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Quantitative Analysis of Novel Chemical and shRNA Based Methods to Increase Survival of Motor Neuron Protein Levels

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QUANTITATIVE ANALYSIS OF NOVEL CHEMICAL AND shRNA BASED METHODS TO INCREASE SURVIVAL OF MOTOR NEURON PROTEIN LEVELS

A Dissertation Presented

By

Matthew C. Evans

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Interdisciplinary Graduate Program

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Interdisciplinary Graduate Program

June 20th, 2011

Dedication

I dedicate this work and all my future endeavors to my daughter, Lily. I can only hope that she will be provided everything she needs to succeed and be happy.

I want to thank my parents, my wife, and my life-long friends for all of their continued support in everything I have set out to achieve. I would not be here without all of your help.

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My committee:

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Zheng Zheng Bao, Ph.D.

Neil Aronin, M.D.

And Michele Maxwell, Ph.D.

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Abstract

Spinal muscular atrophy (SMA) is an autosomal recessive neurodegenerative disorder that is the leading genetic cause of infantile death. SMA is caused by homozygous deletion or mutation of the survival of motor neuron 1 gene (*SMN1*). The *SMN2* gene is nearly identical to *SMN1*, however is alternatively spliced. The close relationship to *SMN1* results in *SMN2* being a very power genetic modifier of SMA disease severity and a target for therapies. In this study we attempt to characterize novel chemical compounds identified as potential activators of the *SMN2* gene. Additionally, we sought to determine the regulatory role individual HDAC proteins use to control expression of full length protein from the *SMN2* gene.

We used quantitative PCR to determine the effects of novel compounds and shRNA silencing of individual HDACs on the steady state levels of a *SMN2*-luciferase reporter transcripts. We determined that the compounds identified in multiple reporter high throughput screens increased SMN protein levels via transcriptional activation of the *SMN2* gene. Other compounds identified in the same screen functioned post-transcriptionally, possibly stabilizing the SMN protein itself by decreasing degradation. Furthermore, we determined that reduction of individual HDAC proteins was sufficient to increase SMN protein levels in a transgenic reporter system. Knockdown of class I HDAC proteins preferentially activated the reporter by increased promoter transcription.

Silencing of class II HDAC proteins maintained transcriptional activity; however silencing of HDAC 5 and 6 also appeared to enhance inclusion of an alternatively spliced exon. This collective work defines a quantitative RNA based protocol to determine mechanism of SMN reporter increase in response to any chosen treatment method. Additionally, this work highlights HDAC proteins 2 and 6 as excellent investigative targets. These data are important to the basic understanding of SMN expression regulation and the refinements of current therapeutic compounds as well as the development of novel SMA therapeutics.

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INTRODUCTION

Spinal Muscular Atrophy

Spinal Muscular Atrophy (SMA) is an autosomal recessive disease that is primarily described by the degeneration of the anterior horn cells of the spinal cord. SMA is the leading genetic cause of infantile death [1, 2]. It is classified into five clinical types delineated by age of onset and developmental milestones that the patient is capable of achieving. The types of SMA in decreasing order of severity are Type 0, acute infantile (Type I), chronic infantile (Type II), chronic juvenile (Type III), and adult-onset SMA (Type IV) [3-5]. There is no evidence of a variability of incidence within geographic or racial boundaries. However, there is a noteworthy preference of males over females born with SMA [6].

Type 0 SMA is classified as a severe form of Type I, where diagnosis is made late in pregnancy and little movement and breathing is present following birth. Acute infantile SMA, also known as Werdnig-Hoffmann disease, has an carrier frequency of 1 in 35 and an incidence of 1 in 10,000 live births [7]. Type I infants can appear healthy and normal at birth, however symptom onset is typically before 6 months of age and the life expectancy of the child is less than two years of age [8]. Skeletal muscle weakness progresses rapidly, resulting in lack of head control and weakened arm and leg movements. Disease severity is compounded by respiratory and feeding complications caused by difficulty swallowing and weakened bulbar muscles. Patients are never able to gain the

ability to sit without assistance or stand. Increased susceptibility to respiratory infection ultimately leads to death as the result of respiratory failure.

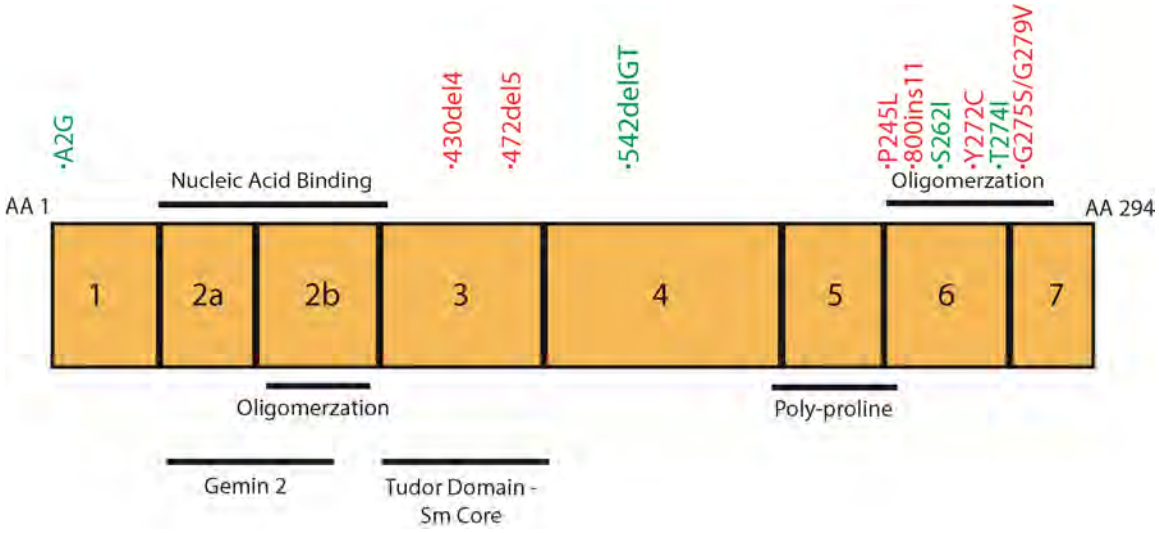
Patients with the less severe forms of SMA all present with similar symptoms as Type I SMA, where the severity of symptoms is inversely proportional to the clinical type. The incidence of Type II and III SMA is approximately 1 in 24,000 births. Chronic infantile SMA is typically first diagnosed prior to 18 months of age and patients are capable of head motor control. The ability to sit independently can be achieved but is significantly delayed. Type II SMA can also result in involuntary movement of the muscles in the arm, leg, and mouth. Patients suffer some level of respiratory and feeding complications, however to a lesser extent than Type I symptoms [5, 9, 10]. Chronic juvenile SMA patients and Type IV patients typically achieve all of the Type II milestones in addition to independent ambulatory movement at some point in their life. The lifespan of these two types can be considered of average length. The line separating Type III and Type IV SMA is highly dependent on age of onset [11]. In the most prevalent forms of SMA, type I through III, the causative genetic element has been linked to genomic locus 5q13 [12-15].

The Genetics of Spinal Muscular Atrophy

Spinal muscular atrophy is linked to homozygous deletion or mutation of the *Survival of Motor Neuron 1 (SMN1)* gene located on chromosome 5 [16]. In humans four genes in the region of chromosome 5q13 are maintained in 2 copies, the result of a large scale inverted gene duplication event [17]. The second copy of the *SMN1* is named *Survival of Motor Neuron 2 (SMN2)*. The protein product of the *SMN2* gene is unstable and incapable of compensating for the loss of *SMN1* [18]. Loss of both genes presumably results in early embryonic lethality. Mice only carry one copy of the *SMN* gene, most equivalent to human *SMN1,* and loss of mutation of this gene is embryonic lethal [19].

SMN1 produces a ubiquitously expressed 38 kDa, 294 amino acid protein localized to both the nucleus and cytoplasm [20-22]. *SMN1* produces a mRNA that encodes 9 exons (1, 2a, 2b, 3-8) with a translation termination codon in exon 7 [11, 16]. The 3-dimensional structure of SMN is unknown; however functional studies have revealed multiple distinct domains (Figure I.1). Exons 2a and 2b encompass a nucleic acid binding domain, a weak oligomerization domain, and the binding site for Gemin2 [23-25]. The carboxy-terminus of SMN there is a second stronger oligomerization domain as well as a poly-proline tract [23, 26]. In greater than 90 % of all SMA cases *SMN1* is homozygous deleted. In the other disease cases there are mutations or deletions within the *SMN1* gene. There is a wide spectrum of the clinical type of SMA associated

Figure 1.1. SMN Protein Domain Architecture. A diagram depicting the domain dimensional structure of the SMN protein. The exon number is indicated within each box. The box size roughly correlates to relative exon sizes. Specific domains are highlighted above and below the diagram. Disease causing mutations in the SMN protein are highlighted in red (Type I) and green (Type II/III). The C-T transition between *SMN1* and *SMN2* at the +6 position is not indicated as this change is translationally silent.


·A2G
Nucleic Acid Binding
·430del4
·472del5
·542delGT
·P245L
·800ins11
·S262I
·Y272C
·T274I
·G275S/G279V
Oligomerzation
AA 1
AA 294
1
2a
2b
3
4
5
6
7
Oligomerzation
Poly-proline
Gemin 2
Tudor Domain -
Sm Core

with each of these mutations. Many of the mutations fall into the Gemin2 protein binding domain, Tudor domain, and the exon 6 oligomerization domain [27]. The Tudor domain in exon 3 mediates protein-protein and possible SMN-RNA interactions [28]. In most cases of *SMN1* mutation disease severity is proportional to the degree the mutation disrupts domain function.

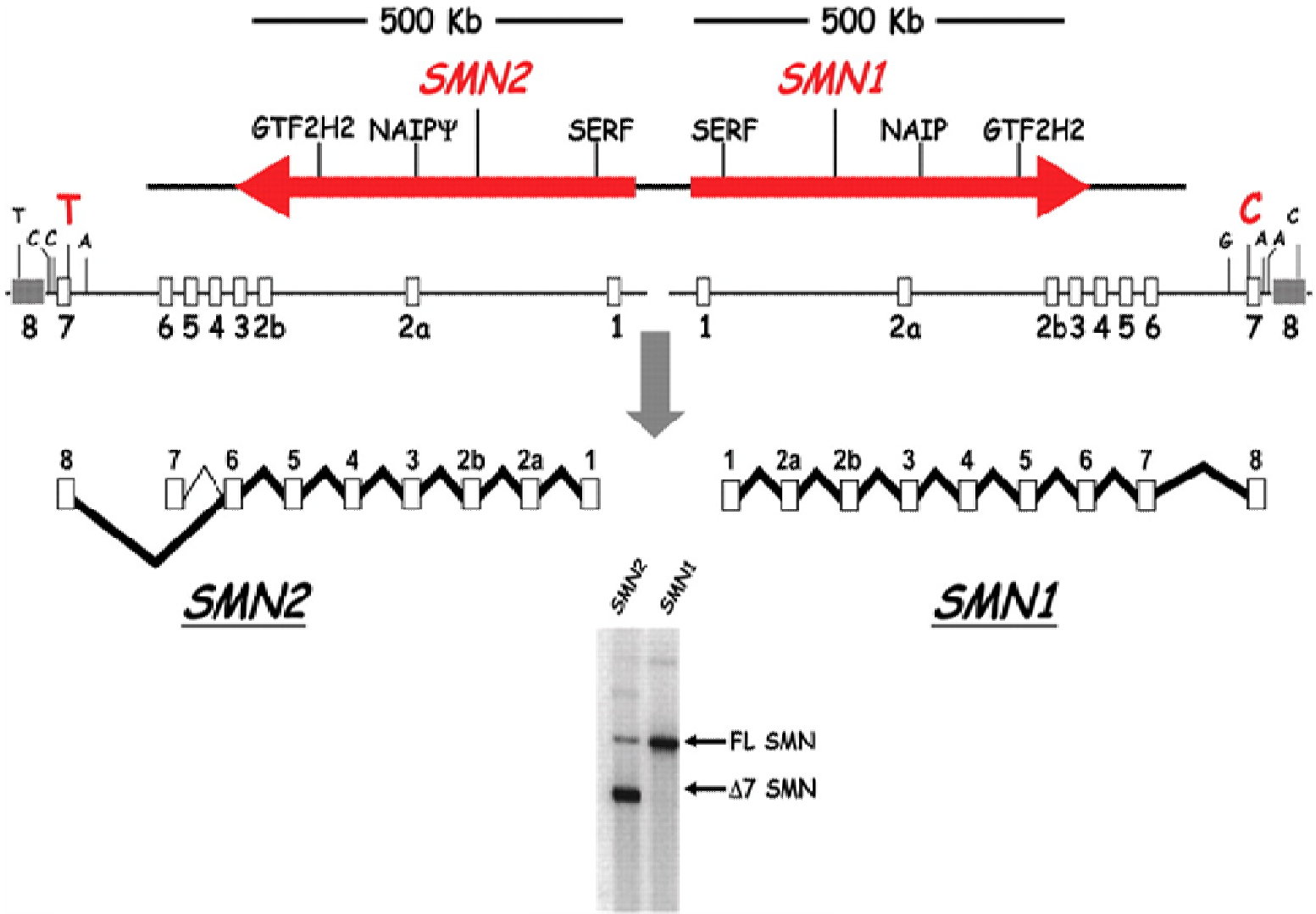
In the case of homozygous deletion of the *SMN1* gene, clinical type is inversely proportional to *SMN2* copy number [29]. The additive levels of appropriately spliced SMN mRNA from the *SMN2* genes can partially compensate for disruption of the *SMN1* locus. In mice it is even possible to completely rescue the SMA phenotype when 8 copies of the human *SMN2* gene are introduced [30]. Increased copy number in patients can also result in some alleviation of symptoms and reduction of the clinical type of SMA. It is for this reason that many believe that *SMN2* is the best drug target for disease therapy.

The *SMN2* gene is 99.9 % identical to *SMN1* starting in the promoter through the coding region [27, 29]. There are 5 silent mutations in the coding region that differentiate *SMN2* from *SMN1*. One of these mutations, a C to T transition at the +6 position of exon 7 drastically changing the mRNA transcribed from *SMN2* [31, 32]. 90% of transcripts derived from *SMN2* are alternatively spliced and lack exon 7. Only 10% of the *SMN2* mRNAs encode the same full length protein dominantly produced from the *SMN1* locus (Figure I.2). SMN exon 6 and 7 contains a critical oligomerization domain that when lacking creates an unstable protein that is rapidly degraded [18, 23, 33]. Therefore, despite

equivalently productive promoters, the *SMN2* gene produces significantly less stable full length product than *SMN1* [34].

Splicing of pre-mRNA is dependent on the proper identification of the 5' and 3' splice sites. A large multiprotein and RNA complex, the spliceosome, utilizes both cis- and trans-elements that act to enhance or silence the inclusion of exons in the final processed mRNA. These sequences are bound by multiple proteins, but a vast number fall into the classes of serine-arginine-rich (SR) proteins and heterogeneous nuclear ribonucleoproteins (hnRNP). The mutation at the +6 position of *SMN2* exon 7 is thought to concurrently disrupt an exonic splicing enhancer (ESE) and strengthen an exonic splicing silencer (ESS). In the first model, the +6 position of exon 7 is contained within a heptameric sequence that acts as an ESE [35, 36]. When a C is in this position the heptamer it acts as a strong binding site for the SR protein SF2/ASF that promotes exonic inclusion. Once bound to this sequence SF2/ASF most likely exerts its function by recruiting secondary splicing actors such as hnRNP G, SRp30c, and hnRNP Q. This complex is capable of binding and stabilizing U2AF at the intron 6 branch site and subsequently the U2 RNP, strengthening the weak 3' splice site [37-39]. Additionally, ASF/SF2 binds the U1 specific protein 70K, enhancing the primary detection of the 5' splice site of intron 7 by the U1 RNP[38]. The introduction of a T in the +6 position of exon 7 interrupts the ESE and any enhancement given by this site is lost.

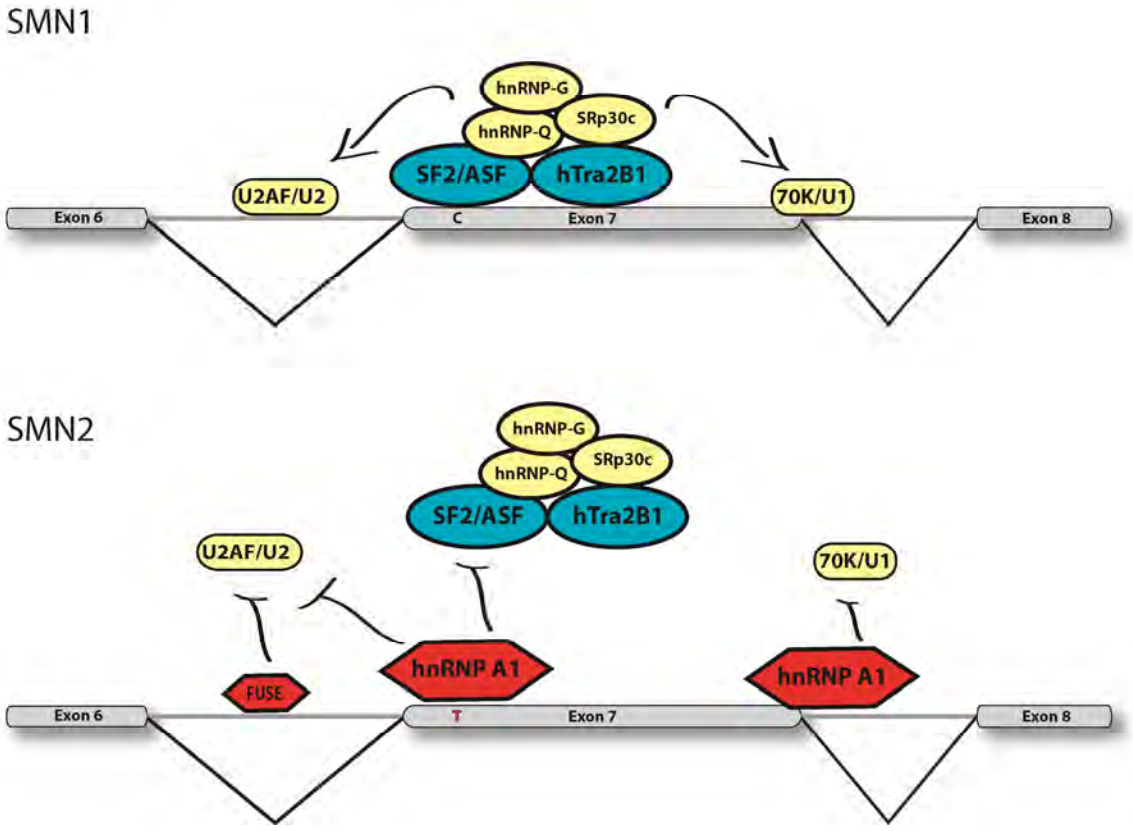
Figure I.2. Alternative Splicing of *SMN2*. A graphical representation of the effect a C to T mutation has on the splicing of *SMN2* exon 7 in comparison to *SMN1*. The 500 Kb genomic DNA region and inverted duplicate are represented. Pre-mRNA with relative sizes of introns and the predominant splicing pattern of *SMN1* and SMN2 are depicted. RT-PCR of *SMN1* and *SMN2* genes show the ratio of exon 7 included (top band) to excluded (bottom band). The majority of transcripts expressed from *SMN1* retain exon 7 while *SMN2* transcripts are inverted. Obtained from Lorson CL et al. Hum. Mol Genet. 2010.


500 Kb
500 Kb
SMN2
SMN1
GTF2H2
NAIPΨ
SERF
SERF
NAIP
GTF2H2
T
C
8 7 6 5 4 3 2b 2a 1
1 2a 2b 3 4 5 6 7 8
SMN2
SMN1
FL SMN
Δ7 SMN

An alternative model conversely suggests that the C to T transition creates an ESS. This site is bound by hnRNP A1 and the weak exon 7 is excluded. The presence of hnRNP A1 inhibits binding of the 5' splice site of intron 7and the intron 6 branch point by splicing RNPs [40, 41]. This is achieved through direct steric interference or secondary disruption of another known ESE downstream of the ESS bound by hTra2β. Additional inhibitory contribution by Far Upstream Element (FUSE) blocking the branch point in intron 6 further promotes exon 7 skipping (Figure I.3) [42]. While both of these models have supporting data, it is entirely likely that exon 7 splicing regulation is not mutually exclusive and is a blend of both scenarios among other influencing factors.

There are numerous secondary protein elements that have been shown to increase inclusion of exon 7 in addition to the ESS and ESE elements already mentioned. At the 3' terminus of exon 7, two negative elements exist that inhibit exonic inclusion. The first of these elements consists of a heptameric sequence, termed the 3' cluster, that when disrupted an increase in exon 7 inclusion is observed [40]. Downstream of this sequence a RNA secondary structural loop element, the terminal stem loop 2 (TSL2), when formed inhibits the proper splicing of the 5' splice site of intron 7[43]. Only in the context of *SMN2*, where the C to T mutation has already created a weak exon, does this inhibitory structure have a striking effect on exon 7 skipping.

Figure I.3 Summary of Protein Elements Controlling Exon 7 Splicing. A graphical representation of the region surrounding SMN exon 7 in *SMN1* and *SMN2.* A C in the +6 position of exon 7 (*SMN1)* is bound by the SR protein ASF/SF2 (blue) which promotes recognition of the branch point on intron 6 as well as the 3' splice site of exon 7. A T in the +6 position (*SMN2)* in bound by hnRNP A1 (red) which inhibits binding of the ASF/SF2 complex as well as the 3' splice site. FUSE/APB protein complex also binds and inhibits the recognition of intron 6 branch point.


