MECHANISM OF ACTION OF HISTONE DEACETYLASE INHIBITORS ON *SURVIVAL MOTOR NEURON 2* PROMOTER

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Bachelors of Science in Biological Sciences with Distinction

Spring 2018

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ACKNOWLEDGMENTS

I would like to acknowledge my thesis director Dr. Butchbach for his wonderful guidance and patience as I worked through my project. He has been an excellent research mentor over the last two years and I am forever thankful that he welcomed me into his lab. His dedication to his work inspires me as an aspiring research scientist. His lessons will carry on with me as I pursue future research in graduate school and beyond.

I would like to thank both current and former members of the Motor Neuron Disease Laboratory: Sambee Kanda, Kyle Hinkle, and Andrew Connell. Sambee and Andrew patiently taught me many of the techniques I utilized in my project, and without them it would not be what it is today. All members of the lab offered me support and encouragement throughout my work on my thesis, and I am grateful that I got to work alongside them.

I would like to thank the other members of my thesis committee, Dr. Galileo, Dr. Laverty, and Dr. Cooper for their time and dedication to supporting me and other undergraduate researchers. The three classes I had with Dr. Galileo played a major role in shaping my future research pursuits, and I am delighted to have had him as a professor as well as committee member.

Finally, I would like to thank my family and friends. Most of them are not biology-oriented individuals, but they listened to and supported me throughout my work on my thesis. Their love helps to keep me going.

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ABSTRACT

Spinal muscular atrophy (SMA) is an early-onset recessive neurodegenerative disease that primarily affects the α-motor neurons in the anterior horn of the spinal cord. The degeneration of these motor neurons leads to gradual muscular atrophy, eventual respiratory complications, and early death in severe types of SMA. Due to deletion or mutation events, patients with SMA lack a functional copy of the *Survival Motor Neuron 1* (*SMN1*) gene. Humans however have a nearly identical copy of *SMN1* known as *Survival Motor Neuron 2* (*SMN2)*, which is retained in SMA patients. The major difference between *SMN1* and *SMN2* is that *SMN2* contains a nucleotide substitution in exon 7 that results in the exclusion of this exon in the majority of mRNA transcripts produced from *SMN2*. This exclusion of exon 7 results in low levels of functional SMN protein. In animal models and within patient populations, it has been shown that increasing *SMN2* copy number results in a less severe SMA phenotype, making *SMN2* an ideal target for SMA therapeutics. Histone deacetylase (HDAC) inhibitors have been extensively studied for treatment of SMA through increasing transcription of the *SMN2* gene; however, several of these HDAC inhibitors demonstrated a highly variable patient response and possible toxicity. In this project, I examine the HDAC inhibitors RGFP106, RGFP109, CAY10433, and HDACi-IV to determine their potential for increasing *SMN2* transcription and develop a *SMN2* promoter dual luciferase assay for studying the potential mechanism of action of these drug compounds. These compounds were selected to study as they have shown positive effects on *SMN2* expression in human SMA fibroblast cell lines. RGFP106

and RGFP109 increased transcription of *Smn* in NSC-34 motor neuron-like cells. The preliminary test of the *SMN2* dual luciferase assay showed that all test compounds activated the *SMN2* promoter, but this data was possibly skewed due to concurrent up-regulation of the *TK* promoter control. My drug compounds of interest failed to significantly increase promoter activation in a *SMN2* β-lactamase promoter assay. My findings indicate that these HDAC inhibitors may not act on the *SMN2* promoter through direct transcription factor based activity, thus an alternative mechanism must be explored.

Chapter 1

INTRODUCTION

1.1 Spinal Muscular Atrophy and *SMN*

Spinal muscular atrophy (SMA) is an early-onset recessive neurodegenerative disease in which the α-motor neurons of the anterior horn of the spinal cord and lower brainstem are lost (Crawford and Pardo, 1996). This loss results in the atrophy of the limb and trunk muscles, leading to eventual respiratory complications and a shortened lifespan. SMA has a high incidence rate of approximately 1 in 10,000 live births (Cuscó et al., 2002) and, as a result, is one of the leading genetics causes of infant death. There are five clinical grades of SMA, ranging from the most severe Type 0 to the mildest Type IV, which are assigned on the basis of disease severity and age-of-onset (Zerres and Rudnik-Schöneborn, 1995). SMA has limited treatment options, with only one FDA-approved drug commercially available, the antisense drug nusinersen (Wood et al., 2017). Bioethical concerns have been raised about the efficiency and cost of nusinersen, so there remains a need to develop more medically effective and cost-efficient treatments for SMA.

The *Survival Motor Neuron 1* (*SMN1*) gene is the disease-determining gene for SMA (Lefebvre et al., 1995). *SMN2* is a nearly identical copy of *SMN1* that resulted due to a large inverted duplication event on chromosome 5 (Lefebvre et al., 1995; Courseaux et al., 2003). Generally in SMA patients *SMN1* has been lost due to deletion or gene conversion events (Lefebvre et al., 1995; Burghes, 1997), while at least one *SMN2* is retained. While *SMN2* and *SMN1* are highly identical, there is an

important difference between the genes in exon 7. *SMN2* contains a C to T transition mutation within exon 7 in a region that normally acts as exonic splice enhancer in *SMN1* (Lorson and Androphy, 2000). Due to this single nucleotide change, <10% of *SMN2* transcripts include exon 7, which is necessary to produce fully functional SMN protein. Full-length SMN (FL-SMN) protein forms a homo-octamer, however SMN lacking exon 7 (SMNΔ7) cannot oligomerize with itself and requires FL-SMN to stabilize it, as shown in Figure 1.1(Lorson et al., 1998; Pellizzoni et al., 1999). The SMN protein plays a role in small nuclear ribonucleoprotein (snRNP) complex assembly, which are vital for splicing of pre-mRNAs. (Burghes and Beattie, 2009).

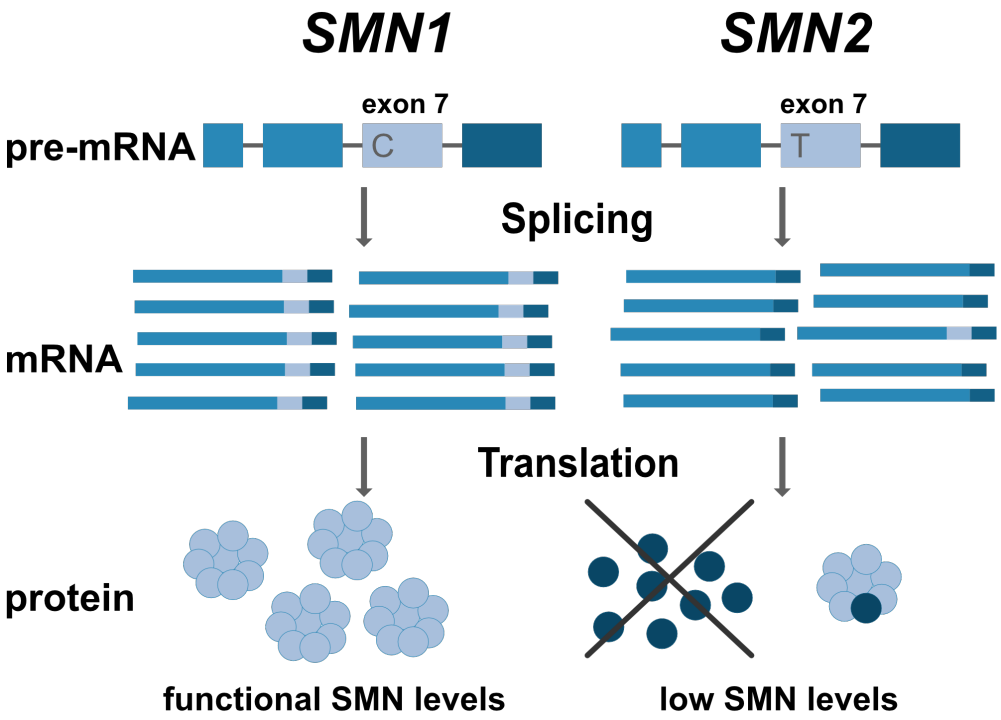

Figure 1.1 Exon 7 inclusion is required for functional SMN protein levels. Adapted from Butchbach, 2016.

