The development of zebrafish (Danio rerio) as a rapid and efficient model system for therapeutic drug screening for Spinal Muscular Atrophy.

THESIS

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Abstract

Spinal muscular atrophy (SMA) is the most common genetic cause of infant mortality. It is characterized by symmetrical weakness and atrophy of the proximal muscles in the limbs and trunk due to α-motoneuron loss in the spinal cord. Patients with the most frequent and severe form of SMA (Type I) lack the ability to sit or walk, and usually die within the first two years from respiratory failure unless provided with intensive therapy.

SMA is caused by low levels of the full-length Survival Motor Neuron (FL SMN) protein. SMN is transcribed from two nearly identical genes, SMN1 and SMN2. SMA occurs when SMN1 is lost due to deletion or mutation while SMN2 is preserved. The genes are nearly identical, but SMN2 differs by one vital nucleotide that results in approximately 90% of transcripts lacking exon 7 (SMNΔ7). Although SMN is ubiquitous in every cell of the body, motoneurons are specifically sensitive to reduced levels.

A primary objective of spinal muscular atrophy research is to identify therapeutic drugs and determine their mechanism of action. Unlike other vertebrate models, zebrafish are less expensive to maintain and drug screening can be completed in a matter of days. This would significantly decrease the cost of drug development and expedite identification of a therapeutic as non-beneficial or toxic compounds would be removed from study before...
progressing on to mice or pigs. I have verified that zebrafish can be utilized for screening potentially therapeutic drugs by examining the effects of Trichostatin A (TSA), which has shown to be beneficial in other models of SMA. TSA significantly increases histone 4 acetylation in WT zebrafish within five hours of treatment, which has been shown to promote *smn* transcription. In concurrence with this, Smn is increased in WT zebrafish after overnight treatment with TSA. In the morphant model, treatment with TSA shows a decreasing trend in the severity of motor axon outgrowth defects. These screening techniques prove that zebrafish models are an efficient tool for examining HDACi and for identifying other compounds capable of increasing levels of Smn.

In addition, I am developing a transgenic zebrafish that is unique from all other SMA animal models available in that it will be used to simultaneously identify and distinguish between drugs that enhance inclusion of exon 7 and those that either promote transcription or stabilize the protein. As quantification of promoter activity and level of FL SMN can be determined *in vivo* and without harm to the embryo, multiple measurements can be taken throughout development or the course of drug treatment. This unique model will be a valuable tool to expedite the identification of a therapeutic for SMA while decreasing the expense.
Dedication

This document is dedicated to my husband Mike for his unflinching belief in my abilities and for his constant encouragement. I also dedicate this document to my children, Samantha Jade and Miya Joy, who have motivated me to succeed, even when doing so meant changing directions.
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Chapter 1: Introduction

The Relationship Between Spinal Muscular Atrophy Disease and the Survival of Motor Neuron Genes.

Spinal muscular atrophy (SMA) is a devastating autosomal recessive neurodegenerative disease that primarily affects infants and young children. It is characterized by muscle atrophy and weakness in the legs, arms and trunk due to α-motoneuron loss in the spinal cord \[^{1,2}\]. Patients with the most common and severe form (Type I, Werdnig-Hoffmann disease) are affected at birth or before they are six months old. Type I patients lack the ability to move on their own, and usually die within the first two years from respiratory failure unless provided with aggressive therapy \[^{2-4}\]. As a result, SMA is the most common genetic cause of infant mortality \[^{1,2}\]. Currently, there is no cure.

Three other types of SMA exist, based on the age of onset and physical ability \[^{2,4}\]. Type II patients are affected after 6 months of age and are able to sit unaided. However, only a few are able to stand and none are able to walk without assistance. Lifespan for Type II patients varies depending on how severely their respiratory system is affected. With therapy, they are able to survive for several decades. Type III and Type IV SMA vary mainly in the age of onset, with Type III occurring in early childhood and Type IV
affecting adults. Both forms are considered mild, as patients are able to walk unassisted at one time and have normal life expectancy. Many patients with Type III will become wheelchair-bound, but some are able to walk unassisted in their adult years.

Humans have two Survival of Motor Neuron (SMN) genes on chromosome 5 (5q12-13). SMN1 is located near the telomere whereas SMN2 is located within an inverted duplication near the centromere [5-6]. SMA is caused by the complete loss or mutation of the SMN1 gene while the SMN2 gene is preserved [1-2,7]. It is estimated that 1 in 35 to 1 in 50 humans carry mutations in SMN1, and approximately 1 in 6,000 to 1 in 10,000 live births are affected with SMA [1,8,9]. The severity of SMA depends on the amount of full-length SMN (FL SMN) protein produced from SMN2 with greater amounts resulting in less severe forms of the disease [10]. The amount of FL SMN transcribed is directly influenced by how many copies of the SMN2 gene are present. Due to the instability of this region of the genome, there are usually between 1-4 copies of SMN2 [11,12].

