in muscle of SMA mice partially restores myomaker and myomixer expression

Current treatment options for SMA aim to increase SMN protein levels through increasing expression and correcting splicing of the *SMN2* gene product to slow disease progression (16). It has been reported that increasing SMN improves the muscle pathology presumably due to slowed denervation, although muscle intrinsic improvements have been described (17; 104). Here, we investigated whether increasing SMN restored myomaker and myomixer expression in SMA model mice. 2 nmol of ASO (MO^{E1v11}), delivered through intracerebroventricular injection, increased SMN levels through splicing correction and improved the motor function of SMA model mice (128). We treated SMA mice daily starting at PND2 with ASO (MO^{E1v11}) and harvested hind limb muscle tissue at PND11 for biochemical analysis. We found increased expression of full-length SMN as well as increased expression of myomixer and myomaker in ASO-treated mice (**Figure 12A**). These data suggest that postnatal restoration of SMN enhances membrane fusion protein expression in SMA model mice.

Overexpressing myomixer improves histopathology and motor function of SMA mice

Given that current treatments for SMA prolong lifespan, but only a small subset of patients have improvements in motor function, we next sought to determine if restoring expression of myomixer improves muscle fiber size and motor function in SMA model mice, alone or in combination with an ASO (MO^{E1v11}). We treated SMA and control littermates with 1×10^{11} vector genomes of self-complimentary AAV9-myomixer through intraperitoneal injection or a combination of ASO (MO^{E1v11}) and AAV9-myomixer at PND2 and examined myofiber diameter and myofiber number at PND11.