Experiments have shown that an increase in the copy number of *SMN2* is inversely correlated with disease severity (reviewed in Butchbach, 2016). For example, a study of type I SMA patients and type III SMA patients revealed that type III SMA patients generally have 3 or 4 copies of *SMN2* while type I SMA patients typically have 2 copies (Mailman et al., 2002). Type II SMA patients fall between these two groups with an average of 3 copies of *SMN2* (Crawford et al., 2012). Patients with the least severe form of SMA, type IV, have been found to have *SMN2* copy numbers between 4 and 6 (Wirth et al., 2006). This effect has also been seen in transgenic mouse models of SMA, with eight copies of *SMN2* rescuing the SMA phenotype (Monani et al., 2000). This inverse relationship between *SMN2* copy number and disease severity suggests *SMN2* is an ideal target for SMA therapeutics. One of the common methods explored for developing SMA therapeutics are compounds that increase *SMN2* transcription.

The promoters of *SMN1* and *SMN2* are highly homologous, and are functionally identical in activity (Monani et al., 1999; Echaniz-Laguna et al., 1999; Germain-Desprez et al., 2001). The region 3.4 kb upstream from the transcription start site of *SMN2* is generally used to represent the *SMN2* promoter, however it has been suggested that the promoter may be at least 4.6 kb (Boda et al., 2001). Experimental evidence suggests that the 3.4 kb promoter contains regulatory DNA elements that are conserved in other species including monkeys and mice (Monani et al., 1999). Previous promoter analysis suggests the *SMN2* promoter is rich with consensus transcription binding sites, which includes the following putative transcription factors: SP1, TFAP2A, H4TF-1, HINFP, AP1, CREB, AP2, YY1, FOXA1, RXRB, and POU3F2 (Monani et al., 1999; Echaniz-Laguna et al., 1999; Germain-Desprez et al.,

2001; Boda et al., 2001). Understanding the roles of these transcription factors in the regulation of *SMN2* expression may be important in understanding how drug compounds are acting to increase overall expression of *SMN2*. As such, further studies of the *SMN2* promoter and its regulatory elements may prove useful in developing targeted therapies for SMA treatment.

1.2 The Histone Code

Chromatin organization is important for gene regulation, and the most basic unit of organization is the nucleosome. The nucleosome unit is made up of DNA wrapped around an octamer of histone proteins: 2 each of H3, H4, H2A, and H2B (Luger et al., 1997). Histones can be dynamically modified on their N-terminus tails in several major ways: acetylation, methylation, phosphorylation, and ubiquitination (Figure 1.2) (Prakash and Fournier, 2018). The sum of these modifications on histone tails is collectively known as the "Histone Code", which functions in regulating gene expression within the cell (Strahl and Allis, 2000). The acetylation and methylation of histones has been extensively studied since the 1960s when these modifications were first discovered by Allfrey et al. (1964) and Phillips (1963). A histone may be methylated on a lysine or an arginine while acetylation occurs exclusively on a lysine (Bannister and Kouzarides, 2011). Methylation of histones is highly complex, as lysine may be mono-, di-, or tri-methylated, while arginine may be mono- or di-methylated (Ng et al., 2008). The addition of a methyl group to an amino acid is mediated by methyltransferases and the removal relies on demethylase enzymes. Similarly histone acetyl groups are added and removed by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively.

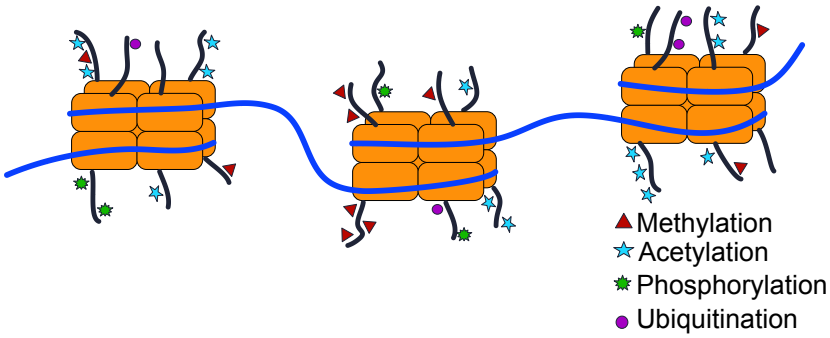

Figure 1.2 Representation of the dynamic modifications of histone tails. Adapted from Kato et al. (2015)

Histone modifications have been experimentally shown to alter gene expression. Methylation is generally associated with decreased transcription due to compaction of chromatin (Allfrey et al., 1964). In contrast, histone acetylation is associated with increasing transcription of genes (Mathis et al., 1978; Hong et al., 1993). The addition of an acetyl group to a lysine neutralizes its positive charge, which in turn changes its affinity for the surrounding DNA and other nucleosomes, reducing compaction (Prakash and Fournier, 2018) Previous experiments have shown that a combination of methylation and acetylation modifications affects overall nucleosome stability, which influences transcription due to nucleosome turnover (Zentner and Henikoff, 2013). HATs and HDACs can also modify non-histone proteins including transcription factors, and the acetylation state alters the stability of these proteins (Spange et al., 2009). Acetylation and methylation of histones both play important roles in maintaining chromatin organization as well as directing other cellular processes allowing for proper gene expression to occur. As a result, histone modification is a process of interest in developing medical therapies because there is

the potential that under the correct conditions genes may be able to be repressed or activated by altering the histone code.

1.3 Histone Deacetylase Inhibitors

Histone deacteylase enzymes are organized into several classes: class I, class II, class III, and class IV. Class I, II and IV HDACs are the "classical" HDACs that rely on a zinc-based mechanism while class III is made up of "sirtuin" HDACs that rely on nicotinamide adenine dinucleotide^{+} (NAD^{+}) (Michan and Sinclair, 2007). The HDACs are organized into classes based on sequence similarity to yeast homologs, due to the yeast HDACs being the first characterized (Morrison et al., 2007). Classical HDACs have been extensively studied in neuronal diseases, while the roles of sirtuin HDACs require further investigation (Thomas and D'mello., 2018; Ajami et al., 2017). The 11 classical HDACs are as follows: class I - HDAC 1, 2, 3, and 8, class II - HDAC 4, 5, 6, 7, 9, and 10, and class IV - HDAC 11 (Gregoretti et al., 2004). Histone deactylase inhibitors disrupt the function of HDACs, resulting in changes to acetylation and methylation patterns. HDAC inhibitors are categorized into several chemical classes including: short-chain fatty acids, hydroxymates, benzamides, and polyphenols (Rajendran et al., 2011). HDAC inhibition of the classical HDACs has shown neuroprotective properties in multiple neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, Huntington's disease, and others (Morrison et al., 2007). Some reported HDAC inhibitor effects in neuronal cells include stimulated synaptogenesis, reduced neurodegeneration, and memory rescue (Morrison et al., 2007; Rumbaugh et al., 2015).

Table 1.1: HDAC inhibitors previously tested for SMA applications.

HDAC inhibitor	Class	Associated HDACs[10, 11]	SMN Effects	
			mRNA	Protein
Sodium Butyrate[5]	Short-chain fatty acid	Class I, IIa	Increase	Increase
Valproic acid[3]	Short-chain fatty acid	Class I, IIa	Increase	Increase
4-phenylbutyrate[1]	Short-chain fatty acid	HDAC1, 2	Increase	Increase
Glyceryl tributyrate[4]	Short-chain fatty acid	N/A	No change	No change
VX563[4]	Short-chain fatty acid	N/A	No change	No change
m-carboxycinnamic acid[8]	Hydroxymate	N/A	Increase	Increase
Suberic bishydroxamic acid[8]	Hydroxymate	N/A	Increase	Increase
LBH589 (Panobinostat)[7, 9]	Hydroxymate	Pan HDACs	Increase	Increase
Scriptaid[9]	Hydroxymate	N/A	Increase	N/A
Oxamflatin[9]	Hydroxymate	N/A	Increase	N/A
Dacinostat[12]	Hydroxymate	N/A	Increase	Increase
Suberoylanilide hydroxamic acid (SAHA)[8, 12]	Hydroxymate	Class I, II	Increase	Increase
Trichostatin A (TSA)[2]	Hydroxymate	Class I, II	Increase	Increase
M344[8]	Benzamide	N/A	Increase	Increase
MS-275[8]	Benzamide	HDAC1, 2, 3, 9	No change	N/A
(E)-Resveratrol[6, 13]	Polyphenol	HDAC8	Increase	Increase
Curcumin[13]	Polyphenol	HDAC8	Increase	Increase
Romidepsin (FL-228)[9]	Cyclic tetrapeptide	HDAC1, 2	Increase	N/A

Compiled from Andressi et al., 2004[1]; Avila et al., 2007[2]; Brichta et al., 2003[3]; Butchbach et al., 2016[4]; Chang et al., 2001[5]; Dayangac-Erden et al., 2009[6]; Garbes et al., 2005[7]; Hahnen et al., 2006[8]; Hauke et al., 2009[9]; Lunke and El-Osta, 2013[10]; Mohseni et al., 2013[11]; Mohseni et al., 2016[12]; Sekla et al., 2008[13].