SMN1 and SMN2, as well as their promoter sequences, are nearly identical with one vital difference. SMN2 has a single nucleotide change in exon 7 that is translationally silent but impacts RNA splicing [13-15]. As a result, about 90% of the protein produced by SMN2 will lack exon 7 (SMNΔ7) [16]. FL SMN self-oligomerizes into a functional complex. The ability to self-oligomerize is compromised in SMNΔ7, leading to rapid protein degradation [17,18]. However, SMNΔ7 is capable of forming heterotypic complexes with FL SMN, though with reduced affinity compared to FL SMN homotypic
complexes\textsuperscript{[19]}. This results in improved SMNΔ7 stability, corrected localization to both the cytoplasm and nucleus, and an increase in survival time in the SMNΔ7 mouse model \textsuperscript{[19]}.

The Current Understanding of SMN Function.

SMN is a highly conserved protein that is ubiquitously expressed in all tissues \textsuperscript{[20]}. It has many protein binding partners, but its role in the SMN complex is best understood in terms of its functions in spliceosome assembly and various aspects of RNA processing \textsuperscript{[20]}. Reduced levels of SMN have been found to cause cell type-specific splicing defects, though these defects do not appear to cause cell death in any other tissue than motoneurons \textsuperscript{[21, 22]}. It is currently unknown why motoneurons are specifically sensitive to reduced levels of SMN. Zhang et al. propose that reduction in the total amount of SMN may be detrimental to motoneurons by causing cell-specific splicing defects in other genes; however these genes have not yet been elucidated \textsuperscript{[21, 23]}. An alternative hypothesis suggests that SMN may have a non-spliceosomal role required for normal motoneuron development, axonal outgrowth and synaptic function \textsuperscript{[24-27]}. In support of this hypothesis, SMN is found in growth cones and branch points of motor axons, and may be involved in the axonal transport of mRNA \textsuperscript{[24, 27-29]}. Unlike FL SMN, SMNΔ7 is restricted to the nucleus \textsuperscript{[27]}. When a cytoplasmic targeting motif was added to SMNΔ7, it was capable of rescuing axon defects but was not able to bind to Smn proteins, which is
necessary for snRNP biogenesis \[^{25}\]. Thus, there may be a role for SMN in motoneurons that is separate from spliceosomal mechanisms.

**Therapeutic Drug Development.**

An important goal of SMA research is to develop therapeutic compounds. Most approaches have focused on increasing the amount of SMN by stabilizing the protein, promoting $SMN2$ transcription, or increasing inclusion of exon 7 \[^{2,16,30-32}\]. A compound, RG3039, also known as Quinazoline495, has recently been found to promote $SMN2$ transcription \[^{31,32}\]. A phase I clinical trial has recently been approved to study the pharmokinetics and safety of RG3039 in humans \[^{33}\]. Other therapeutic efforts are focused on motoneurons themselves, such as developing neuroprotective drugs, replacing $SMN1$ through gene therapy, or restoring motoneurons using stem cells \[^{34}\].

Several potential targets for therapeutic agents have been identified, including transcriptional regulators Stat5 and hnRNP A1, and splicing protein Htra2-β1 \[^{35-38}\]. In addition, an intronic splicing silencer (ISS) and a deactivated exon splicing enhancer (ESE) that directly impact the inclusion or exclusion of $SMN2$ exon 7 have been elucidated \[^{39-41}\]. Another target is the histone deacetylase (HDAC) enzymes that cause condensation of chromatin structure and have been found to negatively impact transcription of $SMN2$ \[^{15,42}\]. Thus, compounds that can modify the expression or activity
of transcriptional regulators, overcome the ISS or activate the ESE, or inhibit HDACs may be therapeutic in SMA.

*The regulation of gene expression through chromatin structure.*

A primary way in which transcription is regulated is through modification of chromatin structure [43, 44]. *In vivo,* DNA is condensed into chromatin through its association with nucleosomes and other packaging proteins. The nucleosome, which is comprised of histones, can be epigenetically modified by histone acetyltransferases (HAT) and histone deacetylases (HDAC) [44]. When histones are acetylated, the chromatin structure relaxes into euchromatin. This relaxed structure gives other protein complexes access to the nucleosomes, which can be removed or moved to produce free DNA [44]. Once freed from the nucleosomes, DNA can be transcribed. Conversely, when acetylation is removed from the nucleosomes, chromatin condenses into heterochromatin, effectively repressing transcription [45]. Heterochromatin is common around centromeres, which often contain DNA repeats such as SMN2 [45]

HDAC inhibiting (HDACi) drugs may provide therapeutic benefit in SMA. The HDACi trichostatin A (TSA), valproic acid (VPA) and phenylbutyrate (PBA) have been shown to promote SMN transcription *in vitro* and *in vivo* [46-50]. VPA and PBA have been used in clinical trials with hopes that increased amounts of SMN will protect remaining
motoneurons and prevent further degeneration \cite{2,30,51}. As these drugs have been used to treat other medical conditions, they were readily available for patient trials.