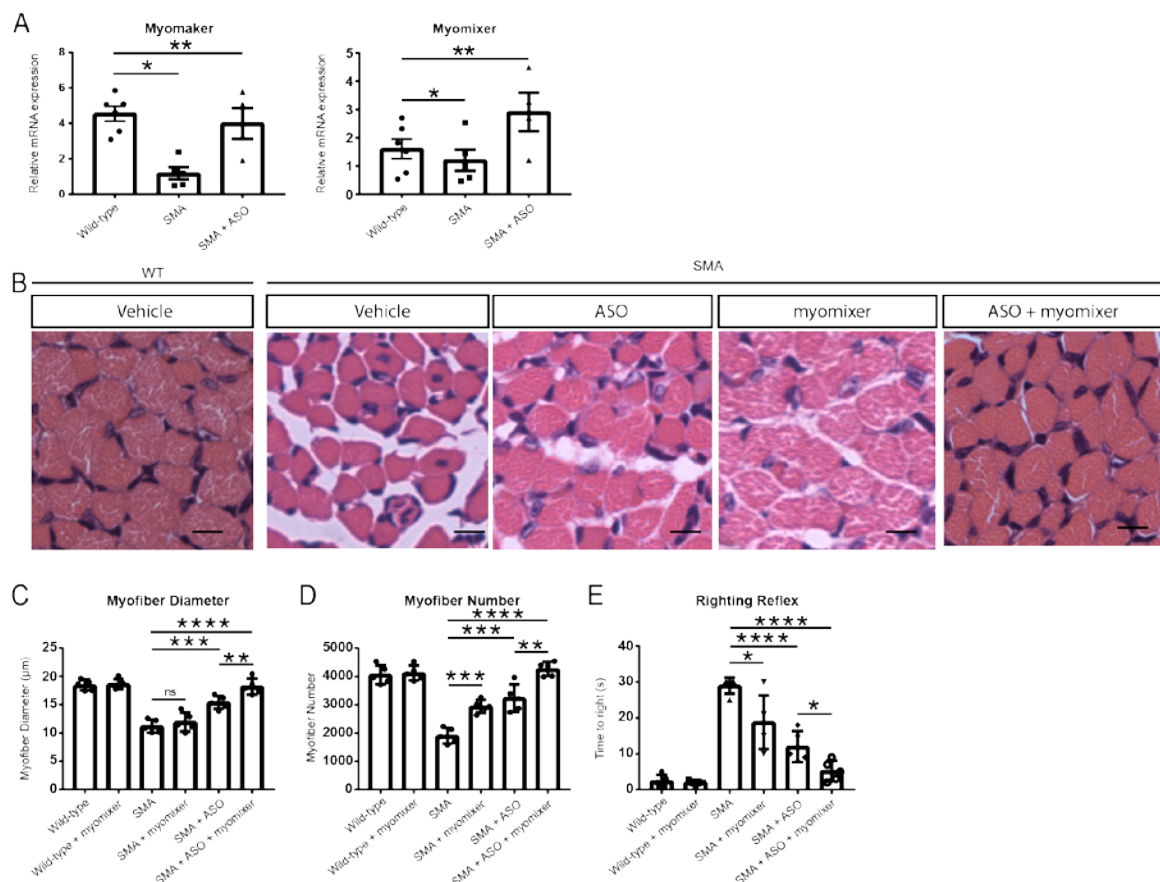


Figure 12. AAV9-delivery of myomixer improve muscle histopathology and motor function of SMND7 mice. (A) Myomaker and myomixer mRNA expression in wild-type and SMND7 mice treated with an ASO. mRNA expression was normalized to untreated SMND7 mice. N = 4-6. (B) H&E stained muscle cross-

sections from wild-type and SMN Δ 7 mice treated with an ASO and AAV9-myomixer. Scale bars: 10 μ m. Myofiber diameter (C) and myofiber number (D) from hind limb muscle, and righting reflex (E) of wild-type and SMN Δ 7 treated with an ASO and AAV9-myomixer. N = 5 for each group. All data represented as mean $\pm$ SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Myomixer expression was confirmed by western blot analysis. We found that ASO increased myofiber diameter and number as previously reported (128). Delivery of AAV9-myomixer improved myofiber diameter and further increased both myofiber diameter and number when co-administered with ASO (**Figure 12B-D**). To determine the functional consequence, if any, of the improved histopathology, we monitored the righting reflex of SMA mice from PND1 to PND10. We found that, consistent with the muscle size data, ectopic myomixer expression improved the righting reflex of SMA mice alone or in combination with ASO (**Figure 12E**). Together, these findings confirm that SMA muscle pathology and motor function can be improved by restoring myomixer expression.

DISCUSSION

SMA patients have fewer myofibers and smaller muscles which could be due in part to the reduced fusion of SMN-deficient muscle cells during development or denervation-dependent atrophy (178). Our previous findings suggested that SMN deficiency impaired myoblast fusion potentially through impaired myogenic gene expression, cell migration, and membrane fusion (21). It remained unclear how loss of SMN impaired these key features of myotube formation. In the present study, we sought to identify the mechanism through which SMN deficiency impairs myoblast fusion.

We found that SMN-deficient myoblasts and muscle fibers and satellite cells from SMA model mice have reduced expression of myomaker and myomixer, two proteins

that are essential for myoblast fusion. Reduction of myomaker and myomixer may explain previous findings that SMA mice have fewer and smaller muscle fibers and why SMN-deficient myoblasts and satellite cells fuse poorly (10; 21; 62; 91; 152). SMN deficiency also alters the expression of the genes responsible for initiating the myogenic program, MyoD and myogenin. Restoration of endogenous SMN did not restore the myogenic programming indicating that current treatments may not be sufficient to ameliorate muscle intrinsic defects. Ectopic overexpression of MyoD or myogenin partially rescues myomaker and myomixer expression 48 hours after transfection even when SMN is reduced suggesting these genes act downstream of SMN in regulating myomaker and myomixer expression. There may be further restoration of myomaker and myomixer expression following MyoD or myogenin overexpression at a later time point.

Additionally, we showed that SMN binds to the promoter region of MyoD suggesting that SMN may directly or indirectly regulate myogenic differentiation at the level of MyoD transcription. We did not detect SMN at the myogenin promoter, indicating that reduced myogenin expression is likely due to reduced expression of MyoD, at an earlier stage of myoblast differentiation (reviewed in (49)). Consistent with this, we showed that restoring myogenin rescues the fusion deficit of SMN-deficient myoblasts. Importantly, we showed that ectopic expression of myomixer increased myofiber number and diameter and improved motor function in a severe mouse model of SMA. To our knowledge, this is one of the earliest pieces of evidence that demonstrate that myoblast fusion can be targeted *in vivo* to improve motor function. Additionally, we showed that myomixer overexpression enhances the effects of an ASO (MO^{E1v11}) that corrects *SMN2* splicing on muscle histopathology and motor function in SMA mice. Our

data indicate that myoblast fusion may be a potential SMN-independent mechanism that can be targeted in combination with current therapies to improve SMA pathology.