As shown in Table 1.1, HDAC inhibitors have also been extensively studied as possible treatments for SMA. The first HDAC inhibitor discovered to increase SMN protein levels was sodium butyrate (Chang et al., 2001). Other HDAC inhibitors including 4-phenylbutyrate and valproic acid (Brichta et al., 2003; Andreassi et al., 2004; Brahe et al., 2005) have been shown to increase SMN protein. However when these HDAC inhibitors moved into clinical trials, they ultimately did not have a significant effect on SMA patients. (Also-Rallo et al., 2011). Various other HDAC

inhibitors have also been tested as potential SMA treatments, but have not moved into clinical trials. One of the key concerns of treating SMA with HDAC inhibitors are the off-target or cytotoxic effects that can occur due to changing the acetylation landscape of cells (Hanen et al., 2006; Dayangac-Erden et al., 2009; Reissland et al. 2006). Inhibition of specific HDAC isoforms may reduce this problem. The HDAC6 specific benzamide M344 increased expression of *SMN2* at low concentrations, however the drug displayed cytotoxic effects at higher concentrations raising concerns about long-term use (Reissland et al. 2006). A similar issue arose from the use of HDAC8 specific inhibitor (E)-Resveratrol as an *SMN2* inducer (Dayangac-Erden et al., 2009). An experiment that down-regulated HDACs in a HEK-293 *SMN2* reporter cell line suggested that *SMN2* transcription is regulated by HDAC 1, 2, 3, 4, 7, 6, and 8 (Evans et al., 2011). Thus these HDACs are all potential targets for the development of HDAC inhibitors as possible SMA therapeutics.

1.4 Project Aims and Hypothesis

The aim of this project was to elucidate a possible mechanism of action of HDAC inhibitors as inducers of the *SMN2* promoter. Earlier researchers suggested that the mechanism of promoter activation from HDAC inhibitors likely was based on increased acetylation surrounding the *SMN2* promoter; however an analysis of the *SMN2* promoter region after treatment with VPA and SAHA revealed that acetylation did not increase markedly (Observed in Lunke and El-Ostra, 2013). I hypothesized that HDAC inhibitors may be inducing the *SMN2* promoter through the altered expression of one or more transcription factor acting in the *SMN2* promoter region. I selected four HDAC inhibitors to study – RGFP106, RGFP109, CAY10433, and HDACi-IV. These HDAC inhibitors have previously shown evidence of increasing

expression of *SMN2* in SMA human fibroblast cells (Andrew Connell and Ryan Kirk, unpublished data). These HDAC inhibitors preferentially inhibit the HDAC1 and HDAC3 isoforms. There is evidence that HDAC1 and HDAC3 have neurotoxic effects, so these isoforms are targets of interest in neurodegenerative diseases (Thomas and D'mello, 2018). This particular set of HDAC inhibitors has also shown promise in other neurological diseases like Frederich's ataxia (Soragni et al., 2012). First I assessed these HDAC inhibitors to determine if these compounds increased transcription of *Smn* in NSC-34 motor neuron-like cells (Durham et al., 1993). In order to study a possible mechanism of action of these HDAC inhibitors, I aimed to develop a *SMN2* promoter dual luciferase assay that can be utilized to study various regions of the *SMN2* promoter. These promoter regions all contain various putative transcription factor-binding sites, and these transcription factors may be relevant to the mechanism of action of our test compounds. I also aimed to determine if these HDAC inhibitors are promoter activating in a previously developed *SMN2* β-lactamase promoter assay to verify in another assay that these HDAC inhibitors were acting as *SMN2* inducers. Thus in this project I explored if these HDAC inhibitors induced the *SMN2* promoter through a mechanism of action that involved the transcription regulators of the *SMN2* promoter.

Chapter 2

METHODS

2.1 Drug Compounds

The HDAC inhibitors RGFP106, RGFP109, and HDACi-IV were obtained from Calbiochem. CAY10433 was obtained from Caymen Chemical and trichostatin A was obtained from Sigma-Aldrich. All drugs were dissolved in DMSO.

2.2 Cell Culture

The NSC-34 cell line, a mouse motor neuron-like model, was maintained in Dulbecco's Modified Eagle Medium (Gibco), 10% EquaFetal (Atlas Biologicals), 2mM L-glutamine (Life Technologies), and 1% penicillin-streptomycin (Life Technologies). NSC-34 clone 11 (Jarecki et al., 2005) was maintained at in 10% EquaFetal, 2mM L-glutamine, 1% penicillin-streptomycin, and treated with 500μg/mL G418 (Santa Cruz) for one week. All cell lines were maintained at 37C in a 5% CO_2 humidified chamber and grown to 80-90% confluence.

2.3 β-Lactamase Reporter Assay

Cells were plated at a density of 5.0 x 10^4 cells/well in a black walled, clear bottom 96-well plate (Santa Cruz Biotechnology). Drugs were dissolved in serum free media at a 1:1000 dilution and added to the wells for a final concentration of 1μM. Plates were incubated for 19 hours then spent media was aspirated and fresh serum-free media added. The GeneBLAzer *in vivo* kit (ThermoFisher Scientific) was used to

determine β-lactamase activity. 20uL of 6xCCF2/AM loading solution was added to each assay well, and the plates were incubated at room temperature for 2 hours. A plate reader (Victor Nico, PerkinElmer) was utilized to obtain two emission signals: a 460nm signal (460/30nm filter) and 530nm(530/10nm filter) signal. Values obtained from the negative control (Serum-free media only) were subtracted from the raw values obtained for samples and a ratio of 460/530 signals was determined. All compounds were tested in quadruplicate.

2.4 Drug Treatment

Cells were plated at densities of 3.2 x 10^4 cells/dish at 24 hours prior to drug treatment. The test drug compounds utilized were RGFP106, RGFP109, CAY10433, and HDACi-IV at concentrations of 1μM, 100nM, and 10nM. Treatment with test compounds occurred over a five-day period and finished with cell harvesting. During the five-day treatment period medium was changed daily and fresh DMSO or drug compound was added at a 1:1000 dilution every 24 hours.

2.5 Quantitative Reverse Transcriptase PCR

Cells were harvested and total RNA was extracted using RNAeast Mini Columns (Qiagen) following manufacturer's protocol. First-strand complementary DNA was generated using the iScript cDNA synthesis kit (Bio-Rad) according to manufacturer's protocol. The quantitative polymerase chain reation utilized SYBR Green PCR master mix (Qiagen) to carry out polymerase chain reaction, and the following primers (Integrated DNA technologies) were used to detect mouse transcripts: *Smn* (F) 5'-ACGGCTGCATTTACCCAGCTA -3'; *Smn* (R) 5'-CAGCTTTGGACTTGCTGTGTGCTT-3'. Data obtained was normalized to the

geometric mean of the following murine reference genes*: glucuronidase beta (Gusb), phosphoglycerate kinase 1 (Pgk1) and ribosomal protein L13a (Rpl13a).* The primers for the reference genes are as follows*: Gusb* (F) 5' - AATGAGCCTTTCCTCTGCTCT-3'; *Gusb* (R)5-'AACTGGCTATTCAGCTGTGG-3'*;Pgk1* (F) 5' -GCAGATTGTTTGGAATGGTC -3' *;Pgk1* (R) 5'-TGCTCACATGGCTGACTTA– 3';*Rpl13a* (F) 5' - ATGACAAGAAAAAGCGGATG-3*'*; *Rpl13a* (R) 5'-CTTTTCTGCCTGTTTCCGTA– 3'. The PCR was carried out in 384-well plates utilizing 10uL of total volume per well, and was performed on a 7900HT Fast Real-Time PCR system (Applied Biosystems). The relative transcript levels were calculated using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak 2008) and adjusted according to primer efficiency (Pfaffl, 2001).

2.6 Transient Transfection of NSC-34

NSC-34 cells were plated in 6-well plates at a density of $3.0x10^5$ cells/well 24 hours prior to transfection. Transient transfection utilized 3µg of total plasmid DNA per transfection. 6µL of LipofectAMINE 2000 (Invitrogen) was used per transfection, and transfection was carried out in 50/50 maintenance media/OptiMEM (Gibco). After 24 hours, media was aspirated and changed to maintenance media.