SMN1
hnRNP-G
SRp30c
hnRNP-Q
SF2/ASF
hTra2B1
U2AF/U2
70K/U1
Exon 6
C
Exon 7
Exon 8
SMN2
hnRNP-G
SRp30c
hnRNP-Q
SF2/ASF
hTra2B1
U2AF/U2
70K/U1
FUSE
hnRNP A1
hnRNP A1
Exon 6
T
Exon 7
Exon 8

SMN Function

The 38 kDa SMN protein localizes to the nucleus and cytoplasm in all cell types [20]. In the nucleus SMN concentrates in structures rich in transcriptional and RNA processing machinery called gems (Gemini of Cajal bodies). These structures often overlap with Cajal bodies but are considered separate structures [44, 45]. There are typically 1-2 gems per nucleus. The number of gems observed in a population of cells correlates directly to SMN protein level and disease severity, with lower counts of gems in the most severe forms of SMA. The SMN protein is essential for gem formation; RNAi depletion results in the loss of detectable gem formations [46]. In the cytoplasm SMN is well distributed with concentration at the growth cone of axons [47-50].

Animal models of SMA have provided insight into the potential roles SMN plays in neuronal outgrowth, neuromuscular junction (NMJ) formation, and synaptic plasticity [51-57]. In the spinal cord SMN is most highly expressed in anterior horn cells, the cell that is also most sensitive to low SMN levels. An SMA model in zebrafish contains motor neurons that are shorter in length than wild-type animals and also display decreased arborization of the motor axon [58]. In contrast, data from the severe SMA mouse model suggest that neuronal pathfinding is normal; however there exists an inability to form and maintain a functional NMJ followed by denervation and cell death [55].

The most characterized SMN complex is responsible for assembly of mature spliceosomal small nuclear ribonucleoproteins (snRNP) [25, 59]. Splicing of cellular mRNAs is dependent on the U1-U7 snRNPs. Each of these snRNPs consists of a RNA element bound by Sm proteins and other proteins unique to each snRNP. Assembly of Sm proteins onto snRNAs will occur spontaneously *in vitro*, however the SMN complex mediates this process to ensure correct, specific, and efficient construction *in vivo[59]*. To do this, SMN binds Gemin proteins to identify snRNAs containing Sm sites and arranges Sm proteins on the RNA into a heptameric ring. The SMN complex consists of Gemin2-8, and UNRIP [25, 60-67]. Gemin2, 3, and 8 are directly bound to the SMN protein while Gemin4-7 bind via the other Gemin proteins. Gemin5 interaction with the complex is most susceptible to high salt concentrations during immunoprecipitation and is therefore considered the weakest [68]. Gemin5 is also rarely detected in gems. Gemin2, 3, and 5 knockouts in organisms from yeast to mouse result in lethality, suggesting the proper assembly of all components of the SMN complex are essential for survival.

Following loading of the Sm proteins on the snRNA and RNA 5' cap processing, the entire SMN-snRNP complex is imported in to the nucleus where the pairing dissolves and SMN returns to the cytoplasm to repeat the cycle. There is a correlation between the ability of the SMN complex to assemble snRNPs and disease severity [69]. Measurement of snRNP formation in the severe (type I) SMA mouse model reveals a significant reduction of mature

snRNPs [70]. There is an analogous, yet diminished, reduction of mature particle formation in models of less severe forms of SMA. Concurrently, the Sm binding capacity of low severity mutant SMN proteins is less than wild-type protein but greater than delta exon 7 SMN [28]. Mutation or disruption of the SMN C-terminal oligomerization domain severely diminishes Sm protein binding and as a consequence affects snRNP assembly. It is believed that multiple SMN proteins complex with the Gemins and interference with oligomerization inhibits the entire complex formation.

SMN has been implicated in multiple cellular processes. However, no specific function of SMN has been directly tied to SMA pathogenesis. The root of understanding the complexity of factors that cause SMA is determining why motor neurons are specifically sensitive to low SMN levels. Two models exist that attempt to explain this conundrum. First, it is possible that SMN function loss is universal to all cell types, however motor neurons, posses unique demands that render them most sensitive. Second, it is also plausible that SMN possess a function that is unique to motor neurons and essential for cell survival.

The first model of SMA pathogenesis predicts that low SMN levels result in a system wide reduction of snRNP formation. Low levels of mature snRNPs can result in a loss of the cell's ability to properly recognize splice sites in mRNA with high fidelity. It is then plausible that in motor neurons this would result in alternative splicing of mRNAs that are essential to cell survival. Exon expression microarrays have been used to detect aberrant splicing patterns of the spinal

cord and other tissues of a SMA mouse model [71, 72]. These studies observed alternative splicing of multiple exons across the genome in both pre- and post-symptomatic mice. With progression of the disease phenotype, there was a parallel increase in deviations from normal splicing patterns. There were also differences in alternatively spliced transcripts between tissue types, supporting the possibility of a motor neuron-specific splicing perturbation leading to cell death. However, it is unclear whether the changes in splicing preceded symptom onset, or whether the effects were a byproduct of the worsening symptoms.

A second and equally compelling hypothesis for SMA pathology theorizes that low SMN levels disrupt a motor neuron specific function of the protein, which results in cell death. SMN complexes in neurites of tissue culture neuronal-like cells show high coincidence with the Gemin proteins while maintaining nearly complete lack of association with the Sm proteins. This data suggests that SMN is imparting a function in the axon of neurons that is mutually exclusive from its better described role in snRNP formation.

Many of SMN's published interacting partners contain RNA Recognition Motifs (RRMs). Despite the lack of Sm protein in the SMN axonal complex, there may be a conserved function of RNP assembly and transport. Specifically, over expression or depletion of the SMN binding partner, hnRNP R, has been linked to increased or decreased neurite outgrowth respectively [73, 74]. Expression levels of hnRNP R have a proportional effect on neurite length in tissue culture. Mutant hnRNP R that lacks either the RRM or SMN interaction domains is

ineffective at modulating neurite length. Additional data suggests that SMN in partnership with hnRNP R forms a complex to transport β-actin mRNA into the neuron. In SMA primary motor neurons the concentration of hnRNP R protein in the axon is reduced and there is a comparable reduction of [48, 75, 76] mRNA and protein at the growth cone. The disruption of β-actin mRNA and protein in the growth cone offers some indication of a potential mechanism for motor neuron sensitivity to low SMN levels. Reduced β-actin protein and mRNA inhibits the cells ability to respond to external stimulus as well as disrupting cytoskeletal structure of the axon. The disruption of actin mRNA may not be a unique phenotype and it could suggest additional mRNAs with disrupted localization could exist in SMA.

Additionally, a neuron specific mRNA binding protein, HuD, binds SMN and actively co-transports into the axon [77, 78]. HuD is a part of a family of mRNA binding proteins expressed in post-mitotic neurons that bind the 3' untranslated region and regulate stability and translation [79, 80]. HuD knockout mouse models have motor defects. Interestingly, one identified target of HuD is β-actin mRNA [81]. Similar to hnRNP R, HuD localization into the axon is disrupted by low SMN levels. In the same study, following SMN knockdown in primary motor neurons the gross localization of poly-A mRNA was reduced in the axon [77].

The SMN protein has also recently been demonstrated to bind the α-subunit of the ER-Golgi coatamer complex [82]. Coatomer is a multiprotein

complex localized to the cytoplasm and is required for COPI vesicle budding. SMN and α-COP co-localize in the axons of primary mouse hippocampal neurons and in chick retinal neurons. Furthermore, fluorescently tagged SMN and α-COP co-traffic within the neurites of cultured differentiated neurite like cells, SH-SY5Y. α-COP depletion also significantly reduced the localization of SMN to the leading edge of the F-actin filipodia. SMN was initially described to traffic in both anterograde and retrograde directions in primary hippocampal neurons prior to the identification of this interaction [83]. The movement was consistent with the fast rate of transport of microtubule based motors.

It is plausible to theorize that the coatamer-SMN complex in conjunction with RNA binding proteins, such as hnRNP R and HuD, identify and traffic mRNAs into the axon of motor neuron. HuD previously reported role in mRNA stability and translation may also suggest a potential added complexity of the role of the SMN-mRNA axonal complex. The complex may stabilize mRNA in the growth cone and delay translation until necessary for cellular response to environmental stimulus. In SMA, low SMN levels may disrupt the formation of the larger complex, resulting in the impaired transport of essential mRNAs to the growing tip of the axon. This defect could handicap the axons ability to adapt and respond to stimulus and in the case of SMA lead to neuronal degeneration.

There is compelling evidence for both theories of motor neuron sensitivity to low SMN protein levels. The reality of SMA pathogenesis most likely lies between both models. There are proven defects in splicing of neuronal specific

factors in SMA, and sufficient evidence in support of a mRNA transport function of SMN. Low SMN levels potential disrupts mRNA biology at multiple levels including processing, translocation, and translation. While it is important to understand SMA pathology to treat the disease, it is just as critical to explore any possible means to treat the disease based on current data.

Spinal Muscular Atrophy Treatment

Despite an incomplete understanding of SMA pathology, viable therapeutic targets have been identified. There are many distinct therapeutic strategies to treat SMA. Gene replacement therapy attempts to directly introduce addition copies of *SMN1* corrects the disease causing mutation or loss of the gene. Other approaches have targeted the neuron with neuroprotective compounds and more recently, neuronal replacement by stem cell therapy in mice has been investigated. However, the most utilized strategy is to increase the expression from the *SMN2* locus or to modify splicing of *SMN2* mRNA. As discussed, *SMN2* gene copy number, or in this case expression, is an excellent modifier of SMA disease severity. Table 1 contains a summary of compounds that have been or are currently in clinical trials for SMA.

Gavrilina [84] *et al.* showed that neuronal expression of SMN alone was sufficient to significantly increase the survival of severe SMA mouse models [84]. Post-natal delivery of the *SMN1* gene by an self complimentary adeno-associated virus (scAAV)-9 vector successfully rescued the severe SMA mouse [85]. The scAAV-9 vector crosses the blood-brain barrier and transduces a high percentage of motor neurons. This same study observed that there was a therapeutic window for scAAV-9 treatment. There was an inverse correlation to the effectiveness of treatment and post-natal time. It is unclear how this time period translates into SMA patients. This may complicate any treatment

Table I.1. Previous SMA Clinical Trials. A brief summary of previous SMA clinical trials highlighting the proposed mechanism of action in addition to the outcome of the studies.

Drug Name	Mechanism of Action	Number of Trials	Type (and #) of Patients	Results	References
Albuterol	β-Andrenoreceptor agonist	1	Type II/III (13)	↑ Strength	[86]
Gabapentin	Neuroprotection	2	Type II/III (204)	Negative / ↑ strength	[87]
Riluzole	Neuroprotection	2	Type I (51)	Inconclusive	[88, 89]
Phenylbutyrate	*SMN2* activation	3	Type I/II (124)	Negative/ ↑ mRNA and strength	[90, 91]
Acetyl-L-Carnitine	Neuroprotection	1	Type I/II (110)	Negative	[92]
Creatine	Neuroprotection	1	Type II/III (504)	Negative	[93]
Hydroxyurea	*SMN2* activation	2	Type I/II/III (>33)	Negative	[94]
Valproic Acid	*SMN2* activation	2	Type I-IV (49)	↑ strength/mRNA	[95, 96]
Valproic Acid / Acetyl-L-Carnitine	*SMN2* activation / Neuroprotection	1	Type II/III (90)	Negative	[97]

considered, as in most cases SMA symptoms are not present at birth. Any delay of diagnosis may reduce the efficacy of disease rescue. The use of a viral delivery method is further complicated by potential immune response by the patient and possible secondary infection if immunosuppresion is necessary. However, these data provide compelling evidence that gene therapy may be highly effective at treating SMA.

Neuroprotective chemical compounds have been used with modest success in treatment of other motor neuron degenerative diseases including Amyotrophic Lateral Sclerosis (ALS) [98-102]. The neuroprotective compounds slow and eventually reverses the death of the motor neuron. Gabapentin, Riluzole, and Acetyl-L-carnitine are all neuroprotective compounds that have been used in current or previous SMA clinical trials [87, 89, 103]. Both Gabapentin and Riluzole control the release of glutamate, reducing the excitability of the motor neuron. Mixed results were observed in two Gabapentin studies, the more successful of the studies showing only low to moderate arm and lower body strength increases [87]. Riluzole showed promising results increasing survival in a mouse model of SMA [103]. In a patient trial, the drug also appeared to increase survival in a small number of patients. However, conclusive results from the study were limited by small sample size [89]. It is not clear the contribution that this class of drugs will have on long term SMA treatment. The moderate improvements observed may not be sufficient alone for treatment, but may provide a valuable added benefit when used in combination

with other therapeutic strategies. One such clinical trial has completed with concurrent treatment with the HDAC inhibitor valproic acid and the neuroprotective compound acetyl-L-carnitine. Unfortunately, the clinical trial resulted in no functional benefit following 6 months of treatment [97].

There are numerous opportunities to increase full length protein produced from the *SMN2* locus. Enhancing transcription from the *SMN2* promoter, increases the total amount of SMN mRNA, both exon 7 included and excluded transcripts. Post-transcriptionally, SMN protein can be increased by stabilizing *SMN2* mRNA, modification of exon 7 alternative splicing, enhanced translation of full length mRNA, or stabilization of SMN protein. Increasing the half-life of SMN mRNA and enhanced translation produces more protein per mRNA transcribed. Modulation of exon 7 splicing is potentially the most attractive method at increasing full length protein levels. *SMN2* is as transcriptionally active as *SMN1*, improving the splicing ratio to increase inclusion of exon 7 could be considered equivalent to *SMN1* gene replacement [34].

RNA based therapies are currently being investigated as a successful method to correct the exon 7 splicing defect. Antisense oligonucleotides (ASO) target sequences within and flanking exon 7 to promote mRNA inclusion. Initial efforts covalently coupled arginine and serine (SR) peptides to the complementary sequence [104]. The SR peptide presumably acted as an artificial ESS to recruit SR-proteins to encourage exon 7 inclusion. These synthetic binding sites successfully increased exon 7 inclusion, however the rate

of inclusion is inefficient. A more stable solution combined the SR-ASO with a U7-snRNA expression vector. By including the U7 backbone, the SR-ASO is incorporated into snRNPs and better promoted inclusion [105]. U7 modification of the SR-ASO is very effective at ameliorating symptoms of the severe SMA mouse when delivered using an adenovirus delivery system similar to the scAAV-9 expression of *SMN1[106].*

An alternative to artificially creating an ESE is to use the oligo to mask intronic splicing silencer sequences. This strategy was very potent, promoting exon 7 inclusion at very low concentrations in primary fibroblasts. The most effective target sequences is the ISS sequences flanking exon 7 [107, 108]. Binding to the mRNA sequence competes for binding with the negative element hnRNP A1. Delivery of similar ASOs into SMA mice significantly increases full length SMN protein in some tissues, however without significantly increasing survival [109]. Increased SMN protein in neuronal tissue is essential for increased survival. One drawback of an ASO treatment strategy is that the oligos do not cross the blood brain barrier, making it very difficult to increase neuronal SMN protein levels. This problem has been circumvented by using intracerebroventricular (ICV) delivery of the oligos directly to the nervous system of SMA mice [110]. Oligos were also demonstrated to be successfully incorporated into non-human primate neurons using similar ICV delivery. This method successfully modifies the splicing of exon 7 in the central nervous system of severe SMA mice and has a significant impact on survival. In a similar study

in type III SMA mice, the ASOs used were very stable and were found to still be present 6 months following the initial treatment. It may be possible to treat SMA patients using ASO and improve disease symptoms with a limited number of treatments. However, more investigation is needed to determine the immune response to ICV injected oligos and the associated safety. Inflammation was observed in some studies in the spinal cord of treatment mice.

Histone deacetylase inhibitors (HDAC) are a class of compounds effective at increasing SMN protein levels. The role of HDACs and HDAC inhibitors will be explored further and more extensively review in the second chapter. Briefly, HDAC inhibitors, phenylbutyrate, hydroxyurea, and valproic acid have all been tested in clinical trials with moderate strength benefit to patients without any enhanced survival. Newer generation HDAC inhibitors have been generated that are proving to be more potent at increasing SMN protein levels. HDAC inhibition is predicted to increase transcription from the *SMN2* promoter as well as increasing the inclusion of exon 7 in the processed mRNA via increased levels of SMN splicing factors.

Preface

Work in this section was supplemented by Jonathan Cherry Ph.D. a current assistant professor at the Indiana University School of Medicine. Portions of this work have been submitted for publication and are currently under revision.

Xiao J, Marugan JJ, Zheng W, Titus S, Southall N, Cherry JJ, **Evans MC**, Androphy EJ, Austin CP. Discovery, Synthesis and Biological Evaluation of Arylpiperidines as Novel SMN Protein Modulators. J Med Chem. 2011 Aug 19. [Epub ahead of print]

Cherry JJ, **Evans MC**, Androphy EJ. Development of an improved cellular reporter assay for SMN protein levels. [submitted JBSC, August 2011]

Cherry JJ, **Evans MC**, Ni J, Ebert A, Osman E, Svensden CN, Lorson CL, Cuny G, Glicksman M, Stein R, Androphy EJ. Identification of small molecules that increase the expression of SMN2 using a new high-throughput screening assay. 2011

Chapter I:

Confirmation and Validation of SMN Activating HTS drug targets

There is a constant need to search for new therapeutic strategies while current treatments are refined. When screening for compounds that activate *SMN2*, two different strategies have been employed previously. Both use artificial plasmids that combine a transgenic protein that is readily detectable with an endogenous element of *SMN2.* One method measures transcriptional activation by placing the reporter gene under the control of the endogenous *SMN2* promoter. If any given compound results in enhanced transcription of the *SMN2* promoter, an increase of the total signal of the reporter protein such as luciferase or bacterial β-lactamase is observed. Similarly, another strategy utilizes a *SMN2* splicing cassette to screen for compounds that modulate SMN exon 7 splicing.

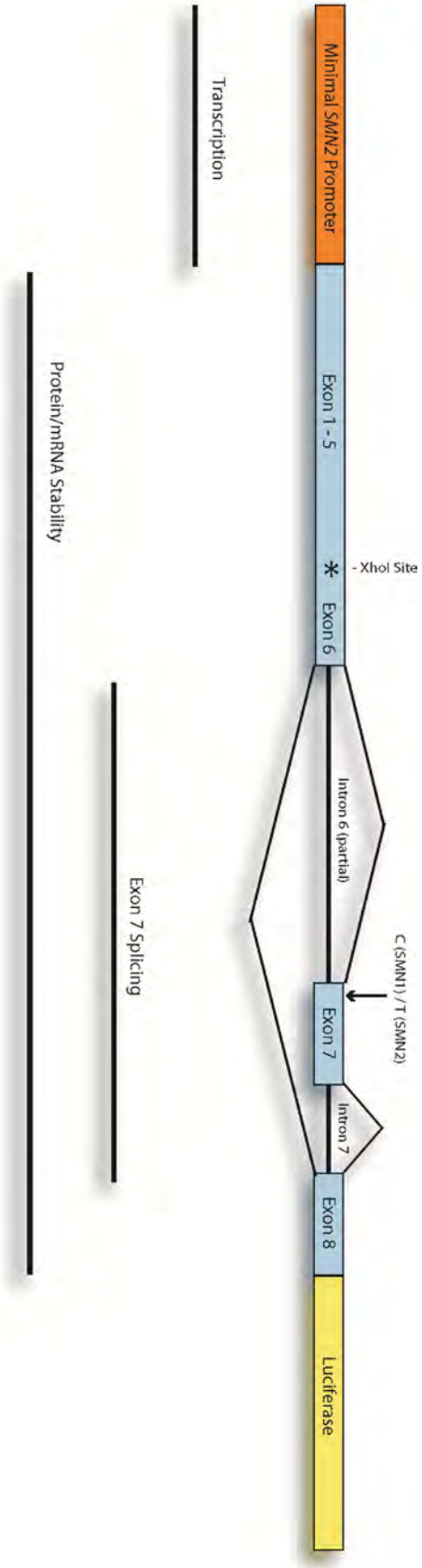
SMN splicing cassettes use the genomic sequence for the c-terminus of either *SMN1* or *SMN2* beginning at exon 6 through the 5' end of exon 8 [111]. The last exon is replaced by a reporter gene early in the sequence. The entire fusion protein is under the control of the abundantly expressing Cytomegalovirus (CMV) promoter. Alternative splicing of exon 7 results in a frameshift downstream and that prevents the translation of the reporter gene as a result of early termination. Reporter signal is only detected if exon 7 is included in the final product.

These two strategies have been successful at identifying new therapeutic compounds, as well as dissecting the mechanics of SMN expression and exon 7 splicing. Drug discovery with either of the two constructs limits the pool of

compounds that have the ability to increase SMN protein levels. The most potent compounds not only increase *SMN2* transcription, but also reduce skipping of exon 7. These two assays completely neglect any compounds that act post-translationally to stabilize SMN because the entire protein is not expressed in either reporter. Moreover, Stable maintenance of the constructs is subject to silencing over time as well as possible negative effects of random integration. It is possible to perform experiments with transient expression, however the normalization between experiments can be difficult and data can be dependent on transfection efficiency.

Dr. Jonathan Cherry constructed an episomal plasmid that combines the utility of each of these systems. The reporter is expressed by the *SMN2* essential 3.4 kb promoter followed by exons 1-5 cDNA cloned in frame with the *SMN2* splicing cassette described previously [112]. In this system the entire full length SMN protein is translated and fused to firefly luciferase when exon 7 is included in the mature mRNA (Figure 1.1). There is also a CMV driven renilla luciferase gene contained in the 20.1 kB pCEP4 episomal based plasmid that can be used for experimental normalization. With this reporter system it is possible to identify increase in SMN levels regardless of the mechanisms utilized. The reporter attempts to mimic the endogenous gene and protein as closely as possible. The reporter will respond to changes in transcription, splicing, translation, as well as mRNA and protein stability (Figure 1.1).