Current treatments for SMA markedly improve lifespan with a small subset of patients having improvements in motor function, especially with early intervention (43; 44; 51; 109; 111). Nevertheless, profound functional deficits remain, with many patients failing to reach normal motor milestones. While slowing motor neuron loss is central to improving the SMA pathology, SMN-independent treatments, such as those that target muscle, may enhance the efficacy of splicing modifiers and SMN gene therapy. Muscle has long been considered a tissue that could be targeted to improve motor function in SMA patients (17; 45; 91; 123; 139). We and others have shown that SMA muscles are small and undergo denervation dependent atrophy during disease progression (22). Some drugs that target skeletal muscle in current clinical trials aim to improve muscle growth by inhibiting myostatin (SRK-015) (NCT03921528) or sensitize the troponin complex to lower the threshold for muscle contractility (CK-2127107) (NCT02644668). Our study identified myoblast fusion as a novel mechanism to improve muscle pathology in SMA that can enhance current approved treatments. Further experiments to identify compounds or biologics to improve myoblast fusion may lead to additional therapeutics for SMA.

Chapter 4: Discussion

INTRODUCTION

SMA is a debilitating genetic neuromuscular disease affecting 1 in 10,000 newborns annually (170). Patients with the most severe form of the disease do not live past their second birthday. Recently approved treatments for SMA, nusinersen and gene therapy, extend survival for patients, but are far from a cure for SMA. Only a small subset of patients see improvements in their motor function. For example, in a clinical trial where SMA type 1 patients were treated with nusinersen, only 22% had full head control, 10% could roll over, 8% could sit unassisted for 30 seconds, and 1% could stand (51). While achieving any of these motor milestones is a major feat for SMA type 1 patients, there remains a need to develop new treatments that can act in combination with current therapies to further improve disease outcomes.

In addition to still having motor deficits following treatment, there are several other issues with these therapies. Autopsy tissue from SMA patients who were treated with nusinersen showed highest uptake of the ASO in the lumbar, sacral, and thoracic regions of the spinal cord with less uptake in the cervical spinal cord (140). Additionally, patients did not have an increase in SMN protein levels in whole spinal cord and there was no increase in full-length *SMN2* transcript or SMN protein levels in liver or skeletal muscle showing the limited effects of nusinersen. There is a growing body of evidence that SMA is a multi-organ disease suggesting that systemic treatments may be required (13; 21; 65; 175). Lastly, administration of nusinersen through intrathecal injection is difficult particularly in SMA type 2 and type 3 patients who have spondylodesis (fusing

of the spine) and advanced scoliosis (53; 159). Treatment with AVXS-101 has the potential to stimulate an immune reaction. Animals treated with AAV9 can create anti-AAV9 antibodies which limits administration of gene therapy to a single dose (136). Some individuals have anti-AAV9 antibodies which prevents them from ever receiving AVXS-101 treatment (136). A recent clinical trial in which AVXS-101 was administered via intrathecal injection to SMA type 2 patients was halted after finding that an intrathecal injection of AVXS-101 caused dorsal root ganglion inflammation and infiltration of mononuclear cells in animals (3). Currently, there is no autopsy tissue data from SMA patients treated with AVXS-101, so it is not clear which cells are incorporating the viral vector and what tissues have increases in SMN protein. Given that nusinersen and AVXS-101 were recently approved in 2016 and 2019, respectively, the safety and long-term effects of these drugs are not known.

Human spinal cord and skeletal muscle autopsy samples have high expression of SMN protein during fetal development (140). SMN protein expression decreases during the perinatal period and remains low postnatally (140). This data suggests that SMN is required early in development and that SMN-increasing treatments, such as nusinersen and AVXS-101, are likely to be most effective when administered early in the course of the disease. Consistent with this, SMA type 2 patients who were treated with nusinersen before the onset of symptoms show the greatest improvement in motor function with 88% of patients having the ability to walk independently (44). Unfortunately, treating patients pre-symptomatically is not always possible as newborn screening for SMA is not routinely performed in every state (cureSMA.org). Therefore, SMA patients may require additional SMN-independent treatments to address defects that are SMN-irreversible and

cannot be fixed by increasing SMN protein levels (68). There is a critical need to develop novel SMN-independent mechanisms, in addition to SMN-increasing therapies, to treat SMA.

Here, we aimed to identify novel mechanisms to treat SMA. Using an RNAi screen, we found that reduction of the RNA processing genes, *WDR33*, *CPSF1*, and *THOC3*, increases SMN protein levels through regulation of nuclear/cytoplasmic RNA transport (**Figure 13**). Additionally, we found that the E3 ligase Nbr1 ubiquitinates and promotes the degradation of SMN (**Figure 13**). Finally, we found SMN regulates myoblast fusion through reduction of the fusion molecules, myomaker and myomixer, and that myoblast fusion can be targeted to improve muscle histopathology and motor function in SMA mice (**Figure 14**). Together, these findings provide novel pathways that can be targeted in combination with current therapies to treat SMA.