2.7 Chroma Glo Luciferase Reporter Assay

NSC-34 cells were transfected with CBR:CBG68 construct in a 1:1 ratio determined by molecular weight, or transfected with just CBR or CBG68 to calculate filter corrections. 48-hours post transfection, cells were harvested into passive lysis buffer (Promega). Cell lysis was carried out for 10min. then the protein lysate was

cleared by centrifugation. Lysate was diluted 1:4 in passive lysis buffer, and then added to a 96-well black clear-bottom plate at a volume of 100µL. 100µL of Chroma Glo reagent (Promega) was added to each well. Light emission for the red signal (615/8.5nm filter) and green signal(535/25nm filter) was obtained using a Perkin Elmer Victor X4 plate reader(PerkinElmers). Negative control (lysis buffer only) was subtracted from raw values. Measured signals were corrected for filter efficiency using the filter efficiency formulas provided by the manufacturer: R'(red signal) = Lrf – [Lgf x (Grf/Gfg)] / [(Rrf/R) – (Rgf/R) x (Grf/Grd)] and G'(green signal) = Lgf – [R' x (Rgf/R)] / (Gfg/G). R = light emission from red luciferase without filter, G = light emission from green luciferase without filter, Rrf = light emission from red luciferase under red filter, Grf = light emission from green luciferase under red fitler, Rgf = light emission from red luciferase under green filter, and Ggf = light emission from green luciferase under green filter. Lrf is the experimental light emission under the red filter, and Lgf is the experimental light emission under the green filter.

In order to measure activation by test compounds, NSC-34 cells were transiently transfected with constructs as described previously. 24 hours post-transfection, cells were plated at a density of $3x10^4$ cells/well in a black-walled, clear-bottom plate using phenol red-free media. The following day cells were treated with drug compound dissolved 1:1000 in media for a final concentration of 1µM. 24 hours after drug treatment, spent media was removed and 100µL of fresh media added. 100µL of Chroma Glo reagent was added to each well. A signal from the red luciferase was first determined (645/75nm filter) then the signal from the green luciferase (530/15nm filter). New filter efficiency corrections were performed as

described previously. Negative control (Phenol red-free media only) values were subtracted from raw data values. Read time is 1.5s per well per filter setting.

2.8 Promoter Analysis

Beginning with the 3.4kb *SMN2* promoter fragment, 400-600 bases fragments from the 5' end of the promoter were utilized for analysis of putative transcription factor binding site using the motif analytical tool FIMO from the MEME suite (Grant et al., 2011). For human transcription factors Jolma2013 (Jolma et al., 2013), HOCOMOCOV10 Human (Kulakovskiy et al., 2016), and SwissRegulon (Packov et al., 2012) were used for motif matrices. Jolma2013 and SwissRegulon additionally contain motif matrices for unique mouse transcription factors .The program relied on returns of p-value < 0.0001, however results reported here also achieved a q-value < 0.05, to adjust for false discovery rate (Grant et al., 2011). Results were additionally refined through elimination of repeat motifs that were marked at the same position due to overlap between databases.

2.9 Statistics

Data reported shows mean ± standard error of the mean (SEM) unless otherwise indicated. Data was analyzed for significance using one-way ANOVA where indicated. All analysis was carried out using Sigma Plot 12.0. Significance for data analysis was set at $p < 0.05$.

Chapter 3

RESULTS

3.1 Effects of HDAC inhibitors on *Smn* mRNA transcript levels

In order to determine if the HDAC inhibitor compounds RGFP106, RGFP109, CAY10433, and HDACi-IV were potential *SMN2* promoter-activating compounds, I tested these compounds in the NSC-34 motor neuron-like cell line. NSC-34 cells have an advantage over the traditionally used human SMA fibroblast cell lines as SMA primarily effects motor neurons, and this cell line displays characteristics of motor neurons (Durham et al., 1993). As NSC-34 cells are derived from mice, I aimed to determine transcriptional activation of *Smn*. As mentioned in the introduction, *Smn* is regulated similarly to *SMN2*, so I predicted that test compounds that increase promoter activation of *Smn* may also activate the *SMN2* promoter. I treated NSC-34 cells with 10nM, 100nM or 1μM concentrations of test compounds or DMSO (vehicle-control) over a course of five days to access effects over an extended treatment period. I found that RGFP106 significantly increased *Smn* transcription with all concentrations tested when compared relatively to DMSO treated cells. In addition, RGFP109 significantly increased transcription beginning at a concentration of 100nM relative to the DMSO treatment. However, CAY10433 and HDACi-IV both failed to increase transcriptional levels at all concentration levels when compared the DMSO control. These results suggest that RGFP106 and RGFP109 are both transcriptional activators for *Smn* at these concentrations, while CAY10433 and HDACi-IV do not demonstrate a similar effect at these concentrations.

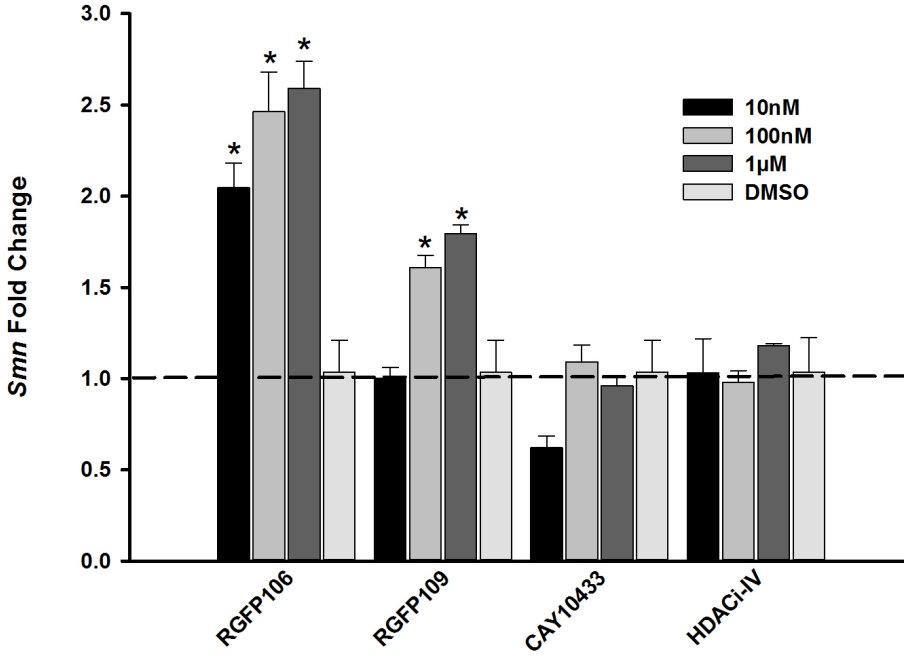

Figure 3.1 Effect of HDAC inhibitors on fold change of *Smn* mRNA levels. Cells were treated for 5 days with 10nM – 1μM concentrations of RGFP106, RGFP109, CAY10433, and HDACi-IV(n=3/treatment group). DMSO served as the control. Transcript levels of *Smn* were normalized to the levels of three reference transcripts, *Pgk1*, *Rpl13a* and *Gusb*. Results are displayed as mean ± SEM. Asterisks indicate significance compared to DMSO control group (*$p < 0.05$, one-way ANOVA).

3.2 Assessment of *SMN2* Promoter Luciferase Constructs

In order to study the mechanism of action of HDAC inhibitors on the *SMN2* promoter, I sought to develop a dual luciferase assay that could be used to study various regions of the *SMN2* promoter (Figure 3.3, A). To begin development of the assay, I selected the control reporter pTKCBG68, which is driven by the constitutively active *thymidine kinase* (*TK*) promoter due. This promoter was selected because it is ubiquitously expressed at low levels in cells, allowing for suitable data normalization.

I determined successful separation of the CBR and CBG68 signals using selected filters (615nm and 535nm, respectively) (Figure 3.2, A and B). In addition, we tested all the *SMN2* promoter constructs for basal activity in the NSC-34 cells, and determined that all *SMN2* constructs had detectable basal activity levels above background levels (Figure 3.3). Using these results, I could move forward in employing these vectors for a dual luciferase promoter assay for studying the *SMN2* promoter.