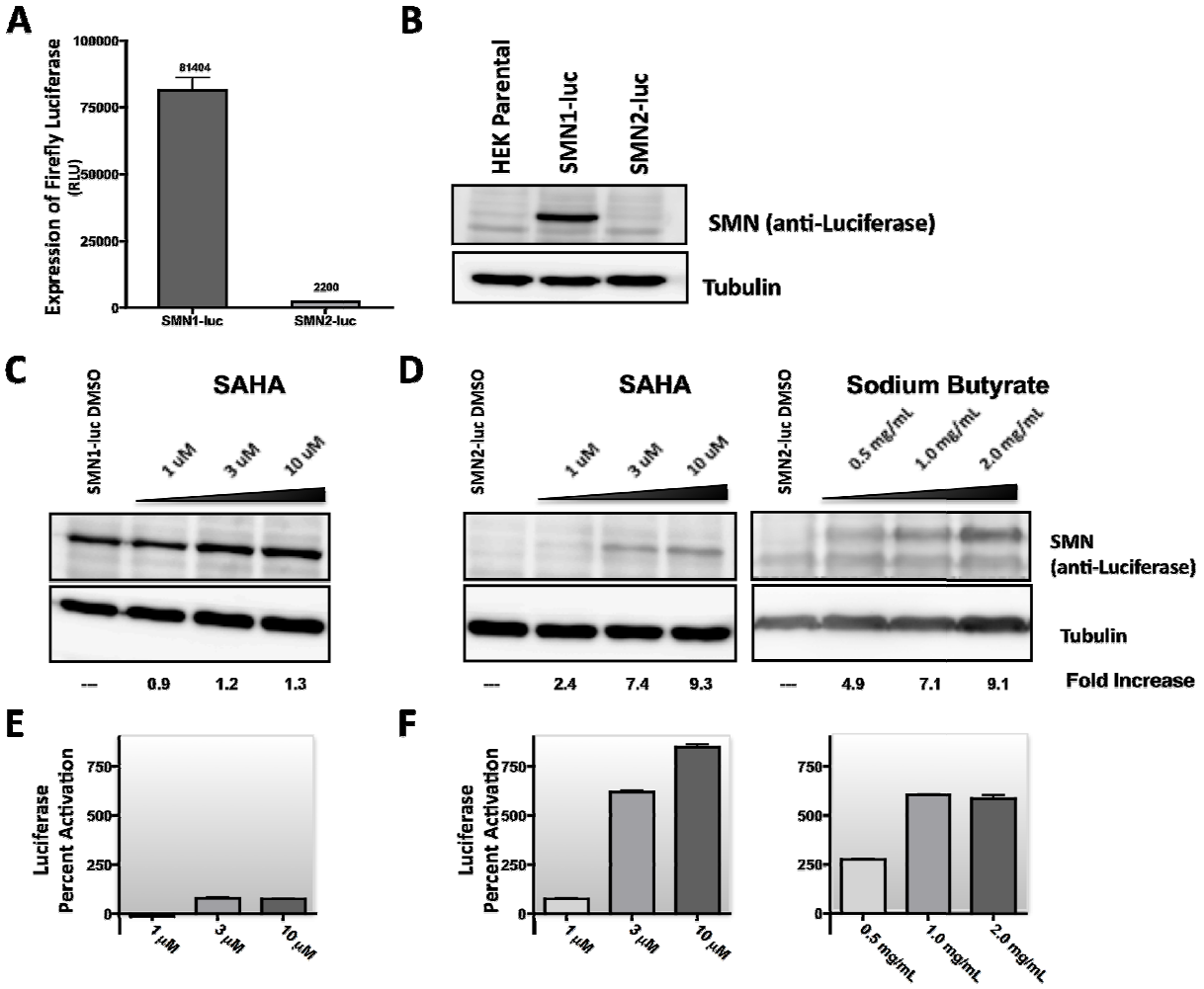
Figure 1.1. Newest Generation *SMN2* Episomal Reporter. Schematic depicting the layout of a *SMN2* reporter expressing full length SMN fused to firefly luciferase under the control of the 3.4 kB *SMN2* promoter.


Minimal SMN2 Promoter
Exon 1 - 5
- XhoI Site
Exon 6
Intron 6 (partial)
C (SMN1) / T (SMN2)
Exon 7
Intron 7
Exon 8
Luciferase
Transcription
Protein/mRNA Stability
Exon 7 Splicing

The plasmid was introduced and maintained in the human embryonic kidney 293 T (HEK293T) cell line. This strain was originally generated from adenovirus transformed kidney cells in culture. Kidney cultures are not uniform in tissue type and it is believed that HEK293 cells are in fact of neuronal origin. There are substantial neuronal specific genes that are expressed in this cell type [113]. Therefore, HEK293T cells are an excellent human cell line to test SMN activating treatments and are exceptionally easy to grow at a scale necessary for high throughput applications. HEK293T cells were transfected with either *SMN1* or *SMN2* episomal reporter constructs and stable cell lines were established. There are a limited number of nucleotides that differ between the *SMN2* and *SMN1* promoters as well as intronic sequences. These changes were conserved throughout the respective reporter constructs.

Stable reporter cell lines were selected based on luciferase fusion expression levels. The *SMN1*-luciferase reporter cell line was observed to have luciferase activity levels approximately 40 fold greater than the *SMN2*-luciferase reporter cell line (Figure 1.2 A, B). The response of the *SMN2*-luciferase reporter cell line was tested at multiple drug concentrations of suberoylanilide hydroxamic acid (SAHA) and sodium butyrate (Figure 1.2 D, F). Treatment of the *SMN1*-luciferase cell line with SAHA did not result in an increase of luciferase fusion protein. The reporter cell lines responded robustly to known SMN activating compounds and therefore should be a viable tool for SMA therapy screening [90, 114, 115].

Figure 1.2. Validation of the *SMN1* and *SMN2* episomal reporters. Western Blot and Luciferase data validating the novel *SMN1* and *SMN2* reporter, A3 and B3 respectively. (A) Relative light units of the SMN-Luciferase fusion protein in A3 and B3 reporter cell under normal conditions. (B) Western-Blot data of the two cells lines using anti-luciferase antibody to identify the fusion protein. (C) SAHA treatment of A3 cells (D) SAHA and sodium butyrate treatment of B3 cells (E, F) Western Blot data for the same cells used in C and D. Data and figure collected by Jonathan Cherry, Ph.D. (submitted data).


A
Expression of Firefly Luciferase (RLU)
100000
75000
50000
25000
0
81404
2200
SMN1-luc
SMN2-luc
B
HEK Parental
SMN1-luc
SMN2-luc
SMN (anti-Luciferase)
Tubulin
C
SMN1-luc DMSO
SAHA
1 uM
3 uM
10 uM
--- 0.9 1.2 1.3
D
SMN2-luc DMSO
SAHA
1 uM
3 uM
10 uM
SMN2-luc DMSO
Sodium Butyrate
0.5 mg/mL
1.0 mg/mL
2.0 mg/mL
SMN (anti-Luciferase)
Tubulin
Fold Increase
--- 2.4 7.4 9.3
--- 4.9 7.1 9.1
E
Luciferase Percent Activation
750
500
250
0
1 μM
3 μM
10 μM
F
Luciferase Percent Activation
750
500
250
0
1 μM
3 μM
10 μM
0.5 mg/mL
1.0 mg/mL
2.0 mg/mL

Both the *SMN1* and *SMN2* reporter cell lines were used in high throughput screens at multiple collaborating sites to identify and screen approximately 1 million compounds for SMN activation. 5 compounds and chemical analogs were identified to increase luciferase activity while maintaining specificity and properties amicable to drug development. Three of the HTS hits were confirmed to enhance *SMN2* transcription while the remainder of the compounds utilized mechanisms of action downstream of translation. The drugs were tested in primary SMA patient fibroblasts and confirmed to maintain activity. The development of the episomal reporter yielded many drug targets that will be investigated further as potential SMA therapies.

Materials and Methods

Tissue Culture

Cells were incubated at 37°C with 5% CO_2. HEK293T cells were grown in D-MEM (Gibco, #11995) with 10% fetal bovine serum (Sigma) and 1x Pen-Strep (Gibco, #15140). Reporter cells were grown in the same medium supplemented with 200 ug/mL Hygromycin B (Invitrogen, #10687-010). Primary human SMA patient fibroblasts, 3813, were grown in D-MEM with 15% fetal bovine serum and 1x Pen-Strep.

PCR and RT-PCR

For analysis of SMN-luciferase fusion, compounds were tested at three concentrations that display maximal activity in the luciferase assay. Reporter cells were plated at 750,000 cells in a 6 well dish. 24 hours after plating drug was added to the well and cells were harvested after an additional 24 hours. Cells were harvested by trypsinization, neutralized with trypsin inhibitor, and washed. 10% of each cell pellet resuspended and plated into three wells of a 96-well dish and was used to analyze for luciferase activity with the DualGlo luciferase assay). RNA was isolated from cells using Trizol Reagent (Invitrogen, #15596-026). cDNA was generated using the Improm-II Reverse Transcription System (Promega, #A3801). The forward primer of the SMN amplicon binds the exon 5-6 junction and uses an XhoI site used to create the reporter in order to amplify only reporter transcripts. The primer pair of SMN Xho-Forward and

Luciferase-reverse amplifies both exon 7 included and excluded transcripts. SMN-Xho-Forward and SMN Exon 7-Reverse is specific to exon 7 included transcript. For a normalization standard we amplified a region within the glyceraldehydes-3-phosphate dehydrogenase (GAPDH) transcript.

All reactions were incubated at the same Tm. The conditions were as follows, 5 minutes at 95 ^{o}C followed by a cycle of 30 seconds at ^{95} ^{o}C, 30 seconds at 60 ^{o}C, and 1 minute at 72 ^{o}C. The number of cycles for GAPDH were 25 while SMN reporter transcripts required 32.

Primer Name	Sequence
Exon 5/XhoI – Forward	CATTTCCTTCTGGACCACTCGAG
Exon 5 – Forward	GCCTCCATTTCCTTCTGGACCA
Exon 5/6 – Forward	CATTTCCTTCTGGACCAATAATTCCC
Luciferase-Reverse	ATAGCTTCTGCCAACCGAACGG
Exon 7 – Reverse	TAAGGAATGTGAGCACCTTCCTTC
Exon 8 – Reverse	CTACAACACCCTTCTCACAGG
GAPDH-Forward	ACCACAGTCCATGCCATCAC
GAPDH-Reverse	TCCACCACCCTGTTGCTGTA

Quantitative PCR (qPCR)

qPCR was performed as described in the protocol for iQ SybrGreen Supermix (BioRad, #170-8882) in an Eppendorf Mastercycler ep Realplex 4 real-time PCR machine. Reactions were incubated for a 10 minute 94 ^{o}C hot start followed by 45 cycles of the following: 94 ^{o}C for 45 second, 60 ^{o}C for 15 seconds, 72 ^{o}C for 45 seconds. The Pfaffl method was used to determine the change in transcript levels relative to the DMSO and normalized to glyceraldehyde-3-

phosphate dehydrogenase (GAPDH). A 5 point standard curve for each primer set was included on every plate to determine experimental deviation in primer efficiency. Error bars in graphs depict SEM.

Gem Count Assay

For gem count assays, 3,000 primary 3813 fibroblasts, were plated in each well of an 8 chambered slide (Nunc, #S6815). 24 hours after plating drug or DMSO carrier were added. Media and drug were changed every 24 hours for 72 additional hours. Prior to fixation cells were washed twice with cold phosphate buffered saline (PBS). The cells were then covered with a 1:1 fresh cold methanol:acetone mixture for 5 minutes. Slides were again washed 3X with PBS + .2% Triton X-100 (PBS-T). Slides were blocked overnight at 4 ^{o}C in PBS-T + 2% BSA. Primary antibody (Anti-SMN 4B7) was diluted 1:15 in blocking buffer and added to the cells for 3 hours at room temperature. Cells were washed 5X with PBS-T. Secondary Alexa-488 Goat Anti-Mouse was diluted 1:1000 in blocking buffer and added to the cells for 1 hour at room temperature followed by 5x PBS-T washes. Cells were mounted with prolong Gold with DAPI DNA stain (Invitrogen Cat# P36931).

Results

The *SMN2*-luciferase reporter cells were active in response to the HDAC inhibitors SAHA and sodium butyrate. As discussed, the reporter is highly versatile and effective because of the capability of identifying a diverse population of SMN activating compounds. However, the ability to distinguish between the potential mechanisms of action is critical to validate and develop any treatment. One challenge posed by the reporter cell lines is that the episomal construct is maintained in the HEK293T cell line which is homozygous for both *SMN1* and *SMN2.* Endogenous HEK293T SMN protein does not interfere with the detection of SMN-luciferase activity. However, endogenous messenger RNA (mRNA), can easily contaminate specific identification of reporter mRNA because of the near exact sequence similarity. It is critical not only to measure the levels of total reporter mRNA, but also to accurately track SMN exon 7 inclusion.

Unique sequences found in the reporter were targeted to discriminate against endogenous mRNA species. The most convenient reporter specific feature to exploit was the firefly luciferase gene. However, exclusive RT-PCR amplification of the luciferase transcript would only measure increases in transcription and would neglect any modification of exon 7 splicing. Upstream of luciferase, the only other reporter specific feature was an XhoI restriction enzyme site used to clone the exons 1-5 cDNA to exon 6 of the spicing cassette. This

primer was capable of binding to endogenous exon 5 sequences; however extension was unable to proceed without specific annealing of the XhoI complementary sequence. A second primer was designed using sequence within exon 7 in combination with the XhoI start site to detect only transcripts that included exon 7 for use in SYBR green based qPCR. A summary of these primers and expected products is illustrated in Figure 1.3.

The specificity of the primers described were tested on wild-type HEK293T and *SMN2*-luciferase reporter cell mRNA (Figure 1.4). Primers that amplified exon 5 or the exon 5/6 junction through exon 8 were also tested. Primer pairs specific to endogenous HEK293T SMN mRNA efficiently amplified appropriate products (Figure 1.4 lanes 1, 2, 4, 5). For primer products spanning exon 7 a doublet was observed. All other primer sets that specifically amplified reporter transcripts were void of any PCR product (lanes 3, 6-9). The same set of primers were tested on B3 reporter cell RNA extracts (Figure 1.4 Bottom Panel) Primer sets Exon 5 Forward - Exon 8 Reverse and Exon 5/6 Forward – Exon 8 Reverse, specific to endogenous mRNA, appeared to be less efficient in the reporter cells than in wild-type HEK293T cells (lanes 2,5). This is likely due to quenching of the forward primer by reporter mRNA transcripts, limiting the direct amplification of endogenous mRNAs. Primer sets specific to reporter transcripts were positive for PCR products from the reporter lysates. In contrast, reactions amplifying exon 5 to luciferase in reporter lysates the major product was the faster migrating delta exon 7 transcript.

Figure 1.3. PCR and RT-PCR Primer Pairs. The locations of primers used to amplify genomic and reporter mRNA. Primer pairs that span both intron 6 and 7 amplify both exon 7 included and excluded transcripts (bottom pair). Any primer that does not anneal to reporter derived transcripts is noted by (Genomic Only).

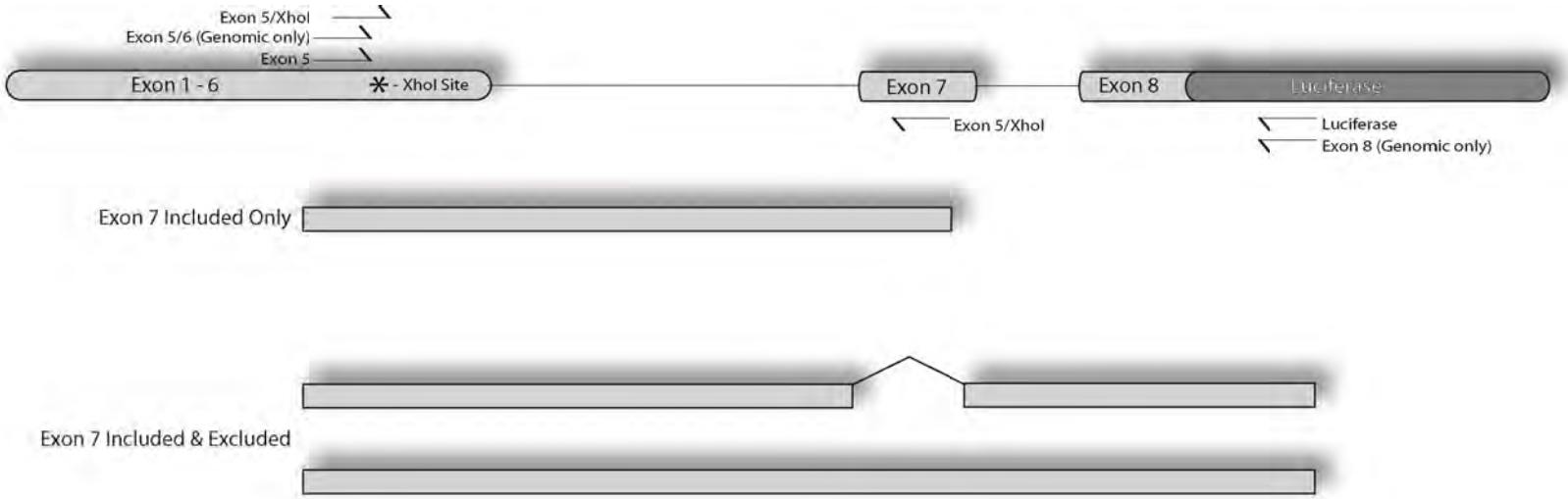
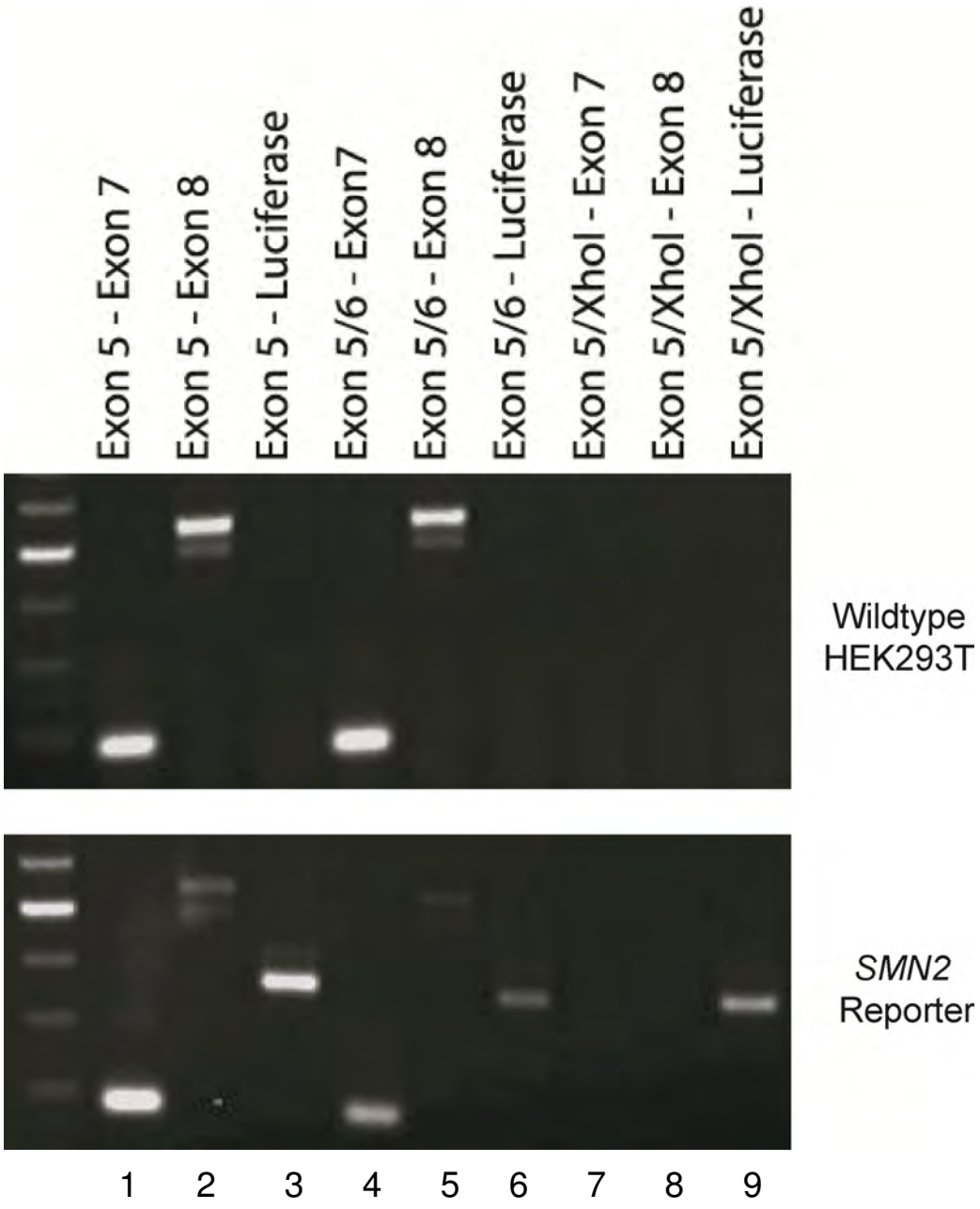

Exon 5/XhoI
Exon 5/6 (Genomic only)
Exon 5
Exon 1 - 6
- XhoI Site
Exon 7
Exon 8
Luciferase
Exon 5/XhoI
Luciferase
Exon 8 (Genomic only)
Exon 7 Included Only
Exon 7 Included & Excluded

Figure 1.4. RT-PCR of Endogenous and Reporter Transcripts. Top Panel: Wild-type HEK293T cells, lanes 1,2,4,5 amplify products from genomic specific primers of expected size. All reactions designed to amplify reporter transcripts are negative. Bottom Panel: Amplification of both genomic and reporter transcripts with appropriate primer pairs.


Exon 5 - Exon 7
Exon 5 - Exon 8
Exon 5 - Luciferase
Exon 5/6 - Exon7
Exon 5/6 - Exon 8
Exon 5/6 - Luciferase
Exon 5/XhoI - Exon 7
Exon 5/XhoI - Exon 8
Exon 5/XhoI - Luciferase
Wildtype HEK293T
SMN2 Reporter
1 2 3 4 5 6 7 8 9

The *SMN2* reporter yielded the appropriate ratio of exon 7 included to skipped mRNA. Quantitative RT-PCR was done to quantify the exact splicing efficiency of the reporter transcripts. To begin the primer efficiency of the primer sets Exon 5-XhoI Forward / Exon 7 Reverse, Exon5-XhoI Forward / Luciferase Reverse, and GAPDH were determined for both *SMN1* and *SMN2* reporter cells. The primer set's efficiencies were determined based on a 5 point dilution curve for each. The efficiencies were consistent between reporters and were 95%, 95%, and 85% respectively. Additionally, a melting point curve was performed for every reaction to confirm the product purity. It is possible to determine the fold difference between total transcripts and exon 7 included transcripts and subsequently calculate the percent inclusion of exon 7. The *SMN1* reporter cells expressed 87% of transcripts containing exon 7 while *SMN2* reporter cells produced 8% of transcripts including exon 7.