REDUCTION OF RNA PROCESSING MACHINERY COMPONENTS INCREASES SMN PROTEIN

Using a genome-wide RNAi screen to identify novel genetic modifiers of SMN, we found that when several components of the RNA processing machinery are knocked down, SMN protein levels are increased. We validated that knockdown of *WDR33*, *CPSF1*, and *THOC3*, all components of the transcription export complex, increases SMN protein levels in SMA patient-derived fibroblasts. Knockdown of *WDR33* increases *SMN2* pre-mRNA, but had no effect on *SMN2* mRNA suggesting that this is likely not the mechanism by which SMN protein levels are increased. *SMN2* pre-mRNA and mRNA were unchanged with knockdown of *CPSF1* and *THOC3*. *SMN2* splicing and mRNA stability were both unchanged with knockdown of *WDR33*, *CPSF1*, and *THOC3*.

There is a slight yet significant increase in cytoplasmic *SMN2* mRNA with knockdown of *WDR33* and *THOC3*, but not *CPSF1*.

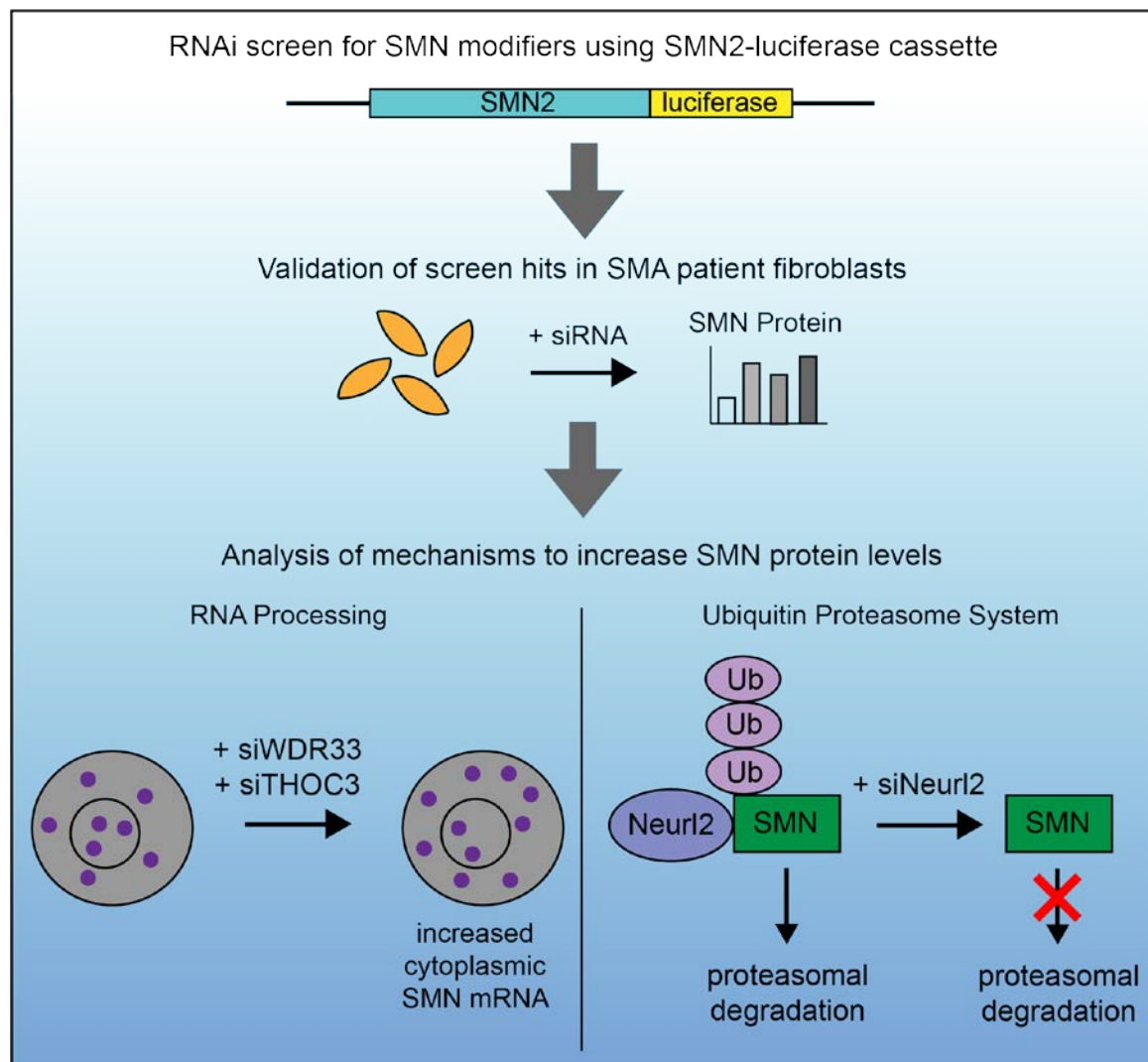


Figure 13. Summary of Chapter 2: A High-Throughput Genome-wide RNAi Screen Identifies Novel Modifiers of Survival Motor Neuron. A SMN2-luciferase cassette was used in an RNAi screen to identify novel modifiers of SMN. Top screen hits were validated in SMA patient fibroblasts. siRNAs to top hits were transfected in SMA patient fibroblasts and SMN protein levels were examined by Western blot analysis after 48 hours. Knockdown of *WDR33* and *THOC3*, RNA processing genes, increased cytoplasmic SMN mRNA to increase SMN protein levels. Neur12, an E3 ligase, ubiquitinates and promotes the degradation of SMN protein. Knockdown of Neur12 stabilizes SMN protein levels.