Unfortunately, clinical trials with VPA and PBA have had limited or negative results \cite{30}. As research has had promising results with HDACi, there is interest in developing newer, more potent HDACi that may be more specific and effective for the treatment of SMA. Two of these compounds are TSA and HDAC-42. TSA has been shown to increase SMN levels, improve motor unit pathology and function, and prolong survival in the mouse model of SMA \cite{46,52}. HDAC-42 has been shown to increase the SMN protein levels in cultured SMA fibroblasts, increase the average lifespan of SMNΔ7 mice, and also reduce motoneuron degeneration \cite{53-55}.

Research Models of SMA

Several models have been developed in order to understand how low levels of SMN result in SMA. The most commonly used are fibroblasts obtained from patient skin biopsies and mouse models \cite{56}. Fibroblasts from patients lack SMN1, are easy to obtain and maintain, and can be used for drug screening. However, as the amount of SMN present varies by tissue type with motoneurons being specifically sensitive to low levels, fibroblasts may not accurately portray the deleterious effects of SMA \cite{57}. Takahashi et al. discovered that fibroblasts can be reverted to a stem cell-like morphology by over expressing specific transcription factors \cite{58}. These cells, termed induced pluripotent stem
cells (iPSCs), are capable of differentiating into cells from all three germ layers and can therefore be used to model cell-specific diseases \[56, 58\]. Using this technique, Ebert and Svendsen have produced motoneuron cell cultures from SMA patient fibroblasts \[56\]. This model may help to identify compounds with potential neuroprotective properties that cannot be identified using fibroblasts \[30, 56\]. Despite these advances, in vitro research is restricted to providing information on how specific cell types respond to reduced levels of SMN. They cannot reflect the complex environment of an animal or human, nor simulate the neuromuscular system, which consists of numerous cell types including neurons, glia, astrocytes, neuromuscular junctions and muscle. This severely limits our understanding of how diseased cells affect one another and how a therapeutic compound would act in the context of the whole organism.

Humans have both the SMN1 and SMN2 genes. All other animals have only one SMN gene that correlates to SMN1 \[59\]. Mice lacking Smn die in early embryogenesis \[60, 61\]. This is not surprising, given the role of SMN in spliceosome assembly and RNA processing \[20\]. Therefore, creation of SMA animal models requires either the manipulation of the endogenous Smn gene to resemble SMN2, or the inclusion of SMN2 with a deletion or disruption of Smn \[19, 61, 62\].

Three mouse models of SMA are most commonly used for research purposes. The first, a double transgenic, was created by adding SMN2 on a Smn\(^{-/-}\) background \[62\]. As with SMA patients, the number of SMN2 copies present determines the severity of the
phenotype, with more copies promoting milder symptoms and a longer lifespan \cite{10, 62}. Mice with only one or two copies of the gene survive approximately one to five days, respectively \cite{19}. However, mice with eight copies do not differ in their phenotype or lifespan from WT mice \cite{62}. Mice with a low SMN2 copy number exhibit symptoms similar to Type I patients including decreased or absent movement, labored breathing, weak intercostals muscles in some mice, and premature death \cite{62}. The second mouse model was created concurrently with the first by another research group \cite{61}. This model also has SMN2 on a Smn−/− background, but it differs from the first in that it was created by interrupting the endogenous Smn gene through removal of Smn exon 7. As with the first model, SMN2 copy number is inversely related to the severity of the phenotype. The third model was created to test the effect of increased SMNΔ7 on an SMN2 model \cite{19}. Increased SMNΔ7 was found to be beneficial to the SMN2 mouse in that lifespan was increased to approximately 13 days. The SMNΔ7 mice are initially able to walk but progressively lose muscle strength and the ability to move \cite{19}. As the severe SMN2 mice have a very short lifespan, SMNΔ7 mice are most often used in therapeutic drug screens \cite{63}.

A porcine model of SMA is currently being created to test therapeutics in an animal that is similar to humans in size and physiology \cite{63}. In this model, the endogenous SMN is targeted with single-stranded DNA modified with recombination sequences. Homologous recombination results in a disruptive deletion of the SMN gene. SMN2 will
then be introduced in order to produce an $SMN^{+/+}$; $SMN2$ model. These animals will be useful for transitioning therapeutics into humans.

*The benefits of using zebrafish to study disease*

Zebrafish provide several unique advantages to study SMA disease progression. The zebrafish is considered an excellent model in which to study vertebrate development and disease regulation as mutants accurately model several characteristics of human diseases [64-67]. Zebrafish develop outside of the mother and are accessible immediately after fertilization. The embryos are optically clear, allowing for microscopic examination of multiple organs including the neuromuscular system [64]. As a result, disease process and effects of treatments can be observed throughout development in the living embryo [64, 66, 67]. Zebrafish are much less expensive to maintain and require less room than mammalian models. They are highly prolific, with a female laying an average of 200 eggs in a single breeding [65-67]. Primary organ systems develop within 72 hours post-fertilization (hpf) and are equivalent to most mammalian organs by 6 days post-fertilization (dpf), reducing experimental time [68, 69]. In addition, many genetic techniques such as transgenesis and gene knockout have been developed for zebrafish, providing tools to study the effects of disease [64, 66, 67].