The increases of the luciferase activity in response to the HDAC inhibitors were validated by measuring the levels of both the total and exon 7 included mRNAs. To begin, we treated *SMN2* B3 reporter cells with HDAC inhibitor compounds valproic acid, aclarubicin, and SAHA. Three concentrations within the active range of each drug were added to the cells and mRNA was harvested and analyzed for changes in total and full length transcripts. Comparison of the mRNA inductions between the two populations of mRNAs revealed potential mechanisms the drug use to increase SMN luciferase activity.

As previously discussed, there are multiple mRNA based mechanisms that increase SMN protein levels. Briefly, these include enhanced transcription of the *SMN2* promoter, mRNA stabilization, and exon 7 splicing modification. A transcriptional increase elevates both exon 7 included and skipped transcripts equally and should be reflected by comparable fold increases of total and exon 7 included transcripts. However, in practice we have observed that this is accurate at low to moderate increase of reporter transcription.

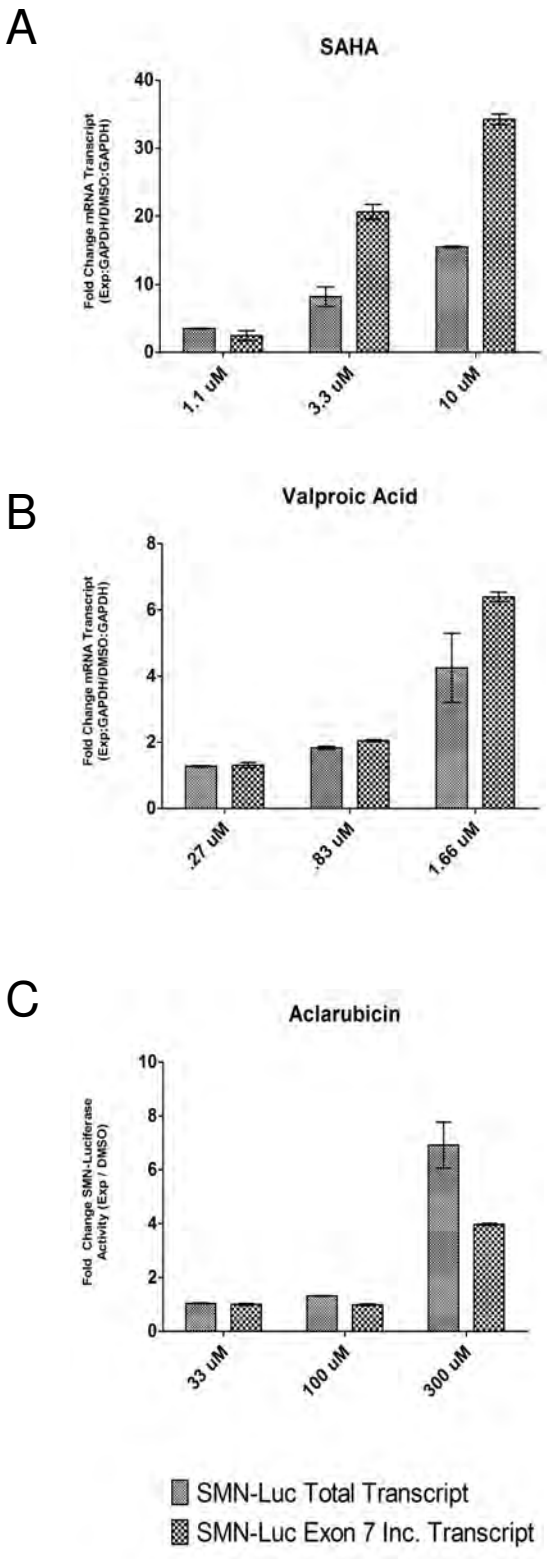
Increased levels of mRNA induction and processing resulted in greater incidences of exon 7 skipping. In this case it is possible that the increase of transcription and rate of transcription negatively affected the recognition of the weak splice sites of exon 7. Recruitment and binding of splicing factors is mediated by RNA polymerase and associated proteins [116]. Increase of the rate of transcription in response to altered histone acetylation negatively impacts the inclusion of weak exons and promotes alternative splicing. Moreover, the presence of nucleosomes and transcription pause sites within weak exons promote exonic inclusion [117]. Increased transcriptional rate can bypass this pause and concurrently cause exonic exclusion. Therefore, it is possible that transcriptional compounds are directly effecting the population of splicing factors associating with the transcriptional complex at the *SMN2* promoter or promoting exclusion of exon 7 via modification of the rate of the RNAP transcription.

The greatest increase of reporter mRNA was observed with SAHA treatment. At the lowest concentration, 1.1 µM, the fold induction in total SMN

reporter mRNA and exon 7 included mRNA were comparable and likely reflect the HDAC inhibitor's effect on promoter transcription. At higher drug dosages there was nearly a 2 fold increase of exon 7 included transcript relative to total transcript (Figure 1.5 a). This suggested that as the concentration of the HDAC inhibitor resulted in an enhanced effect on exon 7 splicing. Presumably, this is the result of increased expression of exon 7 splicing enhancers such as hTra2β or SF2/ASF. SAHA's effects on transcriptional enhancement as well as reducing the percentage of exon 7 skipping resulted in nearly a 35 fold increase of full length transcript. Similar results were observed following reporter treatment with valproic acid (Figure 1.5b). However, the fold increases observed were nearly 3 fold less than the levels obtained by 10.0 µM SAHA. The results observed when *SMN2*-luciferase reporter cells were treated with aclarubicin differ from the other two HDAC inhibitors (Figure 1.5 c). Splicing remains unchanged despite ample activation of the reporter mRNA in response to aclarubicin treatment. Aclarubicin appears to be limited to transcriptional activation.

The HDAC inhibitor LBH-589, a potent activator of SMN mRNA and protein levels has no effect on mRNA turnover [118]. This data in combination between the 99.9% identical mRNA transcripts, the reporter mRNA increases observed are likely to be the byproduct of enhanced transcription rather than mRNA stability. The behavior of the reporter mRNA in response to these compounds confirmed that the *SMN2* reporter accurately reproduced SMN protein level increases consistent with published results.

Figure 1.5. Analysis of Changes of Reporter SMN mRNA Levels with Known SMN Activators. qPCR analysis of SMN-luciferase reporter transcripts following 24 hours HDAC inhibitor treatment (SAHA (A), Valproic Acid (B), and Aclarubicin (C)). (A) SAHA treatment (1.1, 3.3, and 10.0 µM) results in a greater fold increase of exon 7 included transcript in relation to total reporter transcripts. (B) Valproic acid (0.27, 0.83, and 1.66 µM) increased reporter mRNA similar to SAHA, however was approximately 5 fold less potent. (C) Aclarubicin (33, 100, and 300 µM) treatment increased both total reporter transcripts as well as exon 7 included transcript. In contrast to SAHA and valproic acid there was a 1.5 fold greater increase of total transcript relative to exon 7 included transcripts. Data collected by author, figure compiled by Jonathan Cherry, Ph.D.


A
SAHA
Fold Change mRNA Transcript (Exp:GAPDH/DMSO:GAPDH)
40
30
20
10
0
1.1 uM
3.3 uM
10 uM
B
Valproic Acid
Fold Change mRNA Transcript (Exp:GAPDH/DMSO:GAPDH)
8
6
4
2
0
.27 uM
.83 uM
1.66 uM
C
Aclarubicin
Fold Change SMN-Luciferase Activity (Exp / DMSO)
10
8
6
4
2
0
33 uM
100 uM
300 uM
SMN-Luc Total Transcript
SMN-Luc Exon 7 Inc. Transcript

High Throughput Screen Target Validation

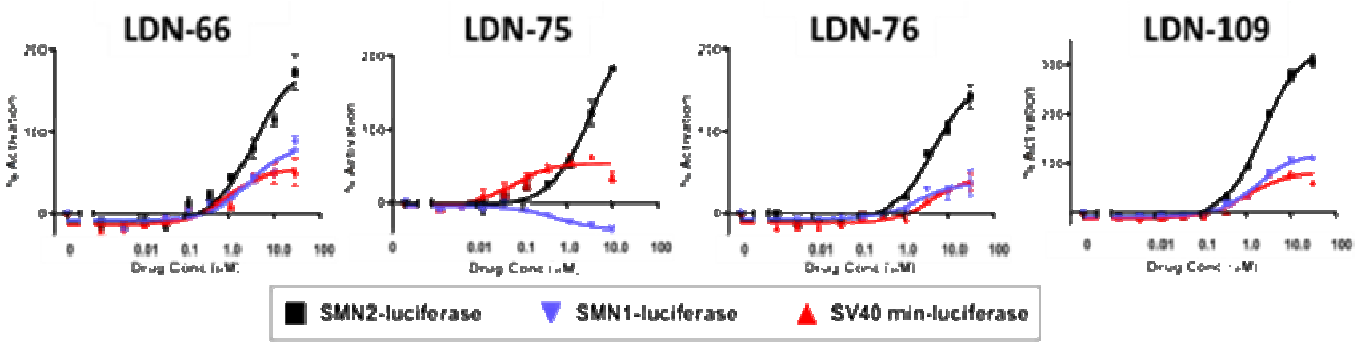
The *SMN2*-lucifease reporter cells line were used in numerous high throughput screens (HTS) at multiple collaborating sites to identify novel compounds that increased SMN protein levels. Over 1 millions compounds were screened for SMN-luciferase activity. A select number of compounds were identified based on assay activity, *SMN2* selectivity over *SMN1,* and ease of medicinal chemistry. Activation was defined at 6 times the coefficient of variation for the entire screen, which corresponds to 60% activity over the DMSO controls. The compounds identified were LDN-66, -75, -76, -109, and analogs of NCGC-533. The LDN and NCGC compounds were identified in two separate screens and were chemically and structurally unique.

Validation of LDN HTS Hits

Following the completion of the HTS, all active compounds were retested at multiple concentrations by Dr. Jonathan Cherry. The compounds were screened for activity in the *SMN1* and *SMN2* reporters as well as an irrelevant cell line that expressed firefly luciferase under the control of the SV40 promoter (Figure 1.6a). All four of the compounds LDN-66, - 75, -76, and -109 preferentially activated the *SMN2* reporter cell line in comparison to each of the other two cell types tested. This was a critical step to determine that the compounds did not non-specifically increase transcription or luciferase activity. Activation of the *SMN1* reporter does not necessarily indicate a poor SMA activating compound as the promoter regions are 99% identical. However, one

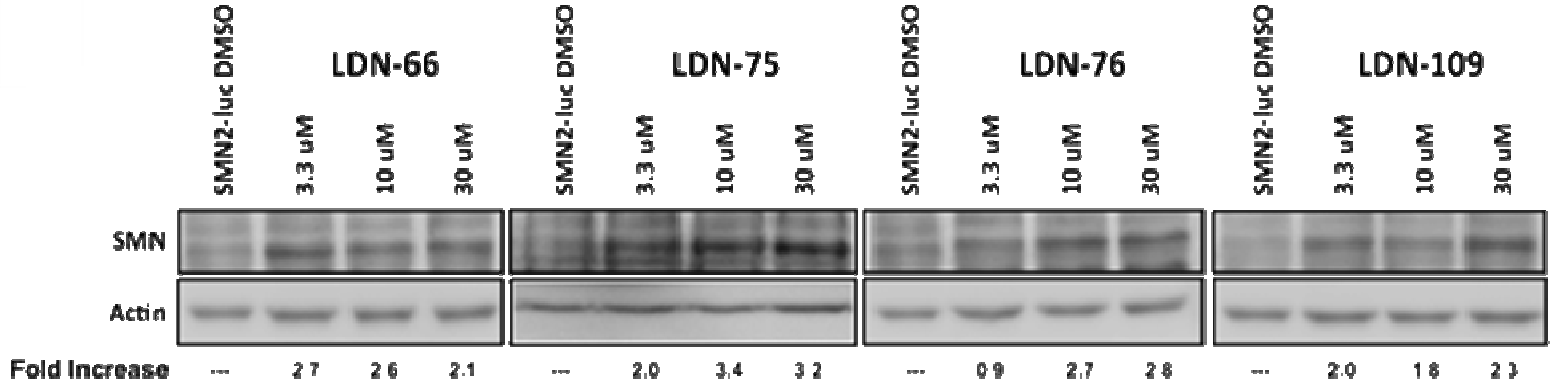
Figure 1.6. Reporter assay validation. (A) Comparison of luciferase activity for equivalent numbers of clonal SMN1-luc and SMN2-luc cells plated in 96 well format and scored as relative light units (RLU). Luciferase activity is nearly 60 times higher in the SMN1-luc reporter than the SMN2-luc reporter. (B) Detection of SMN-luciferase fusion protein in HEK parent, SMN1-luc, and SMN2-luc reporter cells. Lysates were blotted for the fusion protein with an anti-luciferase antibody and compared to α-tubulin as loading controls. (C and E) SMN1-luc cells were treated for 24 hours with increasing amounts of SAHA. The amount of SMN-luciferase fusion protein detected by anti-luciferase antibody (C) is similar to the % increase in luciferase activity over treatment with DMSO carrier in the same samples (E). (D and F) SMN2-luc cells were treated for 24 hours with increasing amounts of SAHA and sodium butyrate. The increase in SMN-luciferase fusion (D) is compared to the % increase in luciferase activity (F) with both compounds. Experiments were performed 3 times. Blots shown are representative. Data and figure courtesy of Jonathan Cherry, Ph.D. (submitted data)

A



B

	LDN-66	LDN-75	LDN-76	LDN-109
Average EC^{50}	1.8 ± 0.5 µM	2.0 ± 0.2 µM	4.0 ± 0.7 µM	2.1 ± 0.2 mM
Average B_{max} (% activation)	169 ± 16	247 ± 15	242 ± 21	274 ± 20



objective of the HTS was to identify therapeutic compounds that were specific to *SMN2* activation and thereby potentially minimizing off target or toxic side effects. HTS hits were screened in an SV40 luciferase cell line to filter out compounds that are potentially acting directly on the luciferase protein and increasing activity independent of SMN.

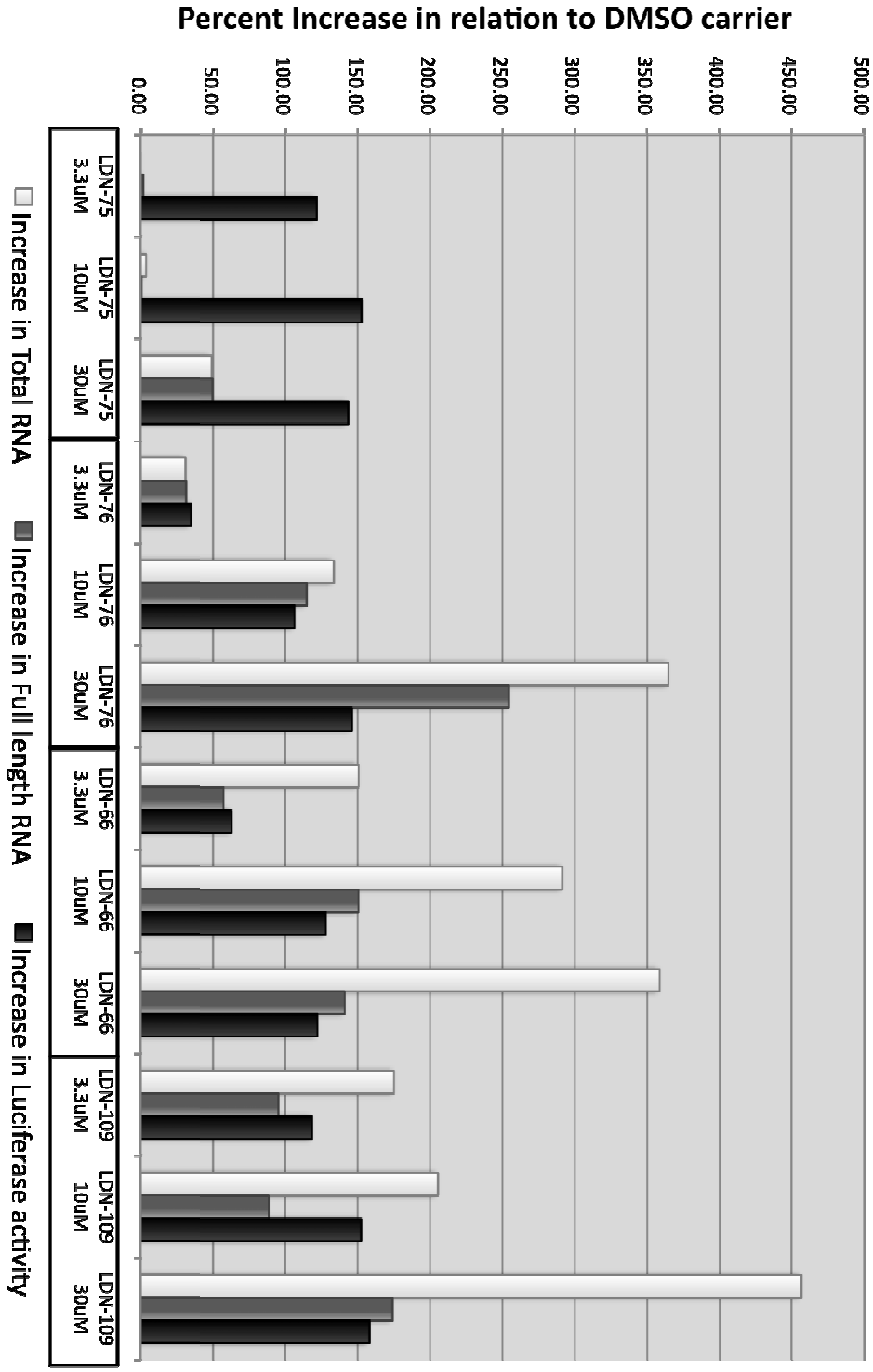
Each of the four compounds had EC_{50} values in the low micromolar range while maintaining *SMN2*-luciferase activity of at least 169% (1.7 fold) (Figure 1.6B). Dr. Cherry measured the protein levels of the SMN-luciferase fusion protein in the B3 reporter cells following drug treatment, by Western Blot using an anti-luciferase antibody (Figure 1.6 c). The fold change of SMN-luciferase were calculated in reference to DMSO controls and normalized to a β-actin well loading control Western blot. The protein increases measured were comparable to the luciferase activity data.

The reporter was designed to identify compounds that increase *SMN2* full length protein production without discrimination based on mechanism of action. Quantification of changes of reporter mRNA levels, or in exon 7 splicing, aids in the identification of the mechanism that the compounds may be exploiting. *SMN2* reporter cells were treated with each of the LDN compounds for 24 hours prior to harvesting RNA and measuring luciferase activity. Three concentrations of drug were chosen that fully encompass the active range of the compounds, 3.3 µM, 10.0 µM, and 30.0 µM.

All four compounds increased activity of the *SMN2*-luciferase reporter (Figure 1.7 – black bars). LDN-76, -66, and -109 increased total and exon 7 included mRNA (1.7 – white and gray bars respectively) with growing efficiency as the drug concentration was increased from 1.1 µM to 10.0 µM. In each of these three cases the fold increase of total SMN reporter mRNA greater in comparison to full length transcripts. Luciferase activity increases more closely mirrored the increases of exon 7 included rather than total transcripts. With increased drug concentration there was a dose response from all mRNA species measured. The pattern of mRNA increase resembled aclarubicin treatment (Figure 1.5C). These data indicated that the most likely mechanism to increase reporter mRNA levels was transcriptional.

LDN-75 did not exhibit the same mRNA induction pattern observed with the other compounds. LDN-75 failed to increase SMN-luciferase mRNA at the 1.1 or 3.3 µM drug concentrations. At the highest concentration, 10.0 µM, there was a 50% or 1.5 fold increase of both total and exon 7 included mRNA. This data contradicted the other compounds where the increase of luciferase activity was directly comparable to full length mRNA induction. In the case of LDN-75 there was a 150% (2.5 fold) increase in reporter activity in the absence of elevated mRNA levels. Western blot data in Figure 1.6C confirmed increased steady state levels

Figure 1.7. *SMN2*-Luciferase Reporter Activity and mRNA Increases. Treatment of reporter cells for 24 hours with LDN-75, -76, -66, and -109 increases reporter activity levels 100% (2 fold) over DMSO carrier treated cells. LDN-76,-66, and -109 increase total SMN-Luciferase mRNA levels (white bars) and exon 7 included mRNA (gray bars). LDN-75 failed to increase reporter mRNA. qPCR was used to calculate mRNA increases. GAPDH was used for normalization. Data collected by author, figure compiled by Jonathan Cherry, Ph.D.


Percent Increase in relation to DMSO carrier
0.00
50.00
100.00
150.00
200.00
250.00
300.00
350.00
400.00
450.00
500.00
LDN-75 3.3uM
LDN-75 10uM
LDN-75 30uM
LDN-76 3.3uM
LDN-76 10uM
LDN-76 30uM
LDN-66 3.3uM
LDN-66 10uM
LDN-66 30uM
LDN-109 3.3uM
LDN-109 10uM
LDN-109 30uM
Increase in Total RNA
Increase in Full length RNA
Increase in Luciferase activity

of the fusion protein. The lack of mRNA induction suggests that the mechanism employed by LDN-75 is downstream of transcription.

Dr. Cherry confirmed each of the LDN compounds increased SMN protein levels in SMA fibroblast cell lines. Validation of the drugs activity on endogenous genes and protein eliminated the possibility that the effects observed were indirect and exclusive to the reporter cell line. Secondary assays were performed; including gem count assays in primary fibroblasts to additionally confirm each of the compounds ability to increase SMN protein levels. Experiments testing the activity of the LDN compounds in a SMA mouse model for SMN protein levels as well as viability are currently pending.

Validation of NCGC HTS Hits

In a separate HTS, 21 chemical compounds were selected as for further investigation. These compounds were selected from a pool of approximately 6000 compounds that were active in the *SMN2* reporter assay. Compounds were eliminated as viable drug targets based on known compound related cell viability data, an *SMN1* reporter counter screen, as well as any evidence suggesting luciferase inhibition activity; Luciferase inhibitors can act to stabilize the protein at lower concentrations, thereby appearing to increase reporter activity in the primary HTS. The entire set of 21 compounds was tested for the ability to increase endogenous protein in primary SMA fibroblasts. From this final set of 21 compounds one compound was selected based on positive SMN

Western blot data as well as favorable chemical structure for medicinal chemistry and structure activity relationship experiments.