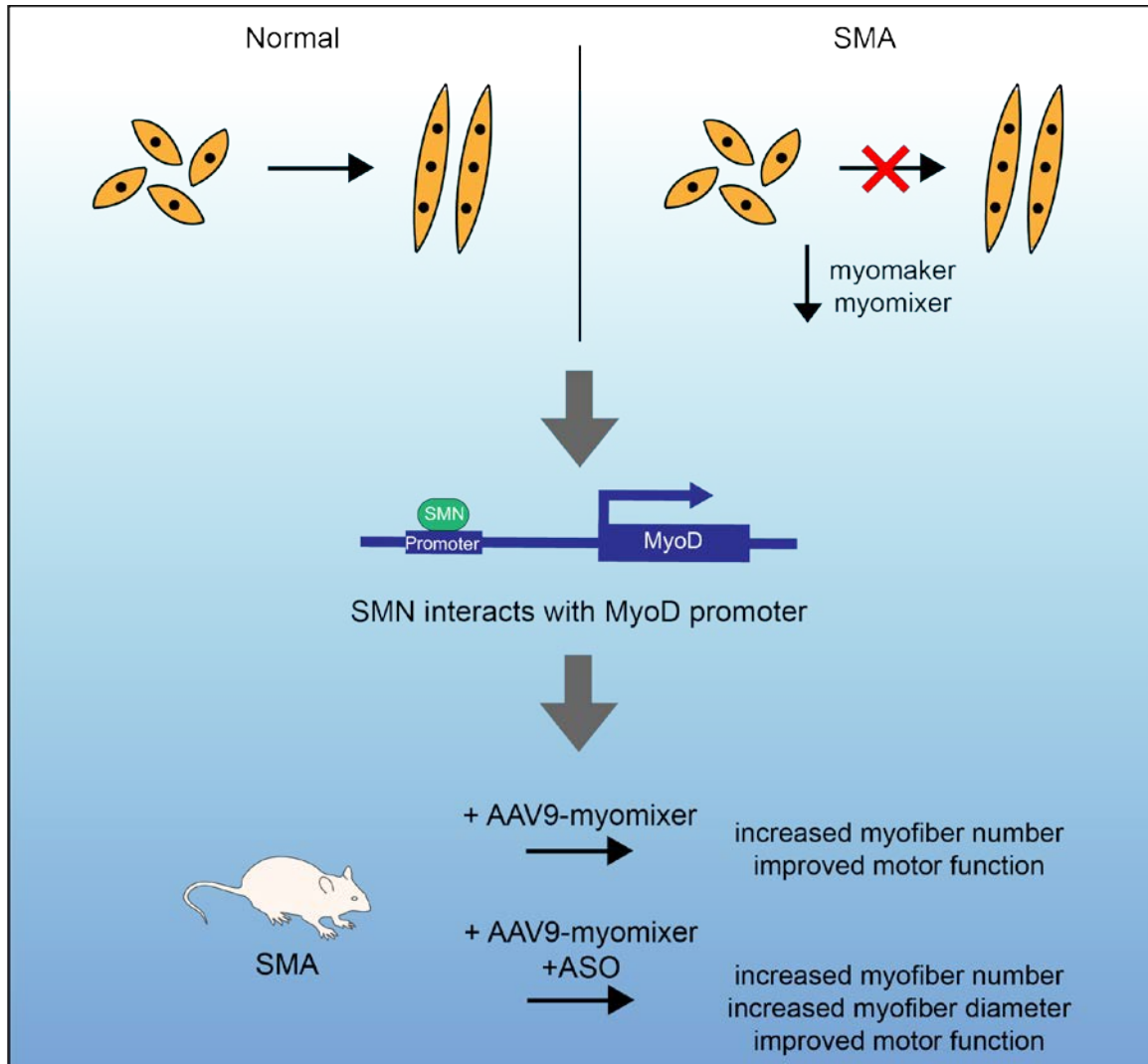


Figure 14. Summary of Chapter 3: SMN Deficiency Slows Myoblast Fusion through Reduced Myomaker and Myomixer Expression. Myoblast fusion is impaired with SMN deficiency. RT-PCR and transcriptome analysis revealed reduced expression of the fusion molecules myomaker and myomixer. ChIP analysis shows that SMN interacts with the MyoD promoter. Given that MyoD is a transcription factor that regulates myogenic differentiation, reduction of MyoD with SMN deficiency leads to reduced expression of myogenin, myomaker, and myomixer to impair myoblast fusion. Ectopic expression of myomixer (AAV9-myomixer) in SMA mice increased myofiber number and improved motor function in the right reflex test. Co-treatment with AAV9-myomixer and an ASO further improves myofiber number and diameter and motor function.

Implications

Increased expression of *SMN2* mRNA in the cytoplasm with knockdown of *WDR33* and *THOC3* suggests that knockdown of these genes leads to increased nuclear

export of *SMN2* mRNA into the cytoplasm, leading to increased translation of *SMN2* mRNA to produce more SMN protein. Previous approaches to increase SMN focus on increasing transcription, correcting *SMN2* splicing, blocking SMN degradation, and gene replacement (4; 12; 52; 74). Here we provide initial evidence of a novel mechanism to increase SMN protein levels which ultimately may lead to new therapies for SMA that can act synergistically with current treatments to increase SMN protein levels.

NEURL2 UBIQUITINATES SMN

Using an RNAi screen, we also found that knockdown of *Neurlized-2* (*Neurl2*) increases SMN protein. We validated that *Neurl2* knockdown increases SMN protein levels in SMA patient-derived fibroblasts. We found that *Neurl2* and SMN interact and that *Neurl2* directly ubiquitinates SMN in an *in vitro* ubiquitination assay. Furthermore, we found that knockdown of *Neurl2* blocks SMN degradation. Lastly, we showed that knockdown of *Neurl2* and *mindbomb-1* further stabilizes SMN.