Zebrafish are vertebrates, and are evolutionally closer to humans than *Drosophila* and *C. elegans*, which are also inexpensive to maintain [67]. Zebrafish have been used to screen
for potentially beneficial drug treatments in such diseases and conditions as polycystic kidney disease, heart disease, leukemia, melanoma, anemia, and cholesterol processing [64-67]. Developing embryos are tolerant to DMSO and easily absorb chemicals from the water [65, 66]. They require only a small amount of space, allowing rapid drug assays in small volumes [65]. This reduces the overall cost by decreasing the amount of drug needed. By using a zebrafish model, toxicity and bioavailability of drugs can be simultaneously determined, the effect of drugs on intact biological pathways can be observed, and sophisticated phenotypes can be studied [64, 66, 67].

The morphant zebrafish model of SMA

We have developed several different, but complimentary, zebrafish models of SMA. The morphant zebrafish model of SMA is obtained by injecting wildtype (WT) with a morpholino against smn. Morpholinos (MO) are synthesized oligomers utilizing morpholine rings in their backbones as opposed to ribose or deoxyribose rings [70]. They function by binding to RNA, blocking translation or altering splicing of the targeted gene. In our zebrafish model, MO injection reduces Smn expression for 10-14 dpf. This is sufficient as organ systems, including the nervous system, develop within that time period [68].

Zebrafish have well-characterized neuromuscular development and organization, providing a means to analyze any defects [71]. Morphant smn larvae exhibit truncated and
branched motor axons that can be easily viewed and categorized \cite{25, 26}. Motoneurons isolated from the \textit{Smn} \textsuperscript{\textminus/\textminus}; \textit{SMN2} mice also show decreased axonal outgrowth \textit{in vitro} \cite{28}. These observations differ from SMA autopsies and \textit{in vivo} examination of the mouse SMA model, which suggest that motoneurons and NMJs develop normally but then undergo denervation \cite{72}. The developmental defects observed in our morphant model may be due to a more simplistic motoneuron developmental pattern than found in mammals or a more severe knock down of Smn. These defects can be rescued by wild-type Smn and a putative modifier of SMA, plastin 3, but not by mutant Smn suggesting that they are a read-out of low Smn levels \cite{25, 73}. Therefore, identifying compounds that promote proper axonal development in the morphant zebrafish model may also promote reinnervation or maintenance of motoneuron junctions (MNJs) in mammalian models and SMA patients.

Although the level of Smn protein reduction is nearly equal between morphants, not every motoneuron is abnormal in \textit{smn} MO-injected embryos, nor is the severity of the defects the same in all muscle segments due to chimeric uptake of the MO \cite{25}. Thus, twenty motor axons, ten on each side of the embryo, are scored and a total fish classification is given \cite{74}. Severe axon defects include missing axons, axons truncated above the ventral nerve cord, and axons that are excessively branched and truncated. Moderate defects include axons that are truncated at the ventral muscle, axons that are excessively branched but not truncated, and axons that innervate neighboring muscle segments. Mild defects include ectopic ventral branching, ectopic ventral spinal roots,
ectopic dorsal branches, and axons that lack stereotypic morphology but do not fall into other categories. The level of expressed Smn can be verified through western blots.

*The mutant zebrafish models of SMA*

The mutant zebrafish models for SMA carry known mutations of the *smn* gene. My lab has three different SMA mutant zebrafish strains: SmnY262stop, L265stop and G264D [75]. The Y262stop and L265stop cause protein truncation just prior to exon 7 or at the second amino acid in exon 7 respectively. The SmnG264D causes a missense mutation located at the first amino acid in exon 7. This mutation is equivalent to the human point mutation SMNG279V, which has been found in some Type I patients [76].

Gene products necessary for embryonic development are initially provided by maternal RNA and proteins deposited within the egg. Embryonic transcription begins as development progresses, and maternal RNA and proteins are degraded [77]. As a result, the level of expressed Smn in the mutants is normal during initial development but declines over time [75]. Fish that are homozygous for the Y262stop or the L265stop mutants have very low levels of Smn and die at approximately 12 dpf whereas fish homozygous for the G264D mutation have higher protein levels and die at approximately 17 dpf [75]. The G264D mutation also differs from the other mutations in that it can weakly rescue motoneuron axon growth defects when co-injected into our
morphant model \cite{75}. Therefore, it is likely that the G264D produces a small but nonsufficient amount of Smn.