To begin chemical analogs were synthesized and tested for activity in the *SMN2* reporter cell line. Seven analogs were tested at four different concentrations, 0.111, 0.333, 1.100, and 3.300 µM by Dr. Cherry. Each of the chemical analogs retained the ability to increase SMN-luciferase activity (Figure 1.8). The maximum percent increase of activity was equivalent with all compounds with the exception of NCGC-931. The drug was more effective at lower concentrations with the EC_{50} in the low nM range. This could indicate that medicinal chemistry on the chemical scaffold may have difficulty enhancing the increase of SMN protein levels. However, further medicinal chemistry could increase drug potency, reducing the amount of compound necessary to increase SMN levels and possibly decreasing off target or toxic side effects.

Quantitative PCR was used to measure increases of reporter total and exon 7 included mRNA for each of the 7 chemical analogs in Figure 1.8. Each of the chemical analogs failed to increase reporter mRNA. Graphs for three of the NCGC compounds, NCGC-533, -564, and -931, are represented in Figure 1.9. The data for these three analogs are representative of all 7 compounds tested. Minor effects on both measured transcripts are consistent between each of the compounds. The increases of luciferase activity observed are most likely due to post-transcriptional and mRNA processing events. These data are most

Figure 1.8. Measuring *SMN2* reporter activity NCGC chemical analogs. *SMN2*-Luciferase reporter cells were treated with 7 NCGC compounds for 24 hours prior to measuring firefly luciferase activity using Dual-Glo Luciferase assay system. Signal was normalized to DMSO carrier treated reporter cells. Cells were treated with 4 different concentrations of compound, 0.111, 0.333, 1.100, and 3.300 μM. Each compound had comparable max activation of the reporter. NCGC – 931 has the lowest EC_{50} in the low micromolar range. Data and figure courtesy of Dr. Jonathan Cherry (submitted data).

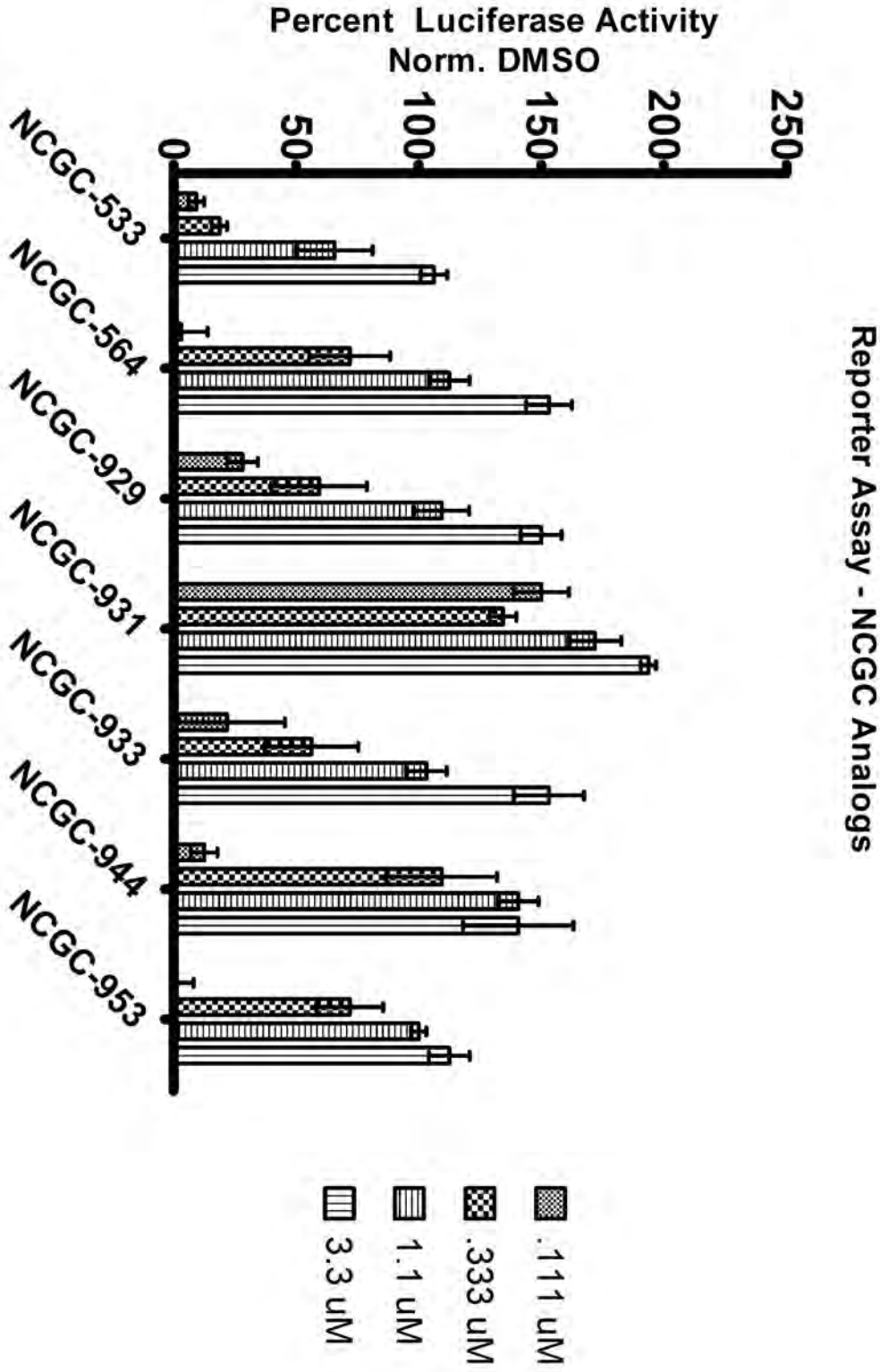
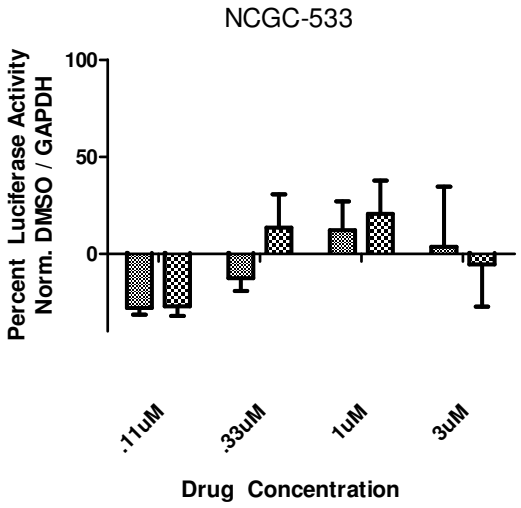
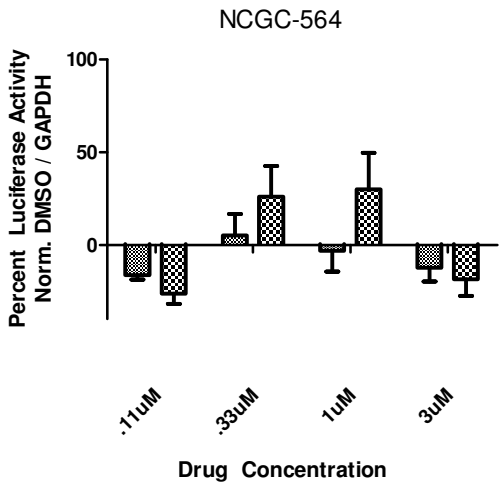

Reporter Assay - NCGC Analogs
Percent Luciferase Activity
Norm. DMSO
0
50
100
150
200
250
NCGC-533
NCGC-564
NCGC-929
NCGC-931
NCGC-933
NCGC-944
NCGC-953
.111 uM
.333 uM
1.1 uM
3.3 uM

Figure 1.9 Measuring SMN-Luciferase mRNA levels by qRT-PCR. NCGC-531, -564, and -931 treatment on *SMN2*-Luciferase reporter cells for 24 hours. Levels of total SMN reporter transcripts and exon 7 included transcripts were measured by qRT-PCR and normalized to GAPDH. Drug treatment failed to increase reporter mRNA despite luciferase activity increase (Figure 1.8). qRT-PCR was done for the other 4 NCGC compounds and mRNA levels were comparable to compounds -531, -564, and -931 shown.

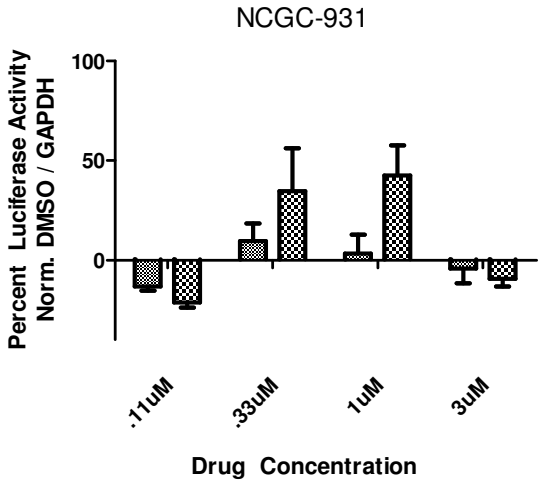
A


NCGC-533
Percent Luciferase Activity
Norm. DMSO / GAPDH
100
50
0
.11uM
.33uM
1uM
3uM
Drug Concentration

B


NCGC-564
Percent Luciferase Activity
Norm. DMSO / GAPDH
100
50
0
.11uM
.33uM
1uM
3uM
Drug Concentration

C


NCGC-931
Percent Luciferase Activity
Norm. DMSO / GAPDH
100
50
0
.11uM
.33uM
1uM
3uM
Drug Concentration

comparable to the results obtained with compound LDN-75. The quantitative PCR data reignited previous concerns that compounds may falsely activate the reporter by stabilizing the luciferase protein independent of SMN.

Therefore, new chemical analogs were developed taking into consideration the reporter data of Figure 1.8. However, all subsequent analysis was performed exclusively in primary SMA fibroblasts to eliminate any luciferase related artifacts. Two methods, Western blot and gem count assays, were used to measure the SMN protein levels in response to the NCGC compounds. The primary fibroblasts selected for these experiments were the 3813 and 3814 cell lines [119]. The 3813 cell line was isolated from a type I SMA patient. It contains two copies of the *SMN2* gene and is homozygous null for *SMN1.* The 3814 cell line was collected from a type I SMA carrier, with direct relationship to the 3813 donor. Initial characterization of these two cell lines reported a 10 fold difference in SMN protein levels [119]. With time and cell passage the protein levels in the 3814 carrier cells are closer to 4 fold greater than the patient 3813 line.

Three compounds, NCGC-8a, -8m, and -9a, were selected for Western blot and gem count experiments in 3813 cells. Cells were treated at 5 concentrations 0.037 µM,0 .111 µM, 0.333 µM, 1.000 µM, and 3.000 µM and compared to DMSO carrier treatment and carrier 3814 cells. Approximately 100 gems per 100 nuclei were detectable in carrier 3814 cells. The intensity of immunofluorescent staining in these cells was intense and gems clearly distinguishable (Figure 1.10). 3813 cells contained 5 fold fewer gems SMN

signal was weak. Treatment with 5 µM SAHA increased the number of gems detected per 100 nuclei; however the staining was still weak in comparison to carrier 3814 cells. Compound NCGC-8a was ineffective at increasing the gem number in 3813 cells while NCGC-8m and -9a treatment resulted in nearly 2 fold increase in counts (Figure 1.11 B). Quantification of Western blot data (Figure 1.11 A) demonstrated remarkable similarity to SMN protein increases quantified by gem count assays.

Figure 1.10. Immunofluorescence of endogenous SMN protein in primary SMA fibroblasts. Immunohistochemistry staining of endogenous SMN protein (Green) with antibody 4F11. Primary SMA fibroblasts 3813 cells were treated for 96 hours with 0.037, 0.111, 0.333, 1.000, and 3.000 µM compound (1.000 µM shown). Genomic DNA was stained with Prolong Gold DAPI mounting media. Gems are labeled with green triangles. Primary SMA carrier fibroblasts, 3814, were used as reference.

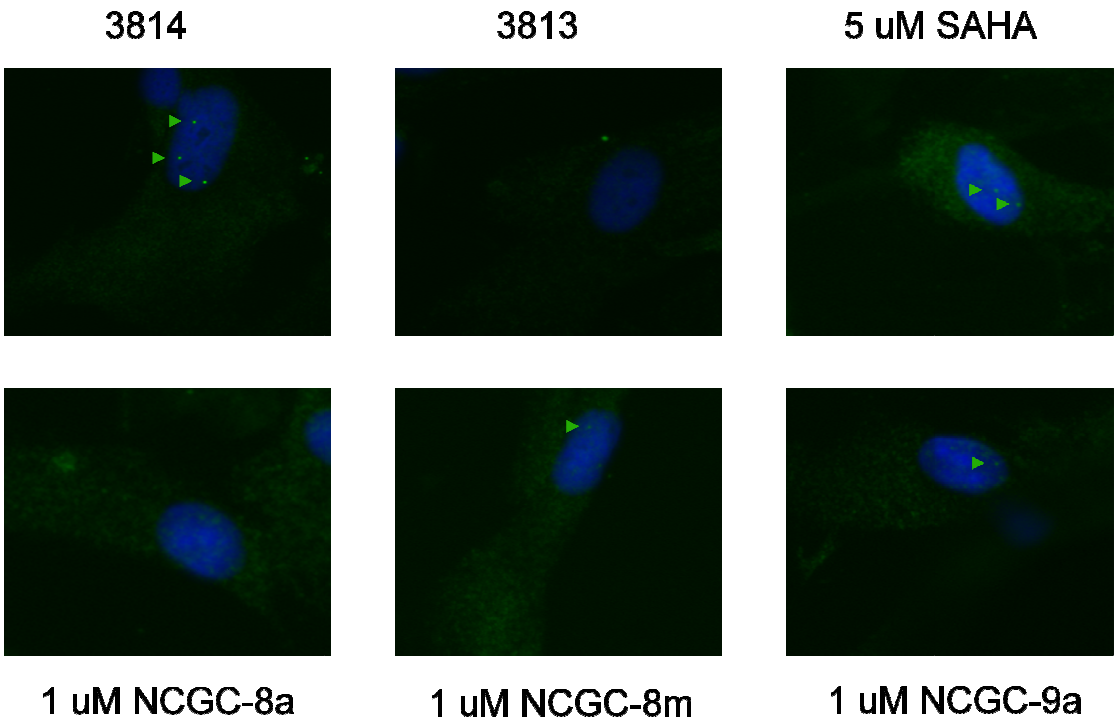
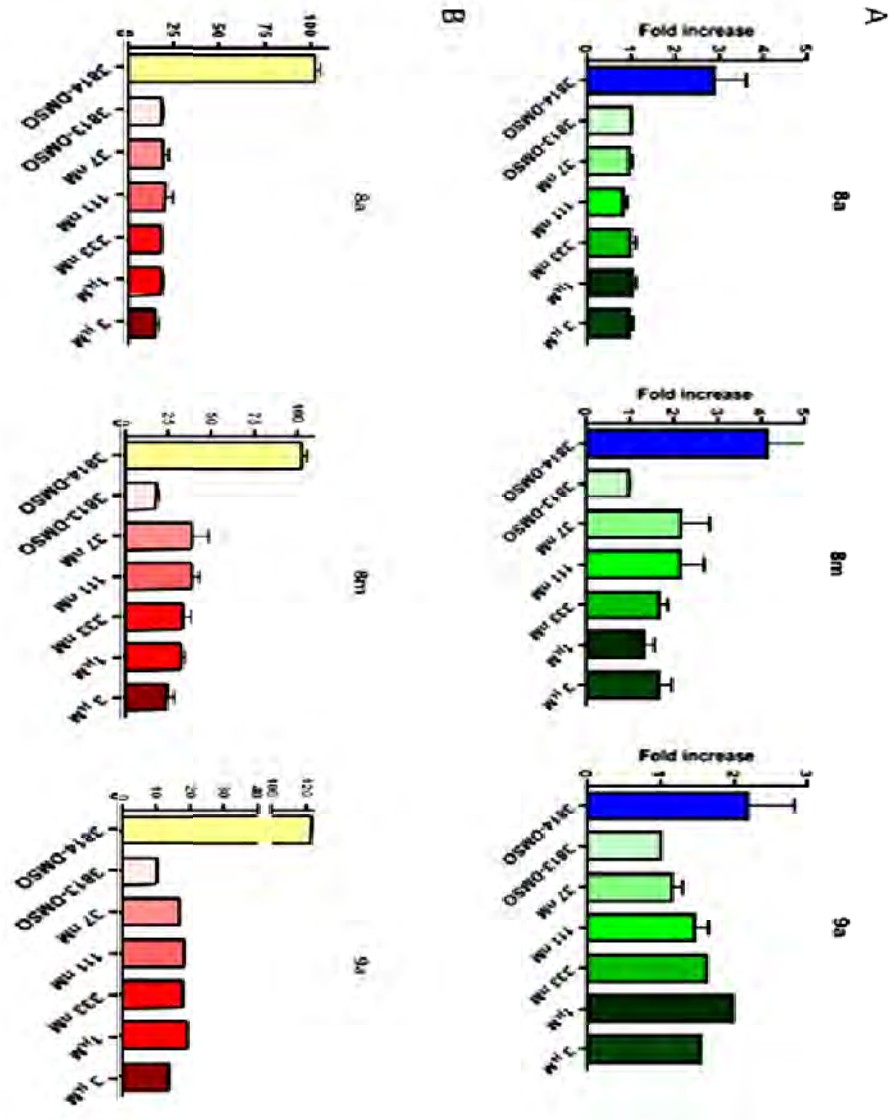

3814
3813
5 uM SAHA
1 uM NCGC-8a
1 uM NCGC-8m
1 uM NCGC-9a

Figure 1.11 Quantification of Gems in primary SMN fibroblasts. Primary SMA fibroblasts 3813 cells were treated for 96 hours with 0.037, 0.111, 0.333, 1.000, and 3.000 µM compound. Quantification of protein increases determined by Western blot and gems counted per 100 nuclei. Increases of protein observed were consistent between Western and gem count assays. Western blot quantification was normalized to tubulin. Fold increase and raw gem count data was shown for Western blog and immunofluoresence data respectively. Experiments were performed in triplicate. Panel A data collected and compiled by Jonathan Cherry, Ph.D.


A
B
Fold increase
8a
8m
9a
3814-DMSO
3813-DMSO
37 nM
111 nM
333 nM
1 μM
3 μM

Discussion and Future Directions

There is a significant effort to identify and develop novel therapies for spinal muscular atrophy. The compounds that have been tested in clinical trials have produced results with little or no significant survival benefit to SMA patients. Previous HTS screening tools focused on compounds that targeted *SMN2* transcription or exon 7 splicing. Dr. Cherry developed a novel luciferase based reporter cell lines that included the minimal promoter driving SMN exon 1-5 cDNA fused to an exon 7 splicing cassette. The final product is a full length SMN protein fused to luciferase only when exon 7 is included. The advantage of this system is that it maintains the potential to identify therapies that increase SMN proteins at various mechanistic levels from gene to protein.

This body of work characterized the episomal reporter mRNAs as well as assisted in the identification, validation, and development of new SMN activating chemical compounds. Cell lines stably maintaining episomal *SMN1* and *SMN2* reporter constructs were found to produce exon 7 included and excluded transcripts in ratios that agreed with published reports. Additionally, the increases of reporter transcripts in response to known SMN activating compounds, SAHA, valproic acid, and aclarubicin were consistent with expected results.

In two separate high throughput screens 5 compounds were identified and selected for further study as potential SMN activating compounds. Quantitative

PCR revealed that three compounds LDN-76, -66, and -109 increased SMN RNA that fits the pattern of transcriptional upregulation. LDN-75 and the NCGC analogs failed to increase SMN transcripts. The effect on SMN protein was confirmed in SMN primary fibroblasts. Therefore, any effect these compounds have on steady state SMN protein levels was independent of any mRNA based mechanism.

LDN-75 and the NCGC compounds likely act to stabilize the SMN protein. An interaction between SMN and ubiquitin carboxyl-terminal hydrolase L1 (UBCH1) is at least partially responsible for regulating SMN protein levels. Overexpression of UBCH1 results in decreased steady state levels of SMN where chemical inhibition of the protein increased SMN protein and gem numbers. Therefore, it is possible that LDN-75 and the NCGC analogs are functioning in a similar pathway, inhibiting the proteosome degradation of SMN. The HDAC inhibitor LBH-589 has been demonstrated to act directly on protein stability in addition to transcriptional and splicing effects. LBH-589 lowers levels of SMN poly-ubiquitination and thereby reduced the degradation and protein turnover. The increase of SMN protein is comparable to levels achieved by proteosomal inhibition by MG132 [118]. The luciferase reporter is activated between 2 and 3 fold with LDN-75 and the NCGC analogs. This is nearly identical to data obtained following MG-132 treatment. However, there is no direct evidence that LDN-75 or the NCGC analogs function in a similar manner.

Identification of the molecular targets of these compounds would significantly benefit their development towards effective SMA therapies. Knowledge of the drug target's protein structure can reveal potential binding sites and further drive medicinal chemistry to increase drug potency, minimize side effects, and reduce binding to off target proteins. Additionally, discovery of a novel protein in SMA biology could foster investigations that may give insight into disease progression and the susceptibility of motor neurons to low SMN levels. The medicinal chemistry already done and tested in high throughput format has mapped sites on the base chemical structure that can be changes without sacrificing SMN induction activity. At these sites it is possible to covalently link a moiety such as biotin to enable precipitation of the compound and any bound proteins from cell lysates. Multidimensional protein identification technology (MudPIT) mass spectrometry or conventional SDS-PAGE band excision followed by matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry could be used to indentify proteins precipitated from the biotin-drug treated lysates. Without target identification it is still possible to delve deeper into the mechanisms used to increase SMN protein. In particular, LDN-75 and the NCGC analog's pathway to elevated protein levels are largely unknown. Further experiments measuring protein half-life and SMN ubiquitination in response to drug treatment would determine if the compounds target SMN protein degradation.