Implications

Our lab previously showed that *mindbomb-1* ubiquitinates SMN and targets SMN for proteasomal degradation (86). Blocking *mindbomb-1* from ubiquitinating SMN using a small molecule, ML372, increases SMN protein levels and improves the disease phenotype in SMA mice (4). Here, we identified an additional E3 ligase, *Neurl2*, which targets SMN for ubiquitination and degradation by the proteasome. Given that E3 ligases provide specificity to the ubiquitin proteasome system, *Neurl2* is an additional E3 ligase that can be targeted to block SMN degradation to increase SMN protein levels.

Additionally, *Neurl2* is highly expressed in skeletal muscle (124). Therefore, targeting *Neurl2* in SMA animal models and potentially SMA patients, may be particularly

beneficial in increasing SMN protein levels in skeletal muscle and in improving muscle histopathology. This data is consistent with our previous studies showing that the ubiquitin proteasome system regulates levels of SMN protein.

SMN DEFICIENCY REDUCES MYOMAKER AND MYOMIXER EXPRESSION TO IMPAIR MYOBLAST FUSION

SMN regulates Myomaker and Myomixer Expression

Our lab and several others previously showed that SMN deficiency impairs myoblast fusion (10; 21; 152). How loss of SMN impairs this process is poorly understood. Here, we used transcriptome analysis and RT-PCR to identify altered expression of cell adhesion molecules in skeletal muscle of SMA model mice. We found that SMN deficiency leads to reduced expression of myomaker and myomixer (also known as Gm7325, myomerger, and minion), two muscle-specific fusion molecules required for proper muscle development. We generated an SMN-deficient myoblast line and confirmed reduced expression of myomaker and myomixer with SMN deficiency. Consequently, SMN-deficient myoblasts were unable to fuse and form multinucleated myotubes. Reduction of these critical fusion molecules may explain why SMA mice have fewer and smaller muscle fibers and why SMA patients have smaller muscle fibers.