As the maternal RNA and protein provides adequate Smn to the embryo during neurogenesis, no developmental defects in motor axon outgrowth are observed in this model. However, neuromuscular junctions, which develop normally, show deterioration at 11 dpf \cite{75}. This result is similar to SMA patient autopsies as well as the severe SMN2 and SMNΔ7 mouse models in which neuromuscular junctions were either partially or completely denervated at postnatal day 14 \cite{19, 72}.

The transgenic SMN2 and SMA zebrafish models

Our lab has also produced a transgenic zebrafish to determine if SMN2 has similar properties in fish as it does in humans and mouse models of SMA \cite{78}. In our model, SMN2 is able to produce the same splice variants. Likewise, FL SMN can be increased by blocking an intronic splicing site. A zebrafish SMA model was then created by crossing the Tg(SMN2) fish with the SmnY262stop mutant. The inclusion of SMN2 significantly extended survival to 14 days and postponed the onset of presynaptic defects \cite{75, 78}.

Unlike the mouse models of SMA, high SMN2 copy number does not rescue the zebrafish phenotype \cite{78}. When comparing zebrafish with a high copy number to fish
with lower copy numbers, SMN was only slightly increased. This may be due to poor activation of the human promoter in zebrafish \cite{78}. Jodelka et al. has recently reported that there is a feedback loop where a small increase in FL SMN results in an increase in SMN exon 7 inclusion \cite{79}. Thus, the small amount of SMN produced in our model was able to significantly extend survival and can be used to screen drug compounds for those that promote exon 7 during splicing.

Research Objectives

As there is currently no cure for SMA, the development of therapeutics is vital. Compounds identified to provide benefit \textit{in vitro} must be tested in vertebrate models before progressing on to human use. Drug screening in mouse and pig models requires several days to weeks to complete, and large amounts of drugs may be needed. Utilizing zebrafish models of SMA would decrease expense significantly as they cost less to maintain and would only require a small amount of drug compound for testing purposes. More importantly, compounds could be screened in a matter of days due to the speed of embryonic development. Thus, using zebrafish in drug discovery efforts may expedite the identification of a therapeutic as non-beneficial or toxic compounds would be removed from study before being tested in mice and pigs. This would reduce overall cost of drug discovery and would focus future research on more promising compounds.
For this purpose, I have explored the use of zebrafish models of SMA for identifying potentially therapeutic compounds. To validate our models, I began by determining if TSA can be taken up and if its effects on histone acetylation and Smn can be measured. In addition, I examined the effects of TSA on motoneuron axon outgrowth in the morphant model and on survivability of the SmnG264D mutant.

I am also creating a transgenic zebrafish that will be unique from all other models that currently exist in that it will simultaneously identify potentially therapeutic compounds and help to elucidate their mechanism of action. Unlike other SMA models, measurements of activity can be obtained in a living animal. As the fish will not be harmed in this process, multiple measurements can be taken, allowing the effects of drug treatments to be monitored throughout development.

By developing drug screening techniques and a novel transgenic zebrafish model, I hope to present valuable tools for SMA drug discovery efforts.
Chapter 2: The use of zebrafish models to test HDAC inhibitors for therapeutic benefit in SMA

Introduction

Histone deacetylase (HDAC) enzymes promote the formation of heterochromatin, and can thus silence genes by inhibiting transcription\(^4\). As HDAC enzymes have been found to negatively impact transcription of SM\(N2\), HDACi have gained attention as potential therapeutics\(^{15,42}\). Two HDACi drugs, valproic acid (VPA) and phenylbutyrate (PBA), are already FDA approved for medical use. As they promote SM\(N\) transcription \textit{in vitro} and \textit{in vivo}, they have been tested in clinical trials\(^2,30,51\). Unfortunately, these drugs provided limited or no benefit\(^{30}\).

DNA methylation can also silence genes through an indirect association with HDACs\(^{80,81}\). Hauke \textit{et al.} recently found that VPA and PBA are unable to overcome SM\(N2\) methylation\(^{82}\). This may explain why VPA and PBA were not beneficial in clinical trials. TSA, which is a more potent HDACi, is able to overcome DNA methylation and has been shown to increase SM\(N\) levels, improve motor unit pathology and function, and prolong survival in the mouse model of SMA\(^{46,52,82}\). These results have promoted interest in discovering new, more potent HDACi for therapeutic use in SMA.
Utilizing zebrafish as a primary vertebrate model to test HDACi for efficacy and toxicity would reduce overall expense and may expedite the identification and development of a therapeutic. In order to determine if our zebrafish models are suitable for HDACi screening, I examined the effects of TSA in wild-type, morphant, and mutant zebrafish models of SMA.

Results

*TSA can be taken up by zebrafish and its effect on histone acetylation can be measured.*

TSA has previously been shown to affect histone 4 acetylation (H4ac) in zebrafish [83, 84]. In these studies, treatment varied from 100 to 200 nM. In order to determine the optimal dose required for future experiments, I tested different concentrations in 3 dpf zebrafish and examined the effect on H4ac after five hours of treatment.