Moving forward the compounds characterized in this set of data should be tested in mouse models of SMA. Additionally, a concerted effort must be made to identify the drug targets as well as cellular pathways being used to increase SMN protein. However, the groundwork laid out in these experiments demonstrates that the episomal cell lines are valuable tools for future drug screening and validation studies. The reporter mimics the behavior of the endogenous genes more completely than any other system to date. The whole protein fusion protein strategy has identified novel compounds that show extreme promise as potential SMA therapeutic compounds.

Preface

Portions of this work will be submitted for publication.

Evans MC, Cherry JJ, Androphy EJ. Differential Regulation of the SMN2 Gene By Individual HDAC Proteins, [Submitted BBRC, 2011]

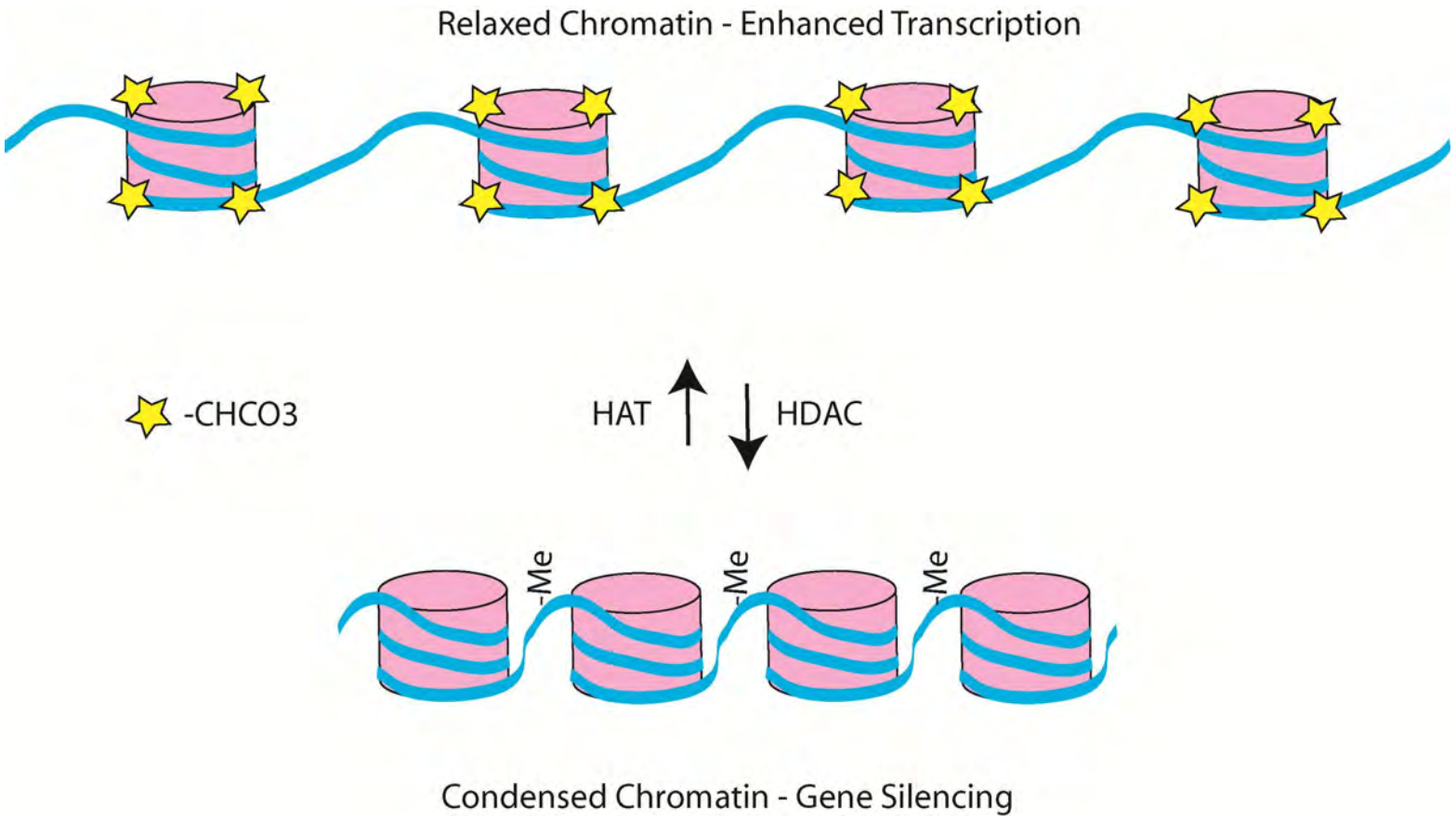
Chapter II:

Silencing of Individual HDAC Protein Differentially Increases SMN-Luciferase

Histone Deacetylase Inhibitors

Histone Deacetylases are a family of proteins that primarily regulate the removal of acetyl (-CHCO3) groups from lysines on histone proteins in a Zn+ dependent mechanism [120]. HDACs work opposing histone acetyl-transferase proteins to determine the balance of histone acetylation. The acetylation state of histone tails regulates the confirmation of chromatin and subsequently regulates the access of transcriptional machinery to the DNA [120, 121]. Briefly, hypoacetylated histones condense chromatin and down regulate transcription while hyperacetylation relaxes the chromatin structure and increases transcription (Figure 2.1). DNA methylation as well as histone tail methylation in cooperation with acetylation modulate transcriptional silencing [122]. *SMN2* is known to be methylated at CpG islands and the level of methylation correlates to the transcriptional activity of the promoter. Treatment with HDAC inhibitors is capable of increasing *SMN2* transcriptional products independent of promoter methylation [123]. HDAC proteins are currently categorized into four classes primarily based on homology to yeast deacetylase proteins [124]. Class I (HDACS 1, 2, 3, and 8) HDACs are similar to the *Saccharomyces cerevisiae* transcriptional regulator RPD3. Class II HDACs (HDACS 4, 5, 6, 7, 9, and 10) are more closely related to yeast HDA1 [124]. Class III HDAC proteins are homologous to yeast SIR1 and are Nicotinamide adenine dinucleotide (NAD+) dependent. The final class of HDAC contains only one protein, HDAC11[125].

Figure 2.1 Schematic Summary of Histone Acetylation. Cartoon diagram showing effect of histone tail acetylation on chromatin confirmation (acetylation – stars). Acetylated histones relax chromatin allowing enhanced transcription factor access to promoter and results in increased mRNA production. Removal of acetyl groups from histone tails condenses chromatin and is associated with increased CpG island methylation (-Me) resulting in gene silencing.

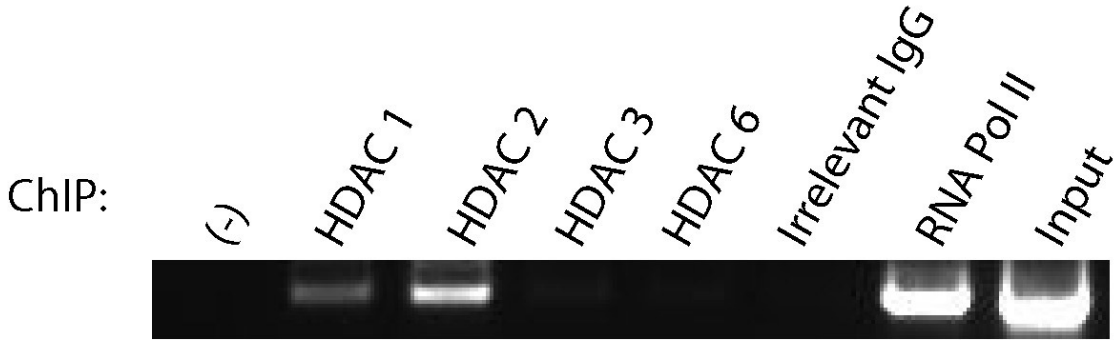

Relaxed Chromatin - Enhanced Transcription
-CHCO3
HAT
HDAC
-Me
-Me
-Me
Condensed Chromatin - Gene Silencing

There is insufficient sequence homology or functional data to categorize HDAC11 in any of the other three classes.

Class I HDACs are almost exclusively nuclear and fairly uniformly expressed in all tissue types, with only HDAC3 occasionally shuttling to the cytoplasm via a nuclear export signal [126]. Regulation of the class I HDAC is largely controlled by secondary proteins affecting deacetylase activity or recruitment to the genome [127, 128]. Class II HDACs localization is dependent on cellular signal and can be either cytoplasmic or nuclear. Class II expression profiles indicate these proteins are more varied between individual tissues. HDAC 5, 6, 7, and 10 distribution is as uniform as Class I, while HDACs 4, 8, and 9 are elevated in tumor tissue and found at variable levels in other tissues. Class II HDAC regulation is achieved by signal induced shutting out of the nucleus, removing the proximity of the HDAC from the histone.

The SMN promoter is non-remarkable relative to histone acetylation. The promoter region surrounding the transcriptional start site is populated by H3 and H4 histones and is conserved between mouse and human samples. Only HDAC1 and HDAC2 have been reported to be bound to the SMN promoter [129]. Both proteins were confirmed to also be bound to the *SMN2*-luciferase reporter promoter (Figure 2.2). Consequently, the episomal reporter is seemingly under the regulation of the same protein as the endogenous gene. SMN binds HDAC2 and Sin3A, a HDAC1/2 co-repressor protein. It is unclear what impact these associations have on SMN expression or SMA biology.

Figure 2.2. Chromosome Immunoprecipitation of HDACs at the *SMN2*-luciferase Reporter Promoter. The SMN promoter was isolated from untreated HEK293T *SMN2*-luciferase reporter cells using chromosome immunoprecipitation. Irrelevant Rabbit IgG and anti-RNA Polymerase CTD antibodies were used for negative and positive controls respectively. The reporter promoter was successfully immunoprecipiated, consistent with published endogenous reporter data. Both HDAC3 and HDAC6 do not appear to bind the *SMN2*-luciferase reporter in the amplified region.


ChIP:
(-)
HDAC 1
HDAC 2
HDAC 3
HDAC 6
Irrelevant IgG
RNA Pol II
Input

HDAC inhibition is thought to increase expression and deacetylate non histone proteins that regulate apoptotic cell death. HDAC inhibitors act by blocking access to the Zn^{2+} ion from the catalytic core thereby inhibiting deacetylase activity. There are multiple clinical trials currently ongoing investigating the role of HDAC inhibition in treating malignancies and solid tumors [130, 131]. The first new generation HDAC inhibitor to be Food and Drug Administration (FDA) approved for the treatment of cutaneous T-cell lymphoma was suberoylanilide hydroxamic acid (SAHA / Vorinostat) [132].

Phenylbutyrate (PB) and valproic acid (VPA) were initially tested to increase SMN levels in tissue culture and mouse models because of in place safety information from FDA approved uses [115, 133, 134]. Both compounds showed 2 to 4 fold increases in SMN mRNA or protein in SMA patient derived cell lines. The success of *in vitro* tests of these compounds prompted clinical trials with SMA patients to be opened. PB was observed to increase SMN levels in blood leukocytes of patients and carriers and displayed some functional benefit. One suspected failing of PB is the short half life of approximately 1 hour. VPA, which has a much longer half life of approximately 8 hours, had variable results in multiple clinical trials. Between studies, and even patients within individual trials, the response to VPA was highly variable [95]. Some patients increased SMN protein levels in leukocytes and experienced some strength benefits. Increased *SMN2* copy number appears to proportionally increase the benefits of VPA administration. As *SMN2* is the target of transcriptional increase

with HDAC inhibitors this result is not surprising, however does provide another variable in the search for a viable SMA therapy.

Newer generation HDAC inhibitors are currently being tested for ability to increase SMN protein levels in tissue culture and mouse models. LBH589 (panobinostat), M344, trichostatin-A (TSA), and SAHA have all shown a capacity to induce higher levels of SMN protein [114, 118, 135-137]. In the case of M344 and LBH589 the fold change of SMN protein in SMA patient cell lines is between 7 and 10 fold greater than control samples. This change is far greater than the levels achieved using PB or VPA in previous studies. However, it is difficult to compare fold changes as cell lines and treatment courses vary between studies. Administration of SAHA and TSA to severe SMA mouse model showed increased strength in addition to increases in motor neuron number and size of neuromuscular junctions. Most importantly, drug treatment increased survival by approximately 30 percent when compared to untreated littermates.

While there are many promising aspects of HDAC inhibitors as SMA therapy, there are still many detracting features associated with this class of compounds. HDAC inhibitor trails for cancer therapy have even suggested that some patients may be immune to the beneficial effects of this class of compounds. An ideal SMA therapeutic compound will effective in all patients regardless of age, weight, and genetics. It is important to identify possible markers that will determine a particular patient's potential response to any given treatment. Secondly, there is an intrinsic toxicity associated with HDAC inhibitor

compounds. The population of genes that are modified following HDAC inhibition is diverse and can also have broad and unpredictable effects within the cell and whole system. Increased expression of SMN in a beneficial outcome of this process, however the extent of other changes is largely unknown. Despite these challenges, HDAC inhibitor are still among some of the most potent SMA therapies and research must continue to understand the effects these compounds have on SMN expression.

In this study we sought to elucidate the role that individual HDAC proteins have on the regulation of SMN expression. We specifically targeted HDACs 1-8 by the expression of short hairpin RNAs (shRNAs) and measured SMN protein levels using a *SMN2* luciferase reporter cell line. We then determined the effects of reduced HDAC levels on *SMN2* transcription, exon 7 inclusion, and mRNA levels of SMN splicing modulators hTra2β, hnRNP A1, and SF2/ASF. Silencing of individual HDAC mRNA was sufficient to increase the amount of SMN reporter transcripts, and increase reporter activity. Changes in total and full length mRNA were differentially affected by the individual HDAC proteins silenced. While enhancing the basic understanding of SMA biology and *SMN2* gene expression regulation, these data will also identify potential drug targets that may have less toxic properties than pan-HDACi while maintaining sufficient SMN protein induction.

Materials and Methods

Tissue Culture

Cells were incubated at 37 C with 5% CO_2. HEK-293 reporter *SMN2* -luciferase cells were grown in D-MEM (Gibco, #11995) with 10% fetal bovine serum (Sigma) and 1x Pen-Strep (Gibco, #15140) with 200 ug/mL hygromycin B (Invitrogen, #10687-010). Primary human SMA patient fibroblasts, 3813, were grown in D-MEM with 15% fetal bovine serum and 1x Pen-Strep.

shRNA Vectors and Transfection

All shRNA vectors were obtained from openbiosystems via the UMass Medical School RNAi Core Facility. All shRNAs were in the pGIPZ vector also expressing GFP under the CMV promoter. HDAC1 (V2LHS_61809, V3LHS_344564), HDAC2 (V2LHS_132136, V3LHS_382880), HDAC3 (V2LHS_53152, V3LHS_380875), HDAC4 (V2LHS_71327, V3LHS_340831), HDAC5 (V2LHS_68644, V3LHS_321382), HDAC6 (V2LHS_71188, V3LHS_330045), HDAC7 (V2LHS_96401, V3LHS_351666), and HDAC8 (V3LHS_355330, V3LHS_355335), pGIPZ Non-Silencing Control. 1 ug shRNAs were transfected into 200,000 *SMN2*-luciferase reporter cells in a 12 well tissue culture treated dish using Lipofectamine 2000 (Invitrogen) at a 1:4 DNA: Lipofectamine 2000 ratio.

Luciferase Assays

Luciferase was measured 48 hours post transfection by harvesting cells with 1X trypsin (Gibco #15050-065) followed by trypsin inhibitor (Invitrogen #17075-029) followed by PBS collection and centrifugation. 10% of cells by volume were equally distributed into 3 wells of a white tissue culture 96 well plate. The remaining 90% of cells were saved for RNA quantification. Promega Dual-glo luciferase assay system was used to determine SMN-luciferase activity as done previously (Cherry). MS-275 was purchased through SelleckChem (#W12277).

Primary Cell Assays

Lentiviruses expressing HDAC shRNA clones were obtained from the UMass Medical School RNAi Core Facility. Lentivirus was added to 250,000 primary 3813 cells, plated in a 10 cm tissue culture dish, at a multiplicity of infection (MOI) of 10 in the presence of polybrene. Growth medium containing 10% FBS and 1 % p/s was replaced 24 hours following infection and allowed to grow an additional 48 hours before harvesting RNA by Trizol preparation. Infection was confirmed prior to the media change by way of visual confirmation of GFP marker expression. Reduced mRNA steady state levels were confirmed by semiquantitative endpoint PCR with the same primer sets and conditions as the reporter cell assays.

PCR and qRT-PCR

RNA was isolated from cells using Trizol Reagent (Invitrogen, #15596-026). cDNA was generated using the Improm-II Reverse Transcription System

(Promega, #A3801). The primer sets used are the same as described in chapter 1. Briefly, Primer sequences: Exon5/Xho-forward: catttccttctggaccactcgag, Exon6-forward: gcccaaatctgctccatggaac, Luciferase-reverse: atagcttctgccaaccgaacgg, Exon7-reverse: taaggaatgtgagcaccttccttc, Exon8-reverse: gatctgtctgatcgtttctttagtgg, GAPDH-reverse (G3A): tccaccaccctgttgctgta, GAPDH-forward (G3S): accacagtccatgccatcac.

The primer pairs to determine HDAC mRNA levels and splicing factor mRNA changes were as follows (forward, reverse), HDAC1 (tcggggctggcaaaggcaag, agaccaccgcactaggctgga), HDAC2 (tgctactactacgacggtgatattgga, tcggcagtggctttatggggc), HDAC3 (gccccatcgcctggcattga, tcctcggagtggaagcggca), HDAC4 (cggagaacggtatcgcgccc, ggcaggcccagcgtgatgtt), HDAC5 (tgaactctcccaacgagtcgga, gggcatggctcttggcagca), HDAC6 (acccacctgctgatgggcct, gcaggggtggtgggtctcca), HDAC7 (ccgttcgccttgccgacagt, gtgtgggggctccccaggat). HDAC8 (cagtgggcagtcgctggtcc, ggaaggtggccatctcctcca), hTra2β (cacatcgaccggcgacagca, ccccgatccgtgagcacttcc), SF2/ASF (ttagatctcatgagggagaaactgcc, ggcttctgctacgactacggc), hnRNP A1 (agggcgaaggtaggctggca, gcttcctcagctgttcgggct).

qPCR was performed as described in the protocol for iQ SybrGreen Supermix (BioRad, #170-8882) in an Eppendorf Mastercycler ep Realplex 4 real-time PCR machine. Reactions were incubated for a 10 minute 94 ^{o}C hot start followed by 45 cycles of the following: 94 ^{o}C for 45 second, 60 ^{o}C for 15 seconds,

72 ^{o}C for 45 seconds. The Pfaffl method was used to determine the change in transcript levels relative to the DMSO or non-silencing shRNA controls and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Chromosome Immunoprecipitation (ChIP)

Formaldehyde was added directly to tissue culture media to a final concentration of 1%. Cells were allowed to incubate at 37^{o} C for 10 minutes. Cells were washed with 1X PBS and collected in 1mL PBS. ChIP protocol was performed as described (Upstate Cell Signaling) using anti-acetyl histone H3 (Upstate # 06-599) and RNA Polymerase II CTD (abcam #ab5095). Antibodies were bound and washed using Protein A Dynabeads (Invitrogen # 10002D) Following reverse crosslinking and Proteinase K digestion, DNA was purified using Qiagen Quick PCR Clean-Up Kit. qPCR was performed as above. Percent input bound was calculated based on a 5 point standard dilution curve of each relative input. Primer Sequences: Forward (Promoter): ggccaccgtactgttccgc, Reverse (Exon 2): ccaaatgtcagaatcatcgctctgg.

Statistical Analysis

An unpaired two-tailed t-test was selected to compare the means of treated and untreated samples. Treated and untreated samples were handled identically and therefore any variations can be consider equal. Statistical significance is determined by a p-value less than 0.05, or less than 5% confidence that the difference between the two samples are sue to random variance.