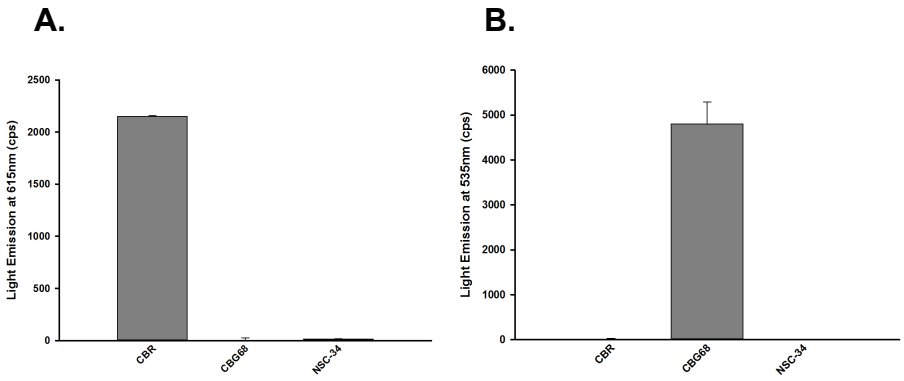

Figure 3.2 Verifying CBR and CBG68 Signaling in NSC-34 cells. NSC-34 cells were transiently transfected with pSMN2(0.75kb)CBR or pTKCBG68 (n= 3/group). NSC-34 cells were included as an un-transfected control. Light emission from each reporter was detected under the 615nm filter (**A**) and 535nm filter (**B**). Data shown represents mean ± SEM.

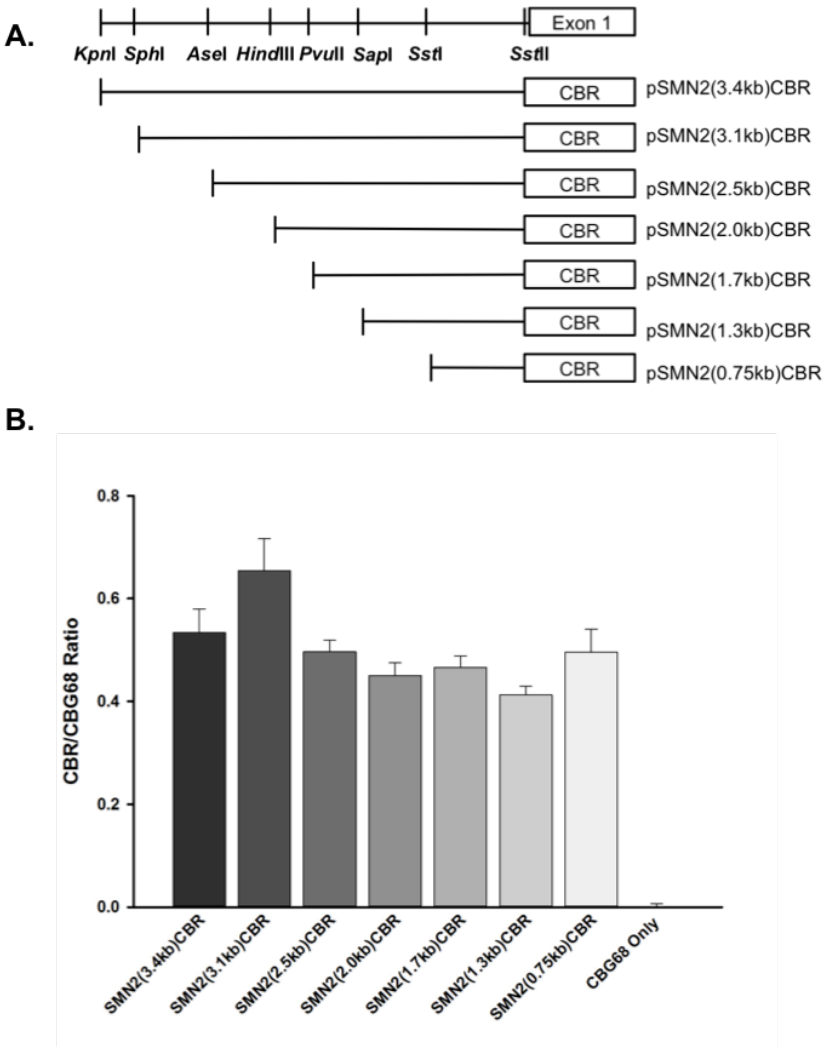

Figure 3.3 Basal activity of *SMN2* Luciferase Constructs in NSC-34s. NSC-34s were transiently transfected with a *SMN2* promoter construct as indicated along with pTKCBG68 (n=3/group). A representative image of the 3.4kb promoter region upstream of SMN2 and the promoter constructs generated from this region are shown in (**A**). Light emission for the CBR and CBG68 reporters was determined using 615nm and 535nm filters, respectively, and a ratio of CBR signal over CBG68 signal was determined (**B**)**.** Results are displayed as mean ± SEM.

3.3 Effect of HDAC inhibitor treatment on *SMN2* Dual Luciferase Assay

To access the effect of HDAC inhibitors on *SMN2* promoter activation, 1μM of each test compound was applied to NSC-34s that were transiently transfected with pSMN2(3.4)CBR and pTKCBG68. Unexpectedly, the emission signals from both the *SMN2* and *TK* constructs were significantly up-regulated by all test treatments, including our positive control trichostatin A (TSA, Figure 3.4). Due to the apparent up-regulation of the *TK* control vector, I could not normalize this data for differences in transfection efficiency using the standard experimental signal/control signal approach as this results in heavily skewed data. Due to this issue, interpretation of these results should be approached cautiously. With this consideration, I can note that both the *SMN2* and *TK* reporters showed substantial up-regulation under all test compounds. This data also suggests a distant difference in CBR signal up-regulation between our test compounds and TSA, suggesting potential differences in mode of *SMN2* activation. In order to find a suitable control as a replacement, I accessed other CBG68 vectors, pCBG68-Basic and pCBG68-Control. pCBG68-Control is a SV40 driven vector, while pCBG68-Basic is a promoter-less vector. My findings indicate that pCBG68-Control is also unsuitable for use as a control reporter, as it was heavily up-regulated by TSA (Figure 3.5). However, pCBG68-Basic produced a signal detectable above background levels, and was less affected by TSA treatment than pTKCBG68 and pCBG68-Control, thus this vector may serve as a suitable control reporter in future dual luciferase experiments.

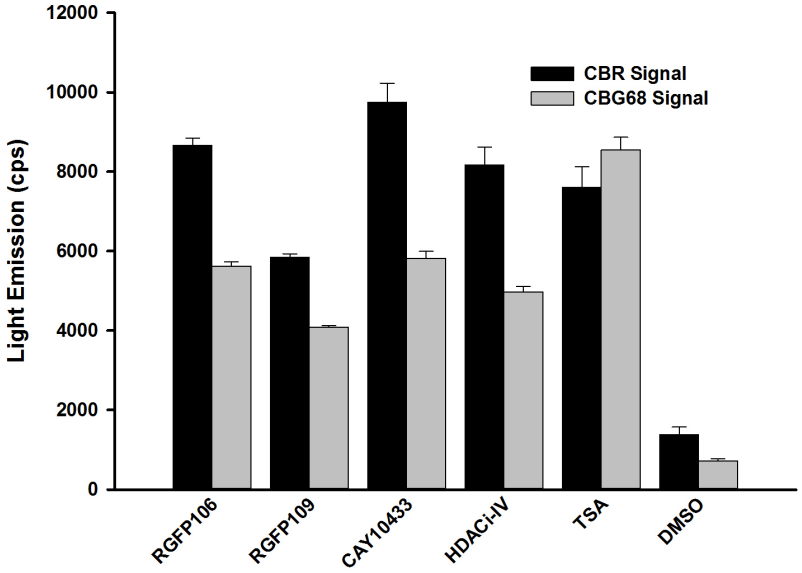

Figure 3.4 Effects of HDAC inhibitor treatment on *SMN2* dual luciferase assay. NSC-34 cells were transiently transfected with pSMN2(3.4kb)CBR and pTKCBG68. Cells were treated with 1μM of RGFP106, RGFP109, CAY10433, or HDACi-IV for 24 hours (n=4/treatment group). DMSO is included as the control. Light emission for the experimental CBR signal was obtained through a red optical filter (645nm/75) while light emission for the control CBG68 signal was obtained through a green optical filter (530nm/15). Results shown are mean ± SEM.

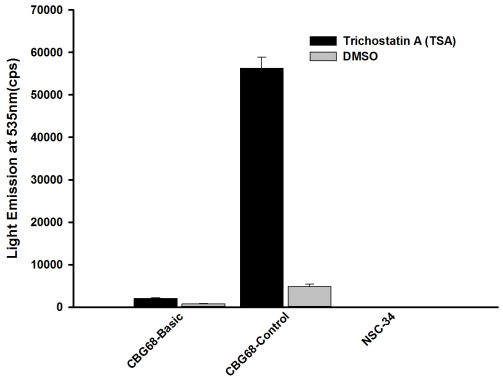

Figure 3.5 Effect of TSA treatment on alternative CBG68 vectors. NSC-34 cells were transfected with pCBG68-Basic or pCBG68-Control, then treated with TSA or DMSO for 24 hours prior to reading. NSC-34 cells served as the un-transfected control (n=4/treatment group). Data shown is after filter efficiency correction was performed as described previously. Results are reported as mean ± SEM.