I originally utilized a fluorometric assay to measure levels of HDAC inhibition. To perform this assay, nuclear protein fractions from treated samples were incubated with an acetylated fluorometric lysine substrate. This substrate is deacetylated by endogenous HDAC. Deacetylation permits a lysine endopeptidase to cleave the substrate, releasing and activating a fluorophore. Fluorescence is then measured to determine the activity of endogenous HDAC. Treating a sample with an effective dose of HDACi will result in
significantly lower fluorescence. Unfortunately, this technique was not sensitive enough for my needs. Thus, I examined nuclear protein extracts by western blotting.

Density analysis was initially performed using UltraQuant 6.0 (UltraLum, Inc.). As my results appeared inconsistent, blots were re-scored to verify my original data. By comparing multiple analyses of each blot, I determined that this protocol introduced a wide-margin of error. This was due to the free-hand method used to isolate each band for comparison. Not only did it require a steady hand, but it introduced user bias as judgment had to be used to determine exactly where the edges of each band were located.

In an attempt to address this issue, I tried using a Photoshop (Adobe Systems, Inc.) protocol that had been used by several different research groups in their published work \[85\]. While this method improved data consistency slightly, it was still impacted by free-hand band isolation. Realizing this, a new ImageJ (NIH) protocol designed to specifically reduce user-bias was used \[86\]. To test this protocol for consistency, I analyzed each blot three times. Results were highly comparable, proving that user bias was dramatically reduced.

In the initial UltraQuant and PhotoShop analyses of my blots, it appeared that 50 nM of TSA significantly increased H4ac in zebrafish larvae versus treatment with DMSO vehicle control. However, data obtained through the ImageJ protocol disputed this conclusion. Instead, I found that the effect of TSA varied between replications (Fig.2.1). This variability persisted even when comparing replications from the same experiment.
Figure 2.1. A comparison between western blots and density analysis for replications of three different lots of TSA.
As this issue was not resolved with repeat testing or optimization, I examined the effects of TSA purchased from two more companies (denoted Lot B and C). The variability persisted with Lot B, apparently due to outliers. Lot C was less problematic in regards to variability, but only two replications were examined. Surprisingly, the average relative densities across TSA concentrations were nearly identical between lots (Fig. 2.2A). However, the high variability found within replications resulted in large standard deviations (Fig. 2.2B). As I was concerned about the outlying replication in Lot B (Fig. 2.1), I re-analyzed the data with this replication removed (Fig. 2.2C and D). While this adjustment caused the relative mean densities to be considerably lower than those of Lot A and C, the only statistical significance was between Lots B and C at 500 nM TSA (p < 0.05).

Each lot was then examined to determine the minimal concentration required to significantly increase the level of H4ac (Fig. 2.3). Despite a rising trend in relative density, H4ac was not significantly increased at any of the concentrations tested for Lots A and B. For Lot C, TSA was able to significantly increase H4ac levels at 250 nM (p < 0.05) and at 500 nM (p < 0.01).

In my experiments, I obtained histones by isolating the nuclear protein fraction. The concentrations of my lysates were determined and then equal amounts were probed with anti-H4ac. Collas et al. was interested in obtaining H4ac and associated chromatin [83]. Therefore, they utilized an immunoprecipitation protocol with anti-H4ac. This allowed
Figure 2.2. A) A comparison of the mean relative densities for three lots of TSA at different concentrations. B) A bar graph representation of (A) with standard deviations noted. There are no significant differences between lots for any of the concentrations tested. C) A comparison of the mean relative densities following removal of a possible outlier in Lot B. D) A bar graph representation of the data shown in (C) with standard deviations noted. Lot B has a significantly lower relative density at 500 nM than Lot C (p = 0.02).

them to specifically isolate and examine the level of H4ac per sample. By utilizing this method, they determined that the minimal effective dose of TSA in zebrafish was 100 nM \[83\]. Thus, I decided to examine the effects of 100 and 500 nM TSA in future experiments.
Figure 2.3. Dosage curves for the three lots of TSA. There were no significant differences in H4ac levels for any of the concentrations of Lots A and B tested. However, Lot C TSA was able to significantly increase H4ac levels at 250 nM (p < 0.05) and at 500 nM (p < 0.01).

*Zebrafish larvae must be treated with 100 nM TSA for five hours to affect H4ac.*

In order to determine the minimal treatment time required to increase H4ac, I examined the effect of 100 nM Lot B TSA over the course of five hours (Fig. 2.4). Initially, my data suggested that it was necessary to treat larvae for only 2 hours in order to see a significant effect. Upon re-examination of my blots, I determined that several should not have been scored due to overexposure. Therefore, I reanalyzed my data based on the two blots that were appropriate for scoring purposes. These results indicated that it was necessary to treat larvae for 5 hours with 100 nM TSA in order to see a significant effect.
(P < 0.05). This result differed from what I observed in my dosage curve experiment where a minimum of 250 nM was required to significantly increase H4ac (Fig. 2.3). Thus, I compared the mean relative density found for 100 nM in my dosage response assay to the results found in this experiment (Fig. 2.4C). No significant difference was found between the means. The inconsistency of these results is likely due to the limited number of blots available for scoring and high differences in standard deviations.