Results

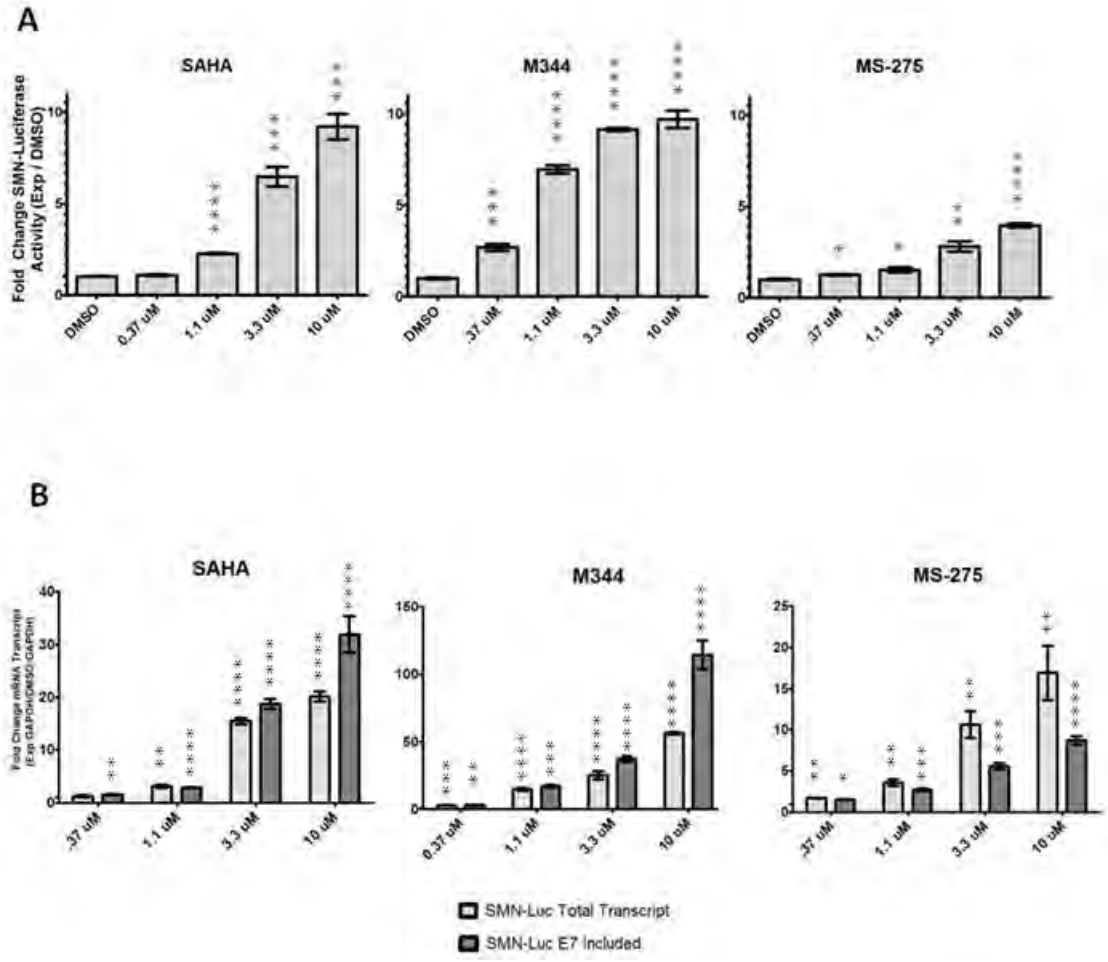
SMN2-Luciferase reporter responds to HDAC inhibitors

We theorized that individual HDAC proteins have distinct contributions to the regulation of SMN expression. We wished to characterize how a class I specific HDAC inhibitor would respond in our reporter cell system [138]. We compared the selective HDAC inhibitor MS-275 (Entinostat) with two pan-HDAC inhibitors, SAHA and M344. Both have been shown to increase SMN levels. MS-275 has previously been tested for activation of SMN levels, however no significant induction was observed in primary SMA patient fibroblasts [139]. The reporter used in this study is expressed from the 3.4 kB *SMN2* promoter followed by exons 1 through 5 cDNA linked to a *SMN2*-luciferase splicing cassette ([111]). The luciferase protein is only properly translated when SMN exon 7 is included in the processed mRNA. When exon 7 is excluded, the exon 6-8 junction shifts the reading frame and translation is prematurely terminated early in the luciferase gene (Figure 1.1) Each of these drugs were tested at four concentrations, .37 µM, 1.10 µM, 3.30 µM and 10.0 µM. Both SAHA and M344 increased SMN luciferase levels up to 10 fold. MS-275 activity was less active, only achieving 4 fold increase of the fusion protein (Figure 2.2A). Activation of the reporter by the class I specific drug MS-275 implied that inhibition of a subset of HDAC proteins is sufficient to increase SMN protein levels.

Analysis of mRNA changes in the *SMN2*-luciferase reporter following HDACi treatment

To further elucidate the mechanism that the individual HDAC inhibitors use to increase the SMN expression, we quantified changes in the reporter mRNA. We used primers specific to the SMN-luciferase splicing cassette transcript to measure the increase of both total transcript and exon 7 included mRNA alone. The primers used to measure the total levels of reporter mRNA span exon 6 through luciferase. Therefore, both exon 7 included and exon 7 skipped transcripts will be measured. Exon 7 included transcripts use a primer set from exon 6 through exon 7. Increases of total reporter mRNA is indicative of an upregulation of transcription at the *SMN2* promoter. An increase of exon 7 included reporter mRNA greater than that observed for total reporter mRNA can be considered a shift in the splicing of exon 7. This can only be inferred because changes in splicing of the reporter mRNA should not affect the amount of mRNA present, only the ratio of total reporter mRNA to exon 7 excluded transcripts. SAHA and M344 induced nearly identical increases in the reporter mRNA. Both total and exon 7 included mRNA were increased following drug treatment. As the drug concentration increased there was a greater difference between exon 7 included transcripts and total mRNA. (Figure 2.2B) This suggests that at lower concentrations the pan-HDAC inhibitors may directly affect the acetylation state of the histones populating the *SMN2* reporter promoter. With the increase of drug, there is also an increased effect on relevant targets potentially including the

Figure 2.3. HDAC inhibition increases SMN-Luciferase Activity and Reporter mRNA Levels. (A) HDAC inhibitors increase SMN-luciferase activity levels. *SMN2* reporter cells were treated for 24 hours with 0.37, 1.10, 3.30, and 10.0 µM SAHA, M344, and MS-275. Pan-HDAC inhibitors increased reporter activity nearly 10 fold at the highest concentration. Class I specific HDAC inhibitor increase reporter activity 4 fold. (B) qRT-PCR was used to measure increases of SMN-luciferase mRNA following treatment with HDAC inhibitors. Fold increase of mRNA was normalized to GAPDH. Pan-HDAC inhibitors SAHA and M344 increase exon 7 reporter mRNA a greater fold than total mRNA. MS-275 appears to increase total SMN-luciferase mRNA preferentially. Two tailed t-test was used to calculate statistical significance. P-values – (**** - < 0.0001, *** - 0.0001 - 0.001, ** - 0.0011 – 0.01, * - 0.011-.05). Error bars are SEM.


A
SAHA
M344
MS-275
Fold Change SMN-Luciferase Activity (Exp / DMSO)
DMSO
0.37 uM
1.1 uM
3.3 uM
10 uM
B
SAHA
M344
MS-275
SMN-Luc Total Transcript
SMN-Luc E7 Included

splicing factors which in turn modifies *SMN2* splicing. The reporter mRNA analysis agrees with previously published reporter stating that these drugs not only increase the transcription activity at the *SMN2* promoter but also increase exon 7 inclusion.

Analysis of mRNA changes following MS-275 treatment revealed a different profile than what was observed with the pan-inhibitors. Class I specific HDAC inhibition increased both total and exon 7 included mRNA. However, the fold increases of the total transcript exceeded that of exon 7 included (Figure 2.2B). Therefore, we propose that MS-275 maintains the ability to positively affect *SMN2* transcription, but is not capable of reducing exon 7 skipping.

Silencing of HDAC proteins increases SMN reporter protein levels

Analysis of mRNA increases with MS-275 and pan-HDAC inhibitors SAHA and M334 revealed key mechanistic differences. Therefore, we targeted individual HDAC proteins to clarify the possible role that each plays in regulating SMN levels. Due to the close homology of all of the HDAC proteins, we targeted a sampling of both class I and class II HDAC proteins using an expression based shRNA construct. This method allowed for direct and specific inhibition of a single HDAC protein, avoiding cross inhibition between HDACs possible with the use of chemical inhibitors. We transfected the *SMN2* reporter cells with shRNAs specific for HDAC1-8 and assayed for changes in the SMN-luciferase activity levels. We confirmed using semi-quantitative endpoint-PCR HDAC mRNA

knockdown (Figure 2.4). Reduced levels of the class I HDAC1, 2, 3, and 8, all increased SMN-luciferase reporter by at least 1.5 fold (Figure 2.5A).

Knockdown of the individual class II HDACs 4, 6, and 7 resulted in an increase of reporter activity, with HDAC6 showing the highest induction. HDAC5 silencing resulted in a small increase in luciferase activity (p-value - 0.0222). While this does indicate with high probability that the increase of reporter activity was not random, the same levels of confidence (p-value < 0.0001) reached by other HDAC knockdown was not achieved. Therefore, at this point of investigation the direct role of HDAC5 on *SMN2* expression may be significantly muted relative to the other HDAC proteins. The level of reporter activation following shRNA transfection was tempered in comparison to the fold changes observed using HDAC inhibitors. However, this could be expected as we measured only the contribution of partial decrease of an individual HDAC protein relative to broader inhibition of HDACs by chemical inhibitors.

Analysis of mRNA changes in the *SMN2*-luciferase reporter following shRNA treatment

We hypothesized that the differences in mRNA expression observed in the presence of class specific HDAC inhibitors would also be observed when individual HDAC proteins were specifically targeted. The increased mRNA expression profiles for in each class of protein mirrored the results from the drug assays. Knockdown of the class I HDAC proteins, HDAC1, 2, 3, and 8, increased total mRNA more than exon 7 included transcript (Figure 2.5B). This is

Figure 2.4. Endpoint RT-PCR Confirms Silencing of Individual HDAC mRNAs. Semi-quantitative PCR of individual HDAC proteins transcripts with non-silencing or specific shRNA treatment. RNA was harvested 48 hours post-transfection of 1 ug of shRNA construct. Percent knockdown of mRNA was quantified using ImageJ to analyze pixel intensity and density. Signal was normalized to GAPDH RT-PCR reactions. Quantification of signal loss is presented to the right of gel data. Error bars are SEM.

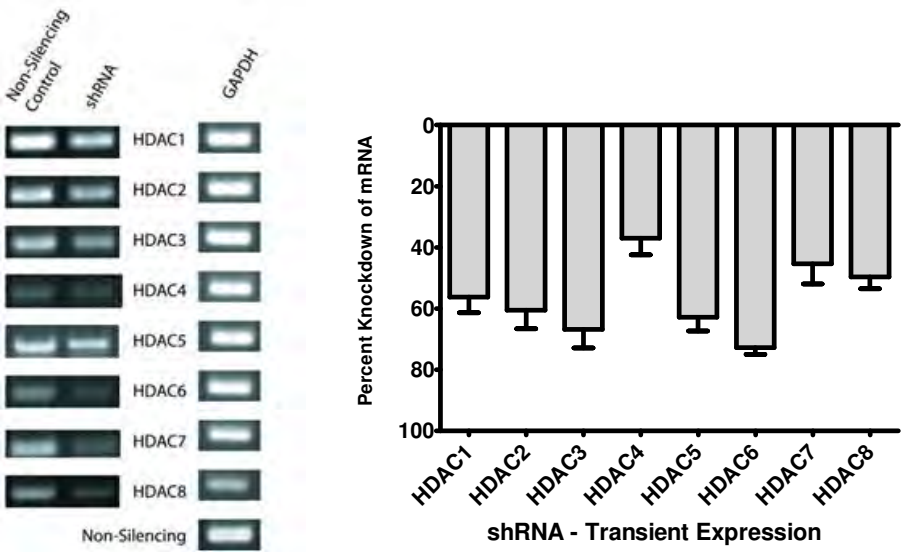
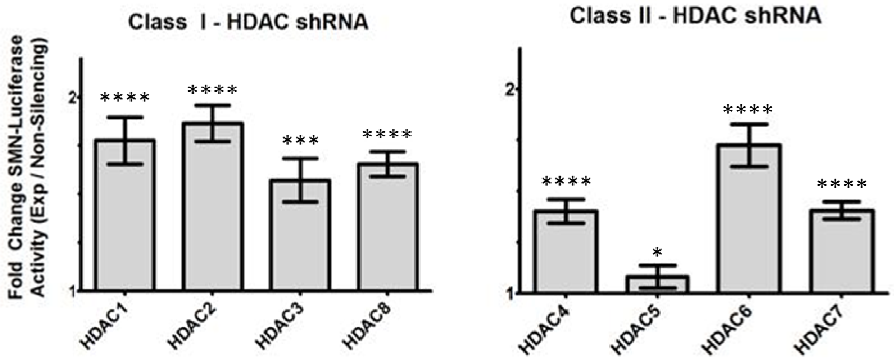
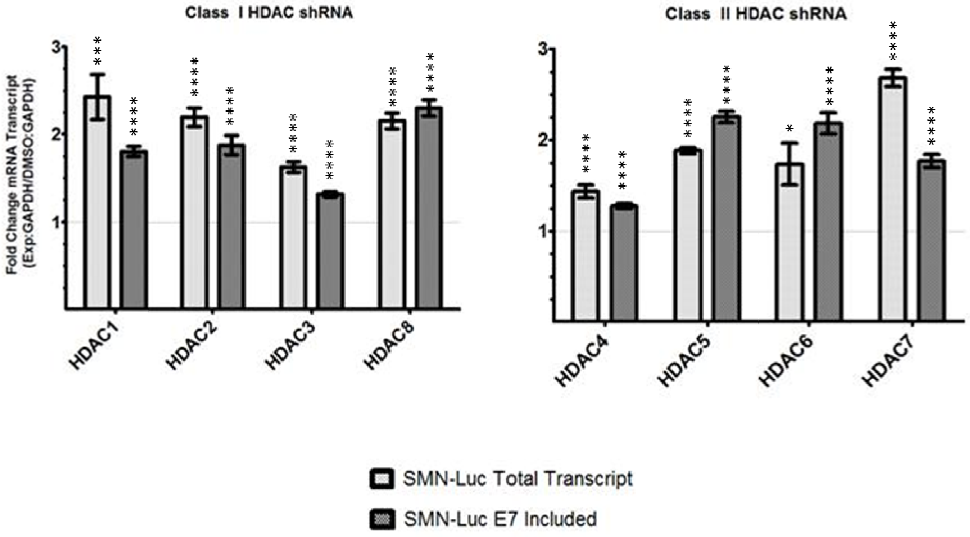

Non-Silencing Control
shRNA
GAPDH
HDAC1
HDAC2
HDAC3
HDAC4
HDAC5
HDAC6
HDAC7
HDAC8
Non-Silencing
Percent Knockdown of mRNA
0
20
40
60
80
100
HDAC1
HDAC2
HDAC3
HDAC4
HDAC5
HDAC6
HDAC7
HDAC8
shRNA - Transient Expression

Figure 2.5. shRNA Silencing of Individual HDACs Increase SMN-Luciferase Activity and mRNA. (A) HDAC shRNAs increase SMN-luciferase activity levels. *SMN2* reporter cells were treated for 48 hours with shRNA. HDAC2 silencing increased luciferase activity the greatest at nearly 2 fold. HDAC5 knockdown appears to have no effect on luciferase activity (B) qRT-PCR was used to measure increases of SMN-luciferase mRNA following treatment with HDAC shRNA. Fold increase of mRNA was normalized to GAPDH. Class I HDAC proteins shRNA treatment increased total reporter mRNA greater than exon 7 included mRNA indicating possible transcriptional activation. HDAC4 and 7 also appear to increase reporter transcription, while HDAC5 and HDAC6 increase exon 7 inclusion in addition to enhancing transcription. Two tailed t-test was used to calculate statistical significance. P-values – (**** - < 0.0001, *** - 0.0001 - 0.001, ** - 0.0011 – 0.01, * - 0.011-.05) Error bars are SEM.

A

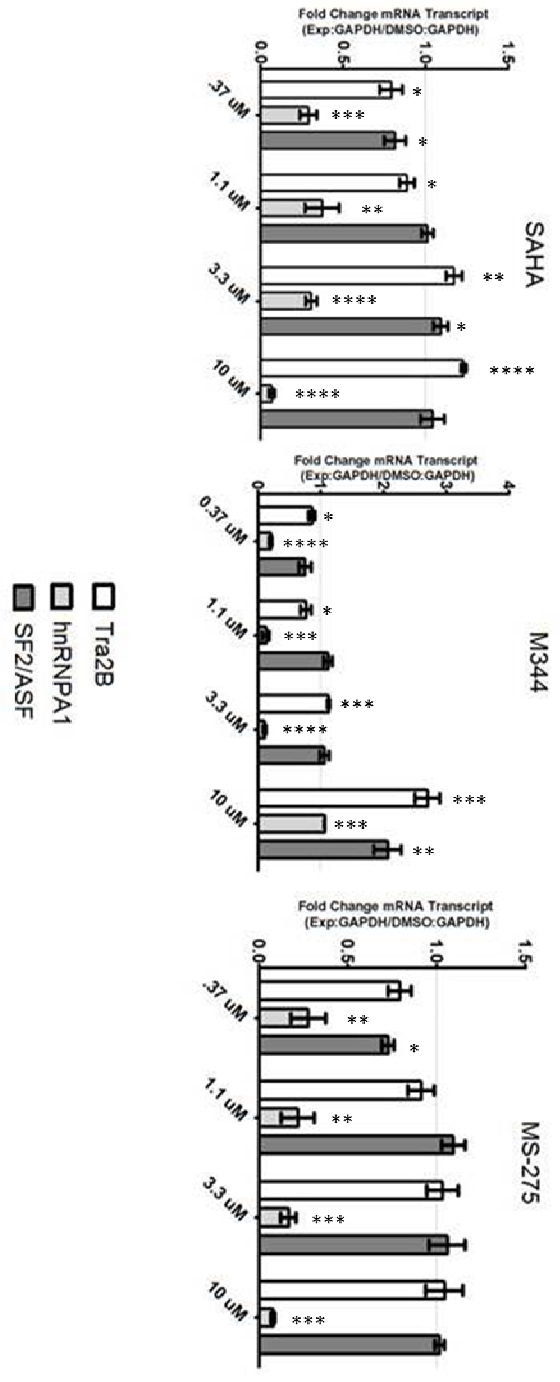

Class I - HDAC shRNA
Fold Change SMN-Luciferase Activity (Exp / Non-Silencing)
HDAC1
HDAC2
HDAC3
HDAC8
Class II - HDAC shRNA
HDAC4
HDAC5
HDAC6
HDAC7

B


Class I HDAC shRNA
Fold Change mRNA Transcript (Exp:GAPDH/DMSO:GAPDH)
HDAC1
HDAC2
HDAC3
HDAC8
Class II HDAC shRNA
HDAC4
HDAC5
HDAC6
HDAC7
SMN-Luc Total Transcript
SMN-Luc E7 Included

consistent with the results from the MS-275 and supports a transcriptional regulation role for these proteins. Analysis of the mRNA increases following shRNA transfection of the class II HDAC protein revealed a more diverse set of profiles. Silencing of HDACs 4 and 7 resulted in a pattern consistent with transcriptional regulation, while HDAC5 and 6 shRNA treatment increased exon 7 included mRNA more than total reporter mRNA. This suggests that these two proteins contribute to the splicing regulation of SMN exon 7 (Figure 2.5B). In comparison to the other proteins tested it is with the least amount of confidence (p-value 0.0107) that HDAC6 has significant effect on *SMN2* transcription. This may be highly relevant due to HDAC6's highly documented role in the cytoplasm. HDAC5 shRNA treatment resulted in an abundant increase of SMN-luciferase mRNA transcripts without any noticeable impact on SMN-luciferase activity (Figure 2.5A). The known function of HDAC5 does not indicate an explanation for this observation. It is possible that silencing of HDAC5 increased reporter mRNA and promoted inclusion of exon 7, but concurrently inhibited translation or caused a premature termination or translational frame shift. We next attempted to target HDAC proteins in the primary SMA cell line 3813 and measure endogenous *SMN2* transcripts. We infected 3813 cells with lentivirus expressing the same shRNA used transiently on reporter cells. We confirmed knockdown of HDAC mRNA using semi-quantitative PCR. Results indicated there were no increases of endogenous *SMN2* transcripts that would not have also been present in an identical population (p-values > 0.1, data not shown).

Figure 2.6. Quantitative PCR of Endogenous Splicing Factors In Response to HDAC Inhibitor Treatment In *SMN2*-Luciferase Reporter Cells. *SMN2*-luciferase reporter cells were treated with 4 concentrations of HDAC inhibitors SAHA, M344, and MS-275. mRNA levels of splicing factors hTra2β (white bars), hnRNPA1 (gray bars), and SF2/ASF (dark gray bars). qRT-PCR was used to measure fold changes in transcript levels relative to DMSO carrier treated cells. GAPDH was used for normalization. Two tailed t-test was used to calculate statistical significance. P-values – (**** - < 0.0001, *** - 0.0001 - 0.001, ** - 0.0011 – 0.01, * - 0.011-.05). Error bars are SEM.


Fold Change mRNA Transcript
(Exp:GAPDH/DMSO:GAPDH)
0.0
0.5
1.0
1.5
.37 uM
1.1 uM
3.3 uM
10 uM
SAHA
Fold Change mRNA Transcript
(Exp:GAPDH/DMSO:GAPDH)
0
1
2
3
4
0.37 uM
1.1 uM
3.3 uM
10 uM
M344
Fold Change mRNA Transcript
(Exp:GAPDH/DMSO:GAPDH)
0.0
0.5
1.0
1.5
.37 uM
1.1 uM
3.3 uM
10 uM
MS-275
Tra2B
hnRNPA1
SF2/ASF

Analysis of changes of endogenous splicing factor mRNAs

Increases in mRNA and protein levels of the known SMN splicing factors Tra2β, hnRNAP A1, and SF2/ASF have been reported to promote inclusion of SMN exon 7 in *SMN2* transcripts. To investigate whether these same mechanisms are operative in the reporter cells; we measured the mRNA levels of these splicing factors following drug or shRNA treatment. hnRNP A1, an inhibitory splicing factor, was decreased following treatment with each of the three chemical HDAC inhibitors tested. At 10.0 μM M344, hnRNP A1 transcripts were elevated relative to the lower levels observed at lower drug concentrations. hTra2β transcript levels were increased in response to both M344 and SAHA, with no effect in response to MS-275 treatment. SF2/ASF was only significantly increased when reporter cells were treated with M344 (Figure 2.6). This is in agreement with data in Figure 2.3 showing a more potent response in fold increase of exon 7 included mRNA in response to M344 over that of SAHA at the same concentration.

Analysis of these same transcripts following shRNA treatments did not reveal any direct pattern associated with the mechanisms determined by SMN transcripts analysis. shRNAs specific to HDAC1 or HDAC2, both suspected transcriptional regulators, show nearly identical effects on total and exon 7 included SMN reporter mRNAs in Figure 2; however, in this experiment low levels of HDAC1 significantly increased hTra2β and SF2/ASF (p-values 0.0425, < 0.0001 respectively), while HDAC2 shRNA treatment was inactive (p-values

0.66, 0.76 respectively) (Figure 2.7). Additionally, silencing of HDAC5 and 6 similarly increase the levels of endogenous splicing factors as HDAC1; yet analysis of the SMN reporter transcripts argues the mechanism of SMN increase differs between the two HDACs. Therefore, there is no direct correlation of these splicing factor mRNA levels to the affect on SMN reporter mRNA. Modulation of SMN splicing is not exclusive to these three proteins measured. The entirety of proteins required to promote exon 7 inclusion may not be sufficiently modified by single HDAC knockdown. Hence, the reason why more potent pan-HDAC inhibitors may be required to directly modify SMN exon 7 splicing.