3.4 Promoter Analysis of *SMN2* constructs

To achieve a comprehensive view of possible regulatory elements acting on the *SMN2* promoter, I conducted a bioinformatics analysis of the *SMN2* promoter constructs. My results returned a large volume of transcription factors that may be acting on the *SMN2* promoter (Table 3.1), suggesting that my selection criteria (P-value < 0.0001, q-value < 0.05) may require more stringent requirements, despite similar criteria being used in other promoter analysis studies (Rich et al., 2016; Wu et al., 2012). Transcription factors acting on *SMN2* are of interest to researchers because these factors may be up-regulated or down-regulated in the mechanism of action of tested drug compounds, and knowing which factors are influencing the *SMN2* promoter can lead to the development of drug compounds that specifically target these transcription factors. Some specific factors that may be of interest for HDAC inhibitor treatment are Sp1, YY1, and c-Jun (JUN in Table 3.1), which have been shown

previously to be associated with HDAC enzymes (de Ruijter et al., 2003; Morrison et al., 2007). Sp1 has been shown to bind the *SMN2* promoter, however the effect of this transcription factor on *SMN2* expression varies depending on other transcription factors active in the cell (Rouget et al., 2005). The results from this analysis could be used in studying regions of the *SMN2* promoter in future uses of the *SMN2* promoter dual luciferase assay.

Table 3.1. List of Putative Transcription Factors in *SMN2* Promoter Constructs.

	Putative Transcription Factors (5'-3')
***SMN2* (3.4kb)** *1-303*	ZNF143, EGR4, KLF16, ZBTB4, YY2, IRX2, HIC2, PROP1, ONECUT1, ONECUT2, MEF2C, MEF2A, PATZ1, PLAG1, PLAGL1, KLF6, REST, ZNF148, ERG1, Egr1, NR0B1, IKZF1, EGR2, TBX15, RREB1, NKX3-2, PAX5, IKZF1, OTX2, PITX2, OTX1, HEY2, JUNB, SMRC1, SRF, PLAG1, IRF5, AIRE, BPTF, TFCP2, CPEB1, FOXL1, BPTF, FOXC2, TBX19, TBX1, MTF1, Hic1, TBX21, TBX20, TLX1, ESR1, LHX2, DDIT3, MNT, KLF15, SP1, ZNF219, GLI1, ZIC1, IKZF1, HMX1, NKX2-2, NKX2-5, PBX2, ONECUT3, BACH1, SMRC1, NFE2, JUNB, TFAP2C, TFAP2A, PAX5, PROX1, PITX1, CRX, RFX5, GFI1, TP53, ZNF173, P73, P63, THAP1, KLF15
***SMN2* (3.1kb)** *304 - 928*	NFIC, CDX1, CDX2, CDX4, YY1, NR2F6, NR2F1, RARB, USF2, RXRB, THA, RARG, NR1I2, THRB, RARA, MITF, TFEC, MAX, BHLHE41, MESP1, ID4, TCF4, TWIST1, SNAI1, SNAI2, MYOD1, TFAP2D, ZNF143, OTX2, TBX1, PITX1, PITX3, TBX3, PITX2, TBX2, FIGLA, MESP1, TCF4, TCF3, ID4, SNAI2, TBX4, MGA, SMAD2, SMAD4, TBX5, ZIC1, ZIC2, MECOM, CPEB1, HEY2, TBX1, POU3F2, POU3F1, POU2F1, POU2F2, POU2F3, POU5F1B, LMX1A, POU1F1, POU5F1B, POU3F4, POU2F3, POU3F3, ZNF384, IRX3, PROP1, Sox1, ARX, LMX1A, FOXD2, FOXB1, POU3F2, POU3F3, POU2F1, CDC5L, NKX6-1, POU6F2, PBX1, FOXP3, CDX1, CDX2, CDX3, MEF2D, MEF2B, ZFX, REST, MTF1, TFCP2, NKX2-8, NKX2-3, HMX1, IKZF1, BHLHE41, CPEB1, SREBF2, ZEP1, NFKB2, NFKB1, MNT, IKZF1, ZNF384, HOXB2, CEBPA, HLF, DBP, CEBPB, CEBPD, CEBPD, POU3F2, POUF3F4, HOMEZ, HMX2, VENTX, HOXB6, HOXB8

Table 3.1 continued.

	Putative Transcription Factors (5'-3')
***SMN2* (2.5kb)** *929 – 1404*	ONEC2, AIRE, LMX1A, FOXJ2, FOXJ3, EVX2, AIRE, HOXC12, HOXC11, HOXD11, HOXA10, CPEB1, FOXO1, FOXO3, FOXO4, HOXC10, IRF5, FOXL1, HOXD10, BPTF, SOX4, FOXJ3, FOXC2, SRY, TBX15, TFCP2, KLF1, HEY2, JUNB, SMRC1, OTX2, ZNF143, IKZF1, TBX1, TBX15, EGR1, TFDP1, MAZ, SP4, SP2, AP2D, TFAP2C, TFAP2A, PAC5, BACH1, ZNF219, RARG, RARB, NR2F6, NR2F1, COT2, ERR2, STF1, ERR3, RARA, NR2E1, ESSRA, NR5A1, NR5A2, NR4A2, RARB, VDR, IRX2, IRX2, Irx3, MEF2B, MEF2A, MEF2C, MEF2D, POU6F1, HOXD8, HOXA4, HOXD4, KLF3, Klf12, TFCP2, SP8, SP1, SP3, KLF1, KLF4, TBX4, TBX5, MGA, TBX21, TBX2, ARNT2, OTX1, MAFG, SRF, MAFF, NKX2-5, ZNF524, ELF2, SP2, VDR, TLX1, TLX3, SMRC1, ZNF143, PAX5, AIRE, ZBTB16, LMX1A, CPEB1, HMGA1, HMGA2, ONEC2, NKX6-1, NKX6-2, FOXG1, FOXL1, HOXC10, CPEB1, FOXO1, FOXO3, FOXO4, FOXC1, SOX4, FOXG1, FOXJ3, FOXC2, BPTF, SRY, ZBTB16, AIRE, AIR3A, FOXL1, FOXO1, FOXO3, FOXO4, SRY, FOXG1, FOXJ3, FOXL1, EVX1, FOXC1, HMGA1, HMGA2, FOXD3, CDX1, CDX2, CDX4, MNX1, EVX1, FOXL1, ALX4, Alx1, DLX, PROP1, ZBTB16, FOXP3, FOXO1, SRY, JUN, TF7L2, SOX17, SOX14, SOX3, HOXC10, HOXA10, Hoxa11, HOXD12, HOXD11, HOXC12, HOXC11, HOXC10, EVX2
***SMN2* (2.0kb)** *1405* - 1732	EVX1, NKX3-1, IRF1, FOXL1, MNX1, FOXC1, CPEB1, HOXD12, FOXG1, FOXJ3, FOXC1, LMX1A, SPDEF, CDX1, CDX1, CDX3, STAT2, IRF1, BPTF, SPDEF, NFIX, PAX5, GTF2I, FOXO6, FOXJ2, FOXJ3, FOXI1, FOXJ2, FOXF1, FOXF2, GATA3, FOXJ2, GATA6, GATA4, NFIA, NFIX, NR3C1, THA, ZBTB16, LMX1A, HMGA1, HMGA2, CDX1, CDX2, CDX4, FOXD3M FOXL1, FOXJ2, EVI1, GATA6, ZBTB16, GATA3, TGIF1, SOX17, IRF9, ZBTB16, HOXC10, Hoxd9, AIR3A, DLX5, POU3F3, FOXB1, FOXD2, FOXC1, POU2F1, FOXC2, POU2F1, CDX1, CDX2, CDX4, POU2F3, FOXL1, EVX1, FOXI1, FOXJ2, FOXD3, NKX3-1, FOXJ3, FOXC1, FOXC2, NKX6-1, NKX6-2, FOXD3, FOXP3, FOXJ2, PAX5, TBX20, TGIF1, JUNB, SRBP2, SRBP1, TLX1, NFIC, NFIA, TLX1, TLX3, SP4, MAZ, DDIT3, SP2, EGR1, ELF2, SP3, CLOCK, HINFP, SP1, WT1, GTF2I, PURA, ELF2, NKX2-5, NKX2-2, NKX2-5, EN1, EN2, SP4, SRF, MAFG, SP2, NFIC, BACH1, NFE2L2, SMRC1, SP1, PAX5, IKZF1
***SMN2* (1.7kb)** *1733 - 2092*	TP73, TBX1, PURA, KLF16, KLF8, NR2E3, MEF2C, MEF2A, MEF2D, HMGA1, HOXA13, CEBPA, CEBPB, ENOA, SMAD1, SMAD7, SMAD9, RARG, NR2F1, RARG, NR2F6, RXRG, RXRA, RARB, RXRB, NR2F1, THA, RARG, NR1I2, THB, ERR3, JUN, NKX2-5, GLI3, PROX1, SRF, ZNF219, ZNF784, TBX15, TLX1, GLI2, ZIC3, ZNF143, IKZF1, OTX2, PITX1, PITX3, CRX, JUNB, SMRC1, HAND1, DLX5, FOXQ1, FOXJ3, LEF1, EVX1, TCF7L1, Tcf7, FOXJ3, FOXQ1, FOXC1, PAX4, DUXA, TEAD1, TEAD4, TEAD3, FOXO6, ZNF713, TBX1, ETV1, MNT, SPIC, EHF, ELF2, PATZ1, FLI1, MNTMZF1, GABP1, EGRI, SPI1,GTF21, HMGA1