Myomaker and myomixer expression is regulated by MyoD and myogenin, two transcription factors that regulate myogenic programming. We found reduced expression of MyoD and myogenin in our SMN-deficient myoblasts which likely explains reduced myomaker and myomixer expression.

SMN Acts as a Transcription Factor

SMN has many functions including a role in pre-mRNA splicing, axonal growth, mRNA localization, and protein synthesis (47; 48; 88; 134). One earlier study found that SMN associates with a nuclear transcription activator suggesting that SMN may also have a role in transcription (160). Here, we used ChIP analysis and found that SMN associates with the MyoD, but not the myogenin, promoter. Therefore, loss of SMN reduces MyoD expression which reduces expression of myomaker and myomixer leading to impaired myoblast fusion. Myogenin expression is regulated by MyoD which likely explains why myogenin expression is reduced with the loss of SMN.

Targeting Myoblast Fusion to Treat SMA

Lastly, we sought to determine whether ectopic expression of myomixer in SMA mice can improve disease outcomes. We treated severe SMA model mice with AAV9-myomixer alone and in combination with MO^{E1v11}, an *SMN2* splicing modifier. We showed that a single IP injection of AAV9-myomixer significantly increased myofiber number and reduces righting time in the righting reflex test. When given in combination with MO^{E1v11}, SMA mice see further improvements in myofiber diameter, myofiber diameter, and motor function. These findings provide initial evidence that myoblast fusion can be targeted *in vivo*. Additionally, we demonstrate that targeting myoblast fusion enhances the effects of a splicing modifier.

Implications

SMA patient autopsy tissue revealed that nusinersen treatment did not increase SMN protein in whole spinal cord or in skeletal muscle. SMA mice treated with AVXS-101 showed major improvements in survival and motor function, but their body weight is only half that of control animals at P90 likely due to low muscle mass. Additionally,

many patients that receive treatment have a prolonged lifespan, but do not see improvements in their motor function. These data suggest that skeletal muscle may need to be targeted by SMN-independent pathways. Given skeletal muscle's major role in movement, improving skeletal muscle pathology is critical for normal motor function.

Current approaches in ongoing clinical trials are inhibition of myostatin and lowering the threshold for muscle contractility. The myostatin pathway regulates muscle mass (26). Myostatin is synthesized as a propeptide which is cleaved and dimerizes to form active myostatin. Mature myostatin binds to one of two activin type II receptors (ActRIIA or ActRIIB) to activate the Smad pathway, induce gene transcription, and inhibit an increase in muscle mass (26). Studies inhibiting the myostatin pathway to increase muscle mass in SMA mice have had contradictory findings. In one study, severe SMA model mice were administered soluble ActRIIB-Fc, which blocks myostatin from binding to its receptor, starting at P2 (163). ActRIIB-Fc-treated SMA mice did not have an improvement in time to right in the righting reflex test, but did have an increase in the number of pulls and had a prolonged latency to fall in the hind limb suspension test. However, this treatment decreased survival compared to vehicle-treated SMA mice (163). Genetically overexpressing follistatin, an inhibitor of myostatin, in a severe SMA mouse model did not significantly increase muscle mass or myofiber diameter and did not improve righting time (163). In a separate study, SMA mice were postnatally treated with recombinant follistatin (145). Follistatin-treated SMA mice had an increased triceps muscle mass beginning at P6, had a decreased time to right in the righting reflex test, and had prolonged survival, but did not have increased cross-sectional area of myofibers or an increase in body weight (145).

Follistatin may not be an optimal target to improve muscle mass in SMA patients. Recently, follistatin was shown to inhibit both myostatin and GDF11, a regulator of skeletal patterning (161). Consequently, follistatin simultaneously increases muscle mass and weakens bone leading to bone fractures (161). SMA type 2 and type 3 patients often have scoliosis and there are additional reports of skeletal system defects in SMA patients suggesting that follistatin may have adverse effects in SMA patients (53; 95). Additionally, SMA patients have reduced myostatin expression and SMA mice have reduced expression of myostatin and increased expression of follistatin (103; 163). Given that myostatin knockout mice have hyperplasia (increased number of myofibers) and hypertrophy (increased myofiber size), altered expression of myostatin and follistatin may be a compensatory mechanism in SMA mice (26; 163). A recent study showed that treatment with an ASO that corrects *SMN2* splicing increases myostatin expression in SMA mice (182). It remains unknown whether nusinersen or gene therapy has a similar effect in SMA patients and whether the combination of an SMN-increasing treatment and a myostatin inhibitor can act synergistically to ameliorate the disease phenotype in SMA patients.