Quantification of Acetyl-Histone H3 and RNA Polymerase II at the reporter promoter

We measured the amount of acetyl-histone H3 and RNA Polymerase II present at the reporter promoter to determine the effects shRNA or HDAC inhibitor treatment imparts on transcriptional modulators. Acetyl-H3 has been reported to populate the *SMN2* promoter surrounding the transcriptional start site [129]. In the same study HDACi treatment increased the bound levels of acetyl-H3 occupying the promoter of primary SMA fibroblasts. We treated reporter cells with a single dosage of each HDACi as well as each shRNA. We performed chromosome immunoprecipitation using an acetyl-histone H3 and RNAPII antibodies and subsequent qPCR to measure the percent of input DNA bound by acetyl-H3 and RNAP.

Figure 2.7. Quantitative PCR of Endogenous Splicing Factors In Response to HDAC shRNA Treatment In *SMN2*-Luciferase Reporter Cells. *SMN2*-luciferase reporter cells were treated with HDAC specific shRNA. mRNA levels of splicing factors hTra2β (white bars), hnRNPA1 (gray bars), and SF2/ASF (dark gray bars). qRT-PCR was used to measure fold changes in transcript levels relative to a non-silencing shRNA control. GAPDH was used for normalization. Two tailed t-test was used to calculate statistical significance. P-values – (**** - < 0.0001, *** - 0.0001 - 0.001, ** - 0.0011 – 0.01, * - 0.011-.05). Error bars are SEM.

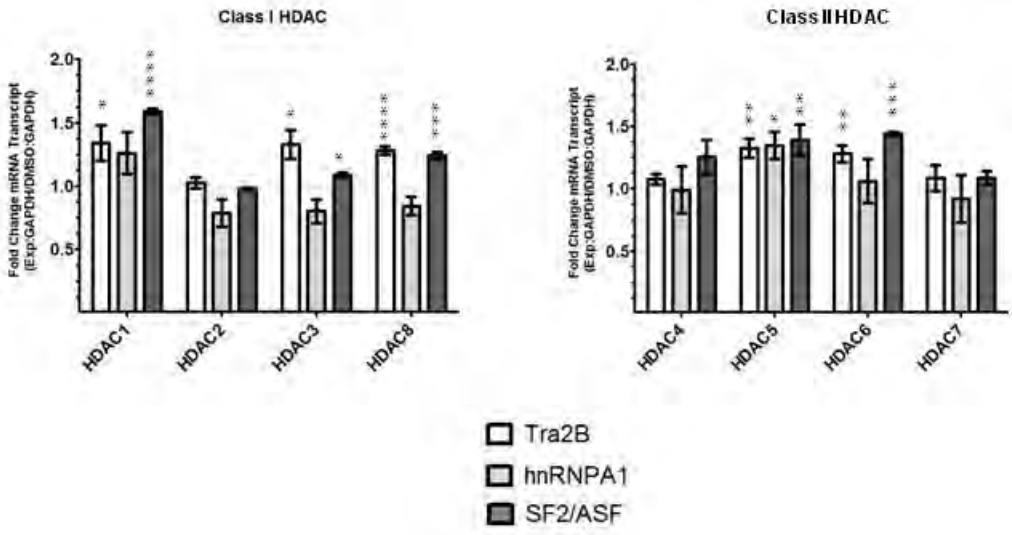
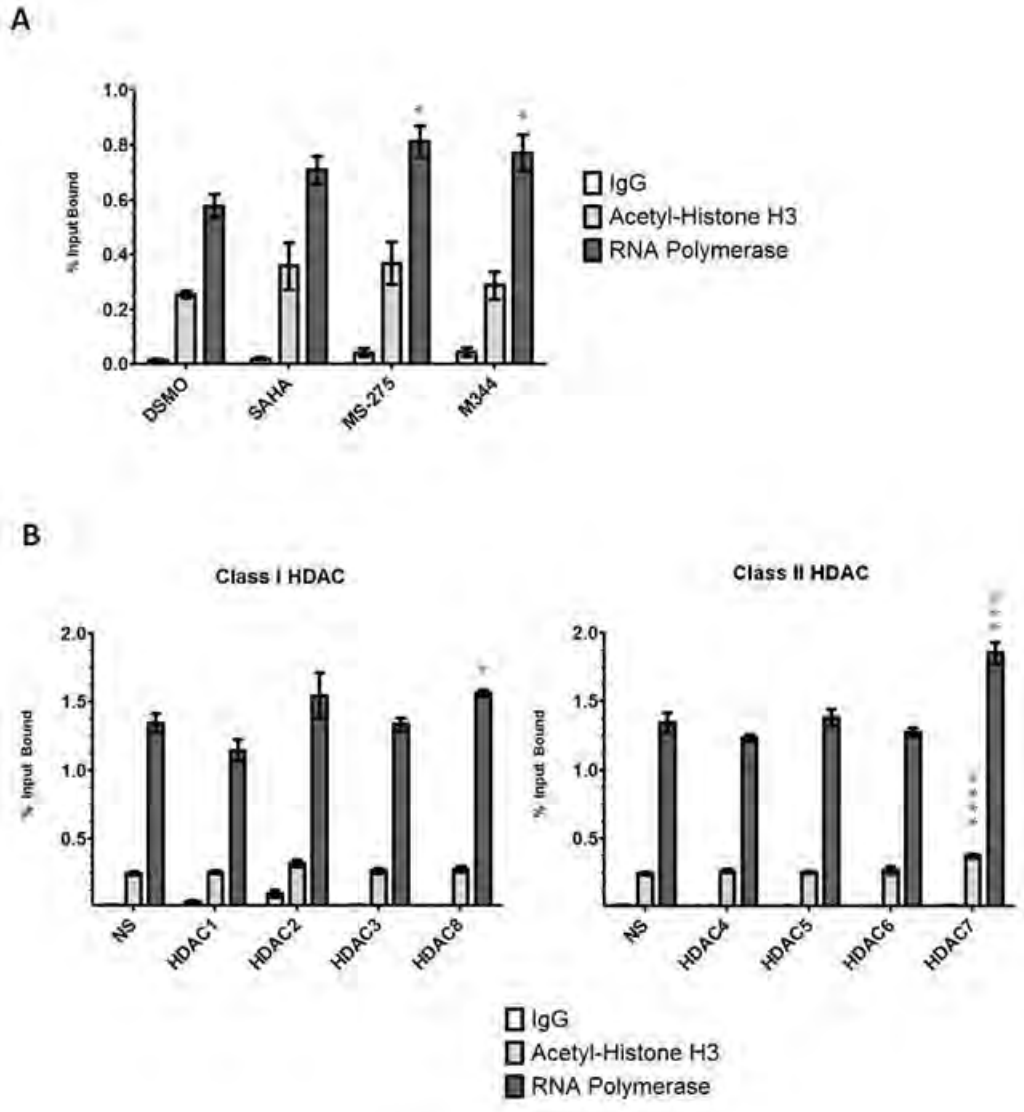

Class I HDAC
Class II HDAC
Fold Change mRNA Transcript (Exp:GAPDH/DMSO:GAPDH)
2.0
1.5
1.0
0.5
HDAC1
HDAC2
HDAC3
HDAC8
HDAC4
HDAC5
HDAC6
HDAC7
Tra2B
hnRNPA1
SF2/ASF

Figure 2.8. Quantitative Chromosome Immunoprecipitation of the *SMN2*-Luciferase Promoter. The SMN promoter was isolated from HDAC inhibitor (A) or HDAC shRNA treated HEK293T *SMN2*-luciferase reporter cells using chromosome immunoprecipitation. Irrelevant Rabbit IgG was used for a negative control. qPCR was used to accurate determine the percent of input DNA bound by anti-acetyl-H3 and anti-RNA Polymerase CTD antibodies. Two tailed t-test was used to calculate statistical significance relative to DMSO or non-silencing shRNA controls. P-values – (**** - < 0.0001, *** - 0.0001 - 0.001, ** - 0.0011 – 0.01, * - 0.011-.05). Error bars are SEM.


A
% Input Bound
1.0
0.8
0.6
0.4
0.2
0.0
DSMO
SAHA
MS-275
M344
IgG
Acetyl-Histone H3
RNA Polymerase
B
Class I HDAC
Class II HDAC
% Input Bound
2.0
1.5
1.0
0.5
NS
HDAC1
HDAC2
HDAC3
HDAC8
HDAC4
HDAC5
HDAC6
HDAC7
IgG
Acetyl-Histone H3
RNA Polymerase

Treatment of the cells with each of the three HDAC inhibitors resulted in increases of approximately 1.5 fold in RNAP ChIP at the reporter promoter (Figure 2.8A). High deviations between experiments revealed that only MS-275 and M344 treatment increased RNAP presence at the reporter promoter greater than random chance (p-values 0.0046 and 0.0257 respectively). All increases observed with Acetyl-Histone H3 ChIP were insignificant relative to DMSO treatment (p-values > 0.05). Increases of acetyl-H3 with HDAC inhibitors were consistent with published reports. HDAC shRNA treatment did not yield any statistically significant increases of acetyl-H3 or RNAP ChIP with the exception of HDAC7 and 8 (Figure 2.8B). HDAC7 silencing increased the amount of Acetyl-Histone H3 and RNAP polymerase ChIP at the *SMN2* reporter promoter approximately 1.4 fold (p-values < 0.0001 and 0.0006 respectively). This result is comparable to drug treatment and supports the data that HDAC7 primarily regulates SMN transcription (Figure 2.5B).

Discussion and Future Directions

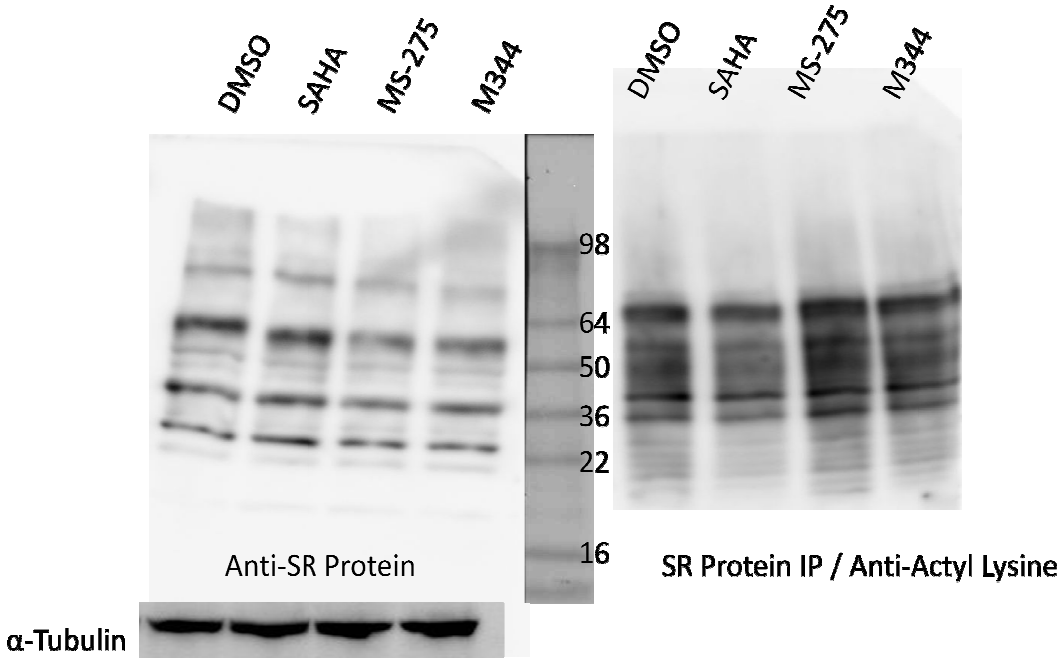
HDAC inhibitors including phenylbutyrate and valproic acid are currently or have been tested in clinical trials for SMA treatments because of evidence of effective increases of full length SMN protein from the *SMN2* gene. The more potent compounds do not discriminate between HDAC classes. These drugs act by globally increasing the acetylation state of histones, and may affect the entire transcriptome. Through this mechanism, the HDAC inhibitors increase expression of the *SMN2* gene and the levels of other modulating proteins such as splicing factors. Ultimately, the goal of increasing full length SMN protein is achieved, which makes this class of compounds attractive SMA therapeutic drugs. To gain a better understanding of the contributions of individual HDAC proteins, we targeted individual HDAC mRNAs and measured the effects of shRNA treatment on *SMN2* transcription and splicing.

The strength of this study was the excellent responsiveness and consistency of the HEK293 *SMN2*-luciferase reporter cell line. We were able to detect changes in *SMN2* mRNA and protein in response to individual HDAC knockdown as well as class I specific HDAC inhibitor MS-275. However, other cell lines, including primary SMA fibroblasts, were less responsive to these treatments. The observed fold changes of SMN protein and mRNA increases in the reporter cells with SAHA and aclarubicin is 2 to 6 fold greater than in primary 3813 cells (Cherry, submitted data). This robust response is most likely due to

the high number of copies of the episomal reporter stably maintained in the cells. Therefore, it is reasonable to expect a muted response with endogenous SMN transcripts in 3813 cells in response to HDAC knockdown. This *SMN2* reporter design appears mimic the endogenous context better than any other system to date. Despite the lack of primary cell data, it is our belief that the reporter cell line is an excellent tool that yields results that accurately represent the behavior of the endogenous SMN genes.

We were able to demonstrate that targeted silencing of individual HDAC proteins was sufficient to increase full length SMN reporter protein. Our experiments support that each HDAC protein individually contributes to increasing SMN protein with some overlap of mechanism. Inhibition of class I HDACs, in addition to class II HDACs 4 and 7, appeared to modulate *SMN2* reporter transcription, while exon 7 inclusion was increased with the class II HDAC proteins 5 and 6. As already stated, delineation of the classes of HDAC proteins is largely based on yeast protein homology as well as subcellular localization. The class I proteins are exclusively retained within the nucleus while class II HDACs shuttle between the cytoplasm and the nucleus. Therefore, it is interesting that silencing of only class II proteins reduced skipping of exon 7. It is possible that these proteins may control the splicing of exon 7 through modulation of the acetylation state of specific splicing factors. We failed to detect increase of SR protein levels or acetylation by Western blot (Figure 2.8).

Figure 2.9 HDAC Inhibitor Treatment Does Not Induce Detectable Changes in SR Protein Acetylation. Total protein levels and acetylation of SR proteins was measured by immunoprecipitation and Western blot. Anti-SR antibody recognizes SRp75, SRp55, SRp40, SRp30a/b (SF2/ASF), and SRp20. Left Panel – Western blot on HDAC inhibitor treated reporter cell lysates. Middle – Protein ladder, Right Panel – Western blot of Anti-acetyl Lysine antibody on Anti-SR immunoprecipiated HDAC inhibitor treated reporter cell lysates. Any increase in total protein was below reliable detection levels. There was no detected acetylation of SR proteins.


DMSO
SAHA
MS-275
M344
98
64
50
36
22
16
Anti-SR Protein
α-Tubulin
DMSO
SAHA
MS-275
M344
SR Protein IP / Anti-Actyl Lysine

Undetectable levels of SR protein acetylation could be sufficient to induce change in splicing activity or effect secondary post-translational regulatory modifications such as phosphorylation.

These data demonstrate that SMN protein levels can be increased through RNAi silencing of individual HDAC proteins. HDAC2 and HDAC 6 may be the most compelling targets for SMA therapy. Kernochan et al. identified HDAC2 as a prime candidate for therapy based largely on its population at the *SMN2* promoter and potential role in developmental regulation of SMN protein levels [129]. Recent works have identified these two HDACs as having neuroprotective properties. Specifically, HDAC2 deficiency resulted in increased synapse number as well as improved memory formation [140]. Interestingly, HDAC2, rather than HDAC1, populates promoters of genes directly associated with synaptic plasticity. This is also consistent with results obtained probing the *SMN2*-luciferase reporter promoter (Figure 2.2). Further highlighting the role of HDAC2 in synaptic plasticity, SAHA was unable to change synapse numbers in HDAC2 deficient mice in contrast to treated wild-type animals [140]. This suggests that the transcriptional effect of SAHA on *SMN2* transcription may be dependent on HDAC2.

HDAC6 is an interesting drug target because a neuron-specific function has been extensively established. HDAC6 is localized exclusively to the cytoplasm and complexes with motor proteins to regulate the acetylation state and thereby the stability of microtubules [127]. Acetylation of tubulin increases

microtubule stability and reduces tubulin dynamics. The growth plate of neurons is normally void of acetylated tubulin. Therefore, regulation of HDAC6 levels or activity could drastically alter a motor neurons ability to respond to external stimuli and either promote or inhibit survival. HDAC6 inhibition has also been shown to enhance neuronal cell survival following injury including promoting regeneration [141]. Conversely, HDAC6 was identified as a critical protein that when overexpressed is capable of rescuing mis-folded protein accumulation related autophagy in neurodegenerative disorders [142, 143]. In some mouse models of SMA there is evidence of neurofilament accumulation, however there is no evidence of related autophagy [144]. HDAC6's role in preventing autophagy is linked to association with poly-ubiquitinated proteins and promoting their degradation. It is possible that inhibition of HDAC6 alters the stability of the SMN protein by countering any mechanism that may promote the proteosome based degradation of SMN.

Interestingly, our analysis revealed that HDAC2 and HDAC6 affect SMN mRNA through differing mechanisms, HDAC2 by way of transcription and HDAC6 by increasing exon 7 inclusion in addition to transcriptional enhancement. In combination with proposed neuroprotective properties, as well as unique mechanisms to increase full length SMN protein, inhibition of one or both of the proteins in combination may have profound implications in SMA therapy.

The advantage of pan-inhibitor compounds is that transcriptional and splicing pathways are being targeted simultaneously. This is most likely why the most potent HDACi drugs currently under investigation for SMA fall into this class of compounds. However, many of these same drugs mentioned, including SAHA, MS-275, phenyl butyrate, valproic acid, and sodium butyrate are currently used or are in clinical trials for cancer therapy. It is this class of drugs propensity to induce toxic effects on cell cycle and also cell death that make them valuable in this context. Compounds that are more specific in could be more effective in SMA therapy and lack potential cytotoxic side effects.

Development of more effective and targeted therapies depends of the complete understanding of HDAC related SMN regulation. This study has shown that inhibition of an individual HDAC is sufficient to increase *SMN2* transcription as well as promote the inclusion of exon 7. Therefore, by decreasing the broad genomic effects of pan-HDAC inhibitors it may decrease drug toxicity while maintaining an acceptable level of SMN activation and possibly other neuroprotective effects. A compound that is capable of achieving this, while maintaining potency would be extremely beneficial to SMA patients and families.

Final Remarks and Conclusions

In this study it was demonstrated that RNAi mediated silencing of individual HDAC proteins was sufficient to increase activity levels of a *SMN2*-luciferase reporter cell line. There were different mechanisms employed to increase steady state levels of the SMN-luciferase fusion protein. Class I HDACs appear to primarily regulate transcription at the SMN promoter. Class II HDACs maintain transcriptional regulation of the promoter, but silencing of HDAC5 and HDAC6 also appear to promote inclusion of exon 7. shRNA response in the luciferase and qRT-PCR assays was not as robust as HDAC inhibitor treatment. This is most likely due to combinatory effects when multiple HDACs are simultaneously targeted by the pan-HDAC inhibitors. Regardless, it is apparent that the individual contributions of HDACs are not equal and suggests multiple, possibly overlapping, pathways are at work. Therefore, it implies that the development of more specific HDAC inhibitors could retain the ability to increase SMN protein levels, while reducing cytotoxic side effects.

This work also used quantitative PCR techniques to characterize the effects on SMN-luciferase mRNA by novel chemical compounds identified in high throughput screens. 5 compounds and additional chemical analogs were identified to activate the *SMN2*-luciferase reporter. qRT-PCR was able to identify transcriptional activation as the most probable molecular mechanism employed

by 3 compounds tested. The remaining compounds failed to activate reporter mRNA and most likely function downstream of protein translation.

Following these studies it is important to ask what greater questions may have been answers. In particularly, it is highly relevant to theorize whether more potent pan-HDAC inhibitors are a better SMA therapy than more targeted selective HDAC inhibitors. Furthermore, would a targeted RNAi based therapy reduce pan-HDAC inhibitor associated toxicity while maintaining acceptable levels of SMN protein increase? The negative side effects of HDAC inhibitors are largely associated with the impact on gene expression and acetylation of factors associated with the cell cycle and cell survival.

In the case of pan-HDAC inhibitors approximately 20% of the transcriptome displays altered expression levels. It is unclear whether or not a more selected inhibitor would drastically modify this number, thereby potentially reducing toxic effects. It is possible the number of genes may not change, but the amplitude of the changes may be muted. Maintaining a therapeutic induction of *SMN2* expression while reducing the global impact of the compound would be most beneficial. The balance of increasing SMN protein levels and SMA symptoms versus possible toxic effects of the therapy must be established.

Oral or IV administration of pan-HDAC inhibitors has a widespread effect in all tissues of the body where the drug can penetrate. Therefore, the chance of a negative side effect of drug treatment is enhanced with the number of body systems and functions affected. In SMA, it has been demonstrated that the

greatest benefit to survival is obtained when SMN levels in motor neurons is increased. There is functional benefit and strength increase observed in muscle, however increased expression in neuronal tissue is sufficient to improve life expectancy. The fold increase of SMN expression in response to shRNA mediated knockdown of HDAC proteins is far less than pan-HDAC inhibitors. However, with adenovirus or neuronal specific promoter targeting of RNAi to the motor neuron it may be possible to enhance quality of life, while minimizing other toxic side effects. Motor neurons are terminally differentiated; so any toxic effects on cell cycle may also be minimized in this tissue in response to HDAC RNAi. Alternatively, direct administration of any compound into the spinal cord may achieve similar results; however the risk of infection and immune response is far greater. The effect of HDAC knockdown on SMN protein levels needs to be studied further and optimized. These results do not indicate any induction of SMN levels in patient fibroblasts. *In vivo* testing of optimized short interfering or short-hairpin RNAi will give a more clear picture of the potential role individual HDACs play in SMA therapy.

These experiments established a system to identify RNA based mechanisms to increase SMN transcripts in novel luciferase reporter cell lines. It utilized this system to dissect the role that individual HDACs use in SMN regulation. The effects of pan-HDAC inhibitors on SMN protein levels were better described via the individual HDACs contributions. This data can be used to further develop current SMA therapeutic drugs as well as drive the discovery and

synthesis of new compounds. Furthermore, we have gained greater understanding of the basic biology of SMN regulation by HDAC proteins.

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