Table 3.1 continued.

	Putative Transcription Factors (5'-3')
***SMN2* (1.3kb)** *2093 – 2686*	SP1, SPZ1, TCF7L1, HNF4A, HNF4G, RXRA, COT1, BCL6B, ZBTB16, FOXL1, POU3F3, PLAL1, TBX1, TBX4, TBX5, TBX2, MGA, TBX21, RUNX2, ARNT2, MGAP, SMAD4, SMRC1, JUNB, SMAD2, TBX3, TBX1, TBR1, TBX21, CLOCK, MESP1, EOMES, T, HMX1, TBX5, TBX4, TBX15, MGA, Meis2, MEIS3, PKNOX1, PKNOX2, TGIF1, ID4, ZEB1, TCF4, TCF3, MESP1, FIGLA, ITF2, SNAI2, SNAI1, TGIF2, PITX1, CRX, OTX2, PITX2, ZNF143, ZFX, GLI1, IKZF1, TBX15, EGR1, MAZ, Tcfap2a, AP2D, AP2B, AP2D, NR2F1, NR2F6, NR1D1, RORA, NR2F1, COT2, ERR3, RARB, RXRB, NR1H4, Nr2f6, RARG, RARA, NR2F1, NR2F6, RARB, NR6A1, ENOA, NFKB2, CEBPA, CEBPB, NFKB1, ZBTB16
***SMN2* (0.75kb)** *2687 - 3432*	KAISO, GLI1, GLI3, ZIC1, ZKSC3, TLX1, THAP1, ZBTB6, FOXC2, FOXL1, BPTF, FOXO3, FOXA3, FOXJ3, FOXM1, FUBP1, FOXF2, FOXA3, FOXJ2, FOXC2, Foxc1, FOXF1, FOXJ3, FOXI1, FOXD3, FUBP1, BPTF, FOXJ2, FOXM1, RHOXF1, ETV1, MAZ, TFDP1, THAP1, PAX5, KLF16, TBX15, ELF2, EHF, FLI1, MNT, E2F6, EGR4, FOXA3, FOXF1, FOXJ2, FOXF2, EHF, FOXG1, FOXL1, FOXG1, FOXF1, FUBP1, FOXA3, FOXJ3, FOXJ2, FOXF2, ZNF148, SP3, MNT, WT1, IRF4, SP8, EGR1, GTF2I, SP1, KLF16, ZNF740, SP1, SPIC, ZNF219, EGR4, MZF1, PATZ1, KLF4, ZIC1, MAZ, FOXB1, FOXI1, FOXJ2, FOXJ3, FOXG1, RREB1, FOXG1, ZSCAN4, EGR3, EPAS1, RREB1, KLF8, TLX1, IRF5, SPZ1, MEF2D, MEF2B, TP73, GMEB2, ARNT2, RFX5, CLOCK, TFDP1, SP2, SP3, RREB1, KLF16, TBX15, TBX1, EGR1, MAZ, MNT, SP4, SP2, PAX5, ZNF219, ARNT2, IZKF1, ZKSC3, HIC1, TLX1, THAP1, HIC1, PAX5, KLF4, FOXC2, FOXL1, AIRE, BPTF, FOXO3, FOXA3, FOXJ3, FOXO1, FOXC2, FOXL1, FOXG1, FOXM1, FUBP1, FOXF2, FOXJ2, FOXF1, RREB1, FOXC1, FOXD3, FOXI1, FOXJ2, FOXL1, FOXO1, FOXJ3, FOXA3, FOXC2, FOXJ2, FOXM1, FUBP1, BPTF, ZNF713, SPI1, ARI3A, IRF3, BC11A, EHF, IRF4,TBX1, TBX21, EGR1, TBX20, EGR4, GLI1, GLI3, ZNF740, ZIC1, MAZ, TBX21, HINFP1, ZBT7B, ELF2, E2F6, EGR4, THAP1, PAX5, ZNF143, TFDP1, AP2D, TFAP2A, TFAP2B, TFAP2C, WTI, ELK1, ELF1, ELF2, GABPA, ETV6, FLI1, ELK4, PAX3, PAX7, SP4, SP4, PLAL1, THAP1, ZIC3, ZIC4, ZIC1, GLIS2, TFAP2C, GLIS3, TFAP2B, TFDP1, MAZ, TBX15, SP2, KLF16, HIC1, NRF1, WTI, MTF1, ZFP161

This table lists the overall putative transcription factors of the 3.4kb *SMN2* promoter in the 5'->3' direction. Transcription factors in the 5' region of each promoter construct are noted alongside their approximate position in the overall 3.4kb promoter. Redundancies in each region are retained as each represents a different putative binding site for that transcription factor.

3.5 Effect of HDAC inhibitor treatment on *SMN2* β-Lactamase Promoter Assay

In order to verify whether these HDAC inhibitors are *SMN2* promoter activating as suggested from the dual luciferase assay, I utilized a previously

developed *SMN2* promoter assay. The NSC-34 clone 11 cell line contains the 3.4 kb *SMN2* promoter fragment driving a β-Lactamase (BLA) reporter (Jarecki et al., 2005). To access promoter activation, these cells were treated with 1μM of each test compound or 100nM of trichostain A (positive control). After calculating the 460/530 ratios, no significant difference was found between cells treated with DMSO control and test compounds despite the positive control (TSA) increasing promoter activation significantly as expected (Figure 3.6). This data suggests that these HDAC inhibitors unexpectedly do not activate the 3.4kb *SMN2* promoter.

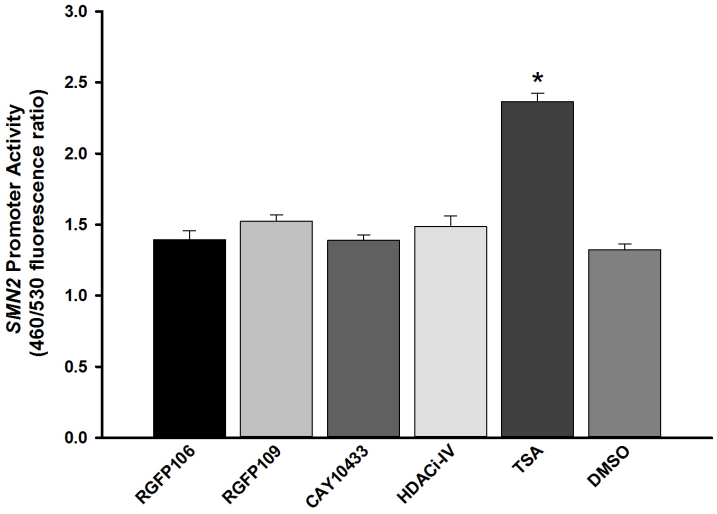

Figure 3.6 ***SMN2* promoter activity under HDAC inhibitor treatment.** NSC-34 clone 11 cells were treated with DMSO or 1μM of RGFP106, RGFP109, CAY10433, or HDACi-IV for 19 hours prior to loading with CCF2/AM BLA substrate (n=4/treatment group). DMSO serves as the control group. Results are displayed as the fluorescence ratio of 460nm signal over 530nm signal. Data shown represents mean ± SEM. Asterisks indicate significance compared to DMSO control group (*$p < 0.05$, one-way ANOVA).