The fast skeletal troponin activator, CK-2127107, increases muscle contractility by sensitizing the sarcomere to calcium (1; 8). In mild SMA mouse models, CK-2127107 increased force output in response to sub-tetanic stimulation frequency (1). It is unknown whether CK-2127107 has a similar effect in severe SMA mouse models and consequently in SMA type 1 patients. Targeting troponin is limited to patients without denervation. Additionally, given that the release of calcium from troponin is delayed with CK-

2127107, the ability of muscle fibers to relax may be impaired with this drug leading to muscle stiffness.

Here, we provide insight into the mechanistic role of SMN in myogenesis, further emphasizing the importance of SMN expression in skeletal muscle. We found loss of myomaker and myomixer with SMN deficiency and demonstrated that ectopic expression of myomixer can act with a splicing modifier to synergistically increase myofiber number and size and improve motor function in the righting reflex test. This some of the first evidence that myoblast fusion can be targeted *in vivo*. Targeting myoblast fusion in SMA mice allows for the formation of multinucleated myofibers which ultimately improves motor function.

Targeting myoblast fusion through ectopic expression of fusion molecules may be beneficial for other diseases. For example, patients with Carey-Fineman-Ziter syndrome have mutations in *myomaker* leading to muscle hypotonia and general muscle weakness (6; 64). Ectopic expression of myomaker in these patients may improve myoblast fusion and reduce muscle weakness.

FUTURE DIRECTIONS

Given the data presented above, we propose the following future experiments:

1. To determine if *WDR33*, *CPSF1*, *THOC3*, and *Neurl2* can be targeted using AAV9-shRNA in SMA animal models to increase SMN protein levels and to examine whether knockdown of these genes prevents denervation and motor neuron loss, increases myofiber number and size, and improves motor function.

2. To screen for small molecules that block the interaction between Neurl2 and SMN. Our lab previously identified ML372, a small molecule that blocks the interaction between mindbomb-1 and SMN. When administered to SMA mice, ML372 increases SMN protein levels and improves the SMA phenotype. Similar experiments can be performed to identify a small molecule that blocks SMN ubiquitination by Neurl2. Furthermore, we can determine if a Neurl2 inhibitor can increase SMN protein levels and improve the disease phenotype in SMA mice.
3. To use ChIPseq to determine if SMN regulates the expression of other genes. We showed that SMN acts as a transcription factor and binds to the promoter region of MyoD. Given that SMN deficiency affects many organ systems, it is possible that SMN regulates the expression of additional genes which may provide further insight into the roles of SMN and ultimately how loss of SMN causes SMA pathology.
4. To further characterize AAV9-myomixer treated SMA mice by examining the number of motor neurons in the spinal cord, denervation of skeletal muscle, muscle atrophy, end plate current amplitude, and survival. Additionally, we can examine whether AAV9-myomaker improves the disease phenotype in SMA mice.
5. To identify small molecules that promote myoblast fusion and assess their ability to improve muscle histopathology and motor function in SMA mice. Given that we found increased myofiber size and number and improved motor

function in SMA mice with AAV9-myomixer, we expect that small molecules that promote myoblast fusion will have a similar effect.

CONCLUSION

SMA is a neuromuscular disease that is one of the leading causes of infant mortality. While current treatment options prolong survival, improvements in motor function are variable. Developing new treatments that either increase SMN protein or target peripheral tissues, such as skeletal muscle, can improve SMA disease outcomes. In these studies, we identified two novel ways to increase SMN protein levels—increasing the export of *SMN2* mRNA to the cytoplasm and blocking the E3 ligase *Neurl2* to prevent SMN ubiquitination and its subsequent degradation. Furthermore, we presented evidence that targeting myoblast fusion improves muscle histopathology and motor function of SMA mice and that AAV9-myomixer treatment can further enhance the effects of a *SMN2* splicing modifier. Ultimately, these novel targets and pathways can act in combination with current therapies for SMA patients and have the potential to modify disease outcomes.

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