Figure 2.4. Time required for 100 nM TSA to affect H4ac. A) Western blots with DMSO as vehicle control. B) 100 nM TSA significantly increases H4ac after 5h of treatment (p < 0.05). C) A bar graph comparing the mean relative densities for 100 nM TSA at 5h.
Overnight treatment with TSA elevated H4ac and Smn levels.

In order to perform survivability assays, I needed to determine how long to treat my larvae in order to maintain elevated H4ac levels. Thus, I examined the effect of treating 3 dpf larvae for 7 hours or overnight with 100 and 500 nM TSA. The larvae were harvested for nuclear proteins 24 hours after the start of treatment. My results demonstrated that overnight treatment was necessary to maintain elevated H4ac levels (Fig. 2.5). I then examined the effect of overnight treatment on Smn. For this experiment, 3 dpf larvae were treated overnight with 0 to 500 nM TSA (Fig. 2.6). Treatment with 250 and 500 nM TSA significantly increased Smn (p < 0.05).

Figure 2.5. Effect of 7 hour and overnight treatment of TSA on H4ac levels at 24h.
Treatment with TSA may decrease the severity of motor axon outgrowth defects in morphants.

In my morphant model, defects in motoneuron axon outgrowth can be rescued by co-injecting wild-type Smn\[^{25,26}\]. Thus, it is reasonable to expect that increased endogenous Smn will also rescue axon outgrowth defects. To test this, I treated morphant embryos with TSA. Unlike my findings in larvae, 50 nM was the optimal dose for embryos as increased concentrations resulted in embryonic death (personal communication, Erin Horan). This suggests that embryos are more sensitive to TSA than larvae. Embryonic death also occurred if TSA was applied prior to 16 hpf. As acetylation patterns differ through development, it may be that TSA interfered with this alteration at vital stages\[^{42}\].

Figure 2.6. The effect of overnight treatment with TSA on Smn level in WT zebrafish. Treatment with 250 and 500 nM significantly increases Smn (p < 0.05) versus vehicle control.
Motoneuron axon outgrowth initiates at approximately 18 hpf in zebrafish\textsuperscript{[87]}. Thus, I was able to treat the embryos prior to this event.

In a preliminary experiment, Erin Horan determined that treatment with TSA reduced the percentage of morphant zebrafish with severe and mild defects, and increased the percentage with no defects (Fig. 2.7A). Additional experiments were performed to validate this finding. In comparison, I found a smaller overall percentage of fish with axon defects after injection with smn MO (Fig. 2.7B). This difference may be due to a MO formulation change, which resulted in higher potency. As a result, there was a narrow margin of error between toxic and non-effective doses of MO. While I also saw a shift towards more mild or no defects, there was no significant difference found between TSA treated and untreated fish.

I have already shown that TSA can increase Smn in WT zebrafish. Therefore, one can expect TSA to increase Smn in the morphant model and thereby reduce axon outgrowth defects. It is possible that 50 nM TSA is not sufficient for axon rescue. However, preliminary results dispute this. Instead, the difficulty in injecting the optimal concentration of MO was most likely impacting my results. It is probable that I did not sufficiently knock down Smn. As this resulted in fewer axon defects, it was unlikely that I could detect a significant level of rescue.
The effect of TSA on the survivability of SmnG264D fish could not be determined.

As TSA was found to increase the level of H4ac and Smn in zebrafish, I next examined if treatment would impact survivability. I selected the SmnG264D model as this mutation correlates with a known Type I mutation in humans\textsuperscript{[76]}. In addition, SmnG264D has a slight but insufficient function in that it can weakly rescue motoneuron axon outgrowth
defects. Unlike the SmnY262stop and SmnL265stop mutants, it is capable of producing a slightly functional protein and survives approximately 5 days longer than the other mutants [75]. Thus, increasing transcription of SmnG264D may provide benefit. The SmnG264D fish are maintained as heterozygotes. In-crossing results in a Mendelian ratio of WT, heterozygotes and mutants [75].

Although I was unable to show a benefit of TSA treatment prior to axon outgrowth, I chose to treat 16 hpf embryos with 50 nM TSA. Twenty-four hours later, the larvae were moved into mesh-sealed PVC tubes and placed into a holding tank containing a higher concentration of TSA. Initially, I used 250 nM TSA as this was the concentration found to increase Smn when treating 3 dpf larvae. However, this concentration was toxic after three days of treatment, perhaps due to an additive effect of the drug or sensitivity of 40 hpf larvae to 250 nM TSA. A second experiment was performed using 100 nM TSA as this was the minimal concentration found to increase H4ac [83]. The tubes allowed me to move the larvae into new holding tanks with fresh preparations of TSA or vehicle control every 24 hours. A total of 116 fish were treated with either TSA or the vehicle control, DMSO. A Mendelian ratio of WT, heterozygote and mutant embryos was not expected as an excess number of embryos were collected from tanks containing more than one breeding pair.