Chapter 4

DISCUSSION

Most drug discovery efforts for treating SMA are focused on targeting *SMN2* through various approaches such as increasing *SMN2* transcription or increasing inclusion of exon 7 (Tisdale and Pellizzoni, 2015). While many drug compounds have been tested and shown to have an effect on *SMN2* transcription, the mechanism through which these compounds act often remains unknown. Understanding the mechanism through which a test compound works may help to develop an even more effective therapy for activation of the *SMN2* gene. In this work, I aimed to determine a possible mechanism of action of HDAC inhibitors acting on the *SMN2* gene. From my results, I found a notable difference between the levels of *Smn* mRNA transcripts following treatment by RGFP106, RGFP109, CAY10433, and HDACi-IV. This may be due to the inherent differences in these HDAC inhibitors. RGFP106 has been shown to be a strong inhibitor of HDAC3, with some inhibition of HDAC1 (Soragni et al., 2012). RGFP109 is a similar HDAC1/HDAC3 inhibitor, however it has been shown to have a higher IC_{50} in comparison to RGFP106 in other cell types, and this may be true in NSC-34 cells as well (Soragni et al., 2015). HDACi-IV and CAY10433 are also both HDAC1/HDAC3 inhibitors, however both of these HDAC inhibitors have been shown to have significantly weaker inhibition ability in other cell types (Herman et al., 2006). My results here suggest that HDAC1/HDAC3 inhibition is useful in activating the *Smn* gene and thus may be useful for promoting transcription of *SMN2*.

I was able to successfully determine that all the *SMN2* promoter luciferase constructs had some basal activity in NSC-34 cells, but I found in performing the actual dual luciferase experiments that the control vector pTKCBG68 was unsuitable to use in this assay because of its apparent up-regulation by test compounds alongside the experimental vector. Unfortunately a recognized disadvantage of dual luciferase assays is that when the control reporter is affected by the treatment, the data will be skewed and not reliable (Shifera and Hardin, 2010). Given that HDAC inhibitors may be affecting various transcription factors, there is a tangible possibility of both control and experimental promoters being affected by treatment, as shown in our results. A possible explanation for the up-regulation of the *TK* promoter is through the transcription factor Sp1, which is associated heavily with HDAC1 and has been shown to up-regulate the *TK* gene in other cell types (Doetzlhofer et al., 1999). Interestingly, Sp1 also has several binding sites in the SV40 promoter, which drives the pCBG68-Control reporter, and this may also explain the large increase in signal shown by this reporter under TSA treatment. The difference between the signal increase of pTKCBG68 and pCBG68-control under TSA treatment may be due to the *TK* promoter containing more regulatory elements than the SV40 promoter (Shifera and Hardin, 2010). My *SMN2* promoter analysis and analysis by previous researchers also show that Sp1 has several binding sites in *SMN2* (Boda et al., 2004; Monani et al., 1999; Lunke and El-Ostra, 2013). Previous researchers have suggested that the mechanism of action of the HDAC inhibitor valproic acid on *SMN2* is related to the Sp1 transcription factor, but this transcription factor has not been explored as a possible mechanism of action for other HDAC inhibitor treatments used for *SMN2* promoter activation (Brichta et al., 2003; Lunke and El-Ostra, 2013). Based on this, it

may be possible to see up-regulation of both *SMN2* and *TK* because of Sp1. This could be further explored by determining if mutations in Sp1 putative binding sites of the *SMN2* promoter have an effect on *SMN2* promoter activation under treatment by these HDAC inhibitors. pCBG68-Basic vector shows promise for use as a control vector as it was minimally affected by TSA treatment, so this vector should be used in future *SMN2* dual luciferase experiments for more accurate determination of *SMN2* promoter activation.

The results from the *SMN2* BLA reporter assay are confounding, as the assay showed no significant up-regulation of *SMN2* promoter activation for any of the test compounds. This result could be explained in several possible ways. It is possible that these compounds specifically are promoter activating on *Smn* but not *SMN2* despite evidence of conserved regulation between the human and mouse genes (Monani et al., 1999). It is also possible that since the 3.4kb *SMN2* promoter fragment may not contain all of the regulatory elements of *SMN2* that I did not see up-regulation due to a missing regulatory element that the native *Smn* retains (Boda et al., 2004). Since the dual luciferase assay possibly suggests that the 3.4kb *SMN2* promoter is up-regulated in NSC-34 cells treated with our test compounds, another possibility is that the lack of promoter activation may be due to the genomic context in which the 3.4kb *SMN2* reporter is integrated. When this reporter cell line was developed, the *SMN2* BLA reporter was randomly integrated and as such the genomic context of the reporter currently is unknown (Jarecki et al., 2005). If certain transcription factors are important to the mechanism of action of these HDAC inhibitors, the genomic context may be important as some regions have more transcriptional accessibility than others due to local chromatin structure (Swygert and Paterson, 2014). This also points to the

important distinction between using assays that are based on transient expression versus stable expression. Since reporters in a transient assay are not integrated into the genome, transcription factors will have different accessibility to the promoter region being studied and thus this can lead to a major difference between promoter activation when comparing transient assays to stable assays. In addition, a transient experiment may have a significantly higher copy number of the reporter as compared to the stable assay, which may also lead to differences in relative signals produced from promoter activation. Experiments with other drug compounds on *SMN2* have shown inconsistency between the results of this *SMN2* promoter assay and the effects seen on native *SMN2* expression (Gentillon et al., 2017), suggesting these HDAC inhibitors should be further studied in additional SMA cell lines to verify if they have an effect on *SMN2* expression.

Chapter 5

CONCLUSION AND FUTURE DIRECTION

My mRNA data showed that RGFP106 and RGFP109 increased the amount of mRNA transcripts from the *Smn* gene. Given the results of the *SMN2* BLA promoter assay, it remains unclear whether these drug compounds would act similarly on the *SMN2* gene. Since the promoter assay may not be accurate to *SMN2* in its native context, these drug compounds still have potential to act on the *SMN2* and should be tested further to determine if the effect seen on *Smn* in a mouse cell line holds true for *SMN2* in human cell lines. Given that RGFP106 had a significant effect at a 10nM dosage, it could be especially valuable in finding a highly potent HDAC inhibitor that may be usable for SMA treatment. Since RGFP106 in particular preferentially targets HDAC3, it may also be valuable to pursue other HDAC3 specific HDAC inhibitors to determine if they may also be useful as potential SMA therapeutics.

Developing a dual luciferase promoter assay to study the effects of HDAC inhibitors on the *SMN2* promoter proved a difficult endeavor. However, given the low activation of the promoter-less vector pCBG68-Basic under TSA treatment, this vector may be the best control to use in future experiments attempting to study the regions of the *SMN2* promoter. The HDAC inhibitor treated dual luciferase promoter assay should be repeated using this vector to determine if the 3.4kb *SMN2* promoter is indeed activated by the test compounds or if the results shown here are simply artifacts of the assay caused by the activation of the pTKCBG68 vector. This dual luciferase

promoter assay may also be useful for studying the mechanism of action of other small molecule drug compounds that have been shown to activate the *SMN2* promoter.

Given the results of the *SMN2* BLA assay, it may be important to determine if this assay is suitable in predicting drug compounds that will activate the *SMN2* promoter. It is possible that a better approach would be to utilize a longer *SMN2* promoter fragment such as the 4.6 kb *SMN2* promoter fragment to determine possible up-regulation of the *SMN2* gene. When the 4.6 kb *SMN2* promoter was compared to a 3.2 kb *SMN2* promoter, the 4.6 kb promoter showed a more significant difference in up-regulation between neuronal and non-neuronal cells possibly due a silencer element in the larger 4.6 kb promoter (Boda et al., 2004). Given that the 3.4 kb and 3.2 kb *SMN2* promoters only differ in approximately 200 bases, it may be useful to determine if the 4.6 kb promoter shows a similar difference in promoter activity when compared to the 3.4 kb promoter, which would suggest additional promoter elements that may be important for mechanism of action studies. Using this promoter fragment should also be taken into consideration for the *SMN2* promoter dual luciferase assay.

Whether my selected drug compounds will act on the *SMN2* promoter remains unclear, and a possible mechanism of action could not be determined. Performing the experiments outlined previously may better determine if these drug compounds may be useful for treatment of SMA, and whether these compounds are likely to act through a promoter based mechanism of action or some other mechanism that remains to be determined. One of the possible alternative mechanisms is that rather than being promoter activating, these HDAC inhibitors may be affecting the stability of mRNA transcripts, as evidenced by the increase in mRNA transcripts from *Smn* following treatment.

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