During the course of the experiment, larvae that did not respond to touch were sacrificed and genotyped. As the genotype results were unclear for five fish, they were not included
in statistical analysis. Nearly all of the fish died by 17 dpf regardless of their genotype or treatment (Fig. 2.8). As wild-type and \textit{smn}G264D\textsuperscript{+/-} fish should have a normal lifespan, my results suggest that daily handling caused significant stress. At the conclusion of this experiment, only five fish treated with vehicle control were alive. Interestingly, three were mutants. There was no significant difference found between treated and untreated fish regardless of genotype. Therefore, I cannot draw any conclusion regarding the effect of TSA on SmnG264D survival.

**Discussion and Conclusions**

Discovery and development of newer, more potent HDACi may result in a future therapeutic for SMA. Here I have shown that zebrafish can be used as a primary vertebrate model to screen HDACi for potential benefit.

I have verified that TSA can be taken up by zebrafish and that its effect on histone acetylation can be measured. Although I was able to detect a significant increase in H4ac by western blotting, this method is not very sensitive. In future screens, H4ac should be measured by immunoprecipitation. I have also demonstrated the effects of TSA over time by determining that fish must remain in treatment for a minimum of five hours before a significant increase in H4ac is detected. In addition, zebrafish must remain in treatment in order to maintain an elevated level of H4ac and to see an increase in Smn. While these results are specific for zebrafish, my screening technique could be used to
Figure 2.8. Survivability of WT, smnG264D+/− and smnG264D−/− fish treated with 100 nM TSA or DMSO vehicle control.

identify more potent compounds that require less time to increase H4ac. Compounds with longer lasting effects could also be identified.

While I was unable to draw conclusions regarding the effect of TSA on motoneuron axon outgrowth, I have demonstrated how the morphant model could be used as a unique drug screening tool. To be effective, the correct concentration of MO must be injected in order to sufficiently knock down Smn without killing the embryos. Alternatively, I can screen HDACi in the Tg(SMN2) model to identify compounds that rescue presynaptic
defects [78]. This transgenic model will also provide a benefit over SmnG264D in survivability assays as it is capable of producing FL SMN without mutations. However, a new technique for drug delivery must be developed in order to minimize handling stress.

Zebrafish are less expensive to maintain than other vertebrate models. As embryos and larvae develop quickly, drug studies can be performed rapidly. Here I have demonstrated that zebrafish can be used to identify HDACi that may provide therapeutic benefit for SMA. I believe that future screens using my techniques, or modifications of them, could significantly reduce initial drug discovery cost and expedite the development of a therapeutic.
Chapter 3: The development of a novel zebrafish model for SMA that is designed to simultaneously identify potentially therapeutic drugs and help to elucidate their mechanism of action.

Introduction

As SMA is the most common genetic cause of infant mortality, there is an urgent need to identify and test potential therapeutics [1, 2]. Cell cultures provide a simplistic disease model that can be used to efficiently screen libraries of compounds. In these high throughput screens (HTS), compounds are selected on very basic criteria; they must not be toxic to the cell type being tested and must improve the phenotype. As cell cultures cannot represent the complex biological environment of an animal, compounds identified in HTS must then be tested in vivo.

Mice are commonly used as the primary vertebrate model for drug testing. The SMN2 and SMNΔ7 mouse models are well characterized and beneficial effects of a compound can be measured through increased lifespan, prevention or delay of motoneuron loss, and the maturation and maintenance of MNJs [19, 62]. The porcine model, being closer to size and physiology of humans, will also be useful for testing therapeutics [63]. While the value of these models in drug screening and testing cannot be disputed, their use is
expensive. Not only are mice and pigs costly to maintain, but drug testing takes several days to weeks and would likely require multiple doses. Depending on the compound being tested, this could significantly increase expense. In addition, it is important to determine the mechanism of action (MOA) for each compound in order to design drugs with a higher specificity while minimizing toxicity. This can take a considerable amount of time, which also increases expense.

An intermediate vertebrate model that is less expensive to maintain, requires less time for testing, and needs only a small amount of drug compound would decrease expenses dramatically. By using zebrafish, compounds can be tested for efficacy and toxicity before progressing to mice and pigs. This would reduce overall expense, and may identify a therapeutic more quickly as non-beneficial or toxic compounds would be removed from study before being tested in mice and pigs. As a result, further research could focus on more promising compounds.

I am creating a transgenic zebrafish model that would expedite drug screening and allow us to measure the effect of a drug compound on the production of SMN in vivo. While developing this model, my design evolved into one that would allow us to simultaneously identify beneficial compounds and help to elucidate their MOA.
Designing the transgene construct

My initial plan was to create a transgenic zebrafish to identify compounds that promote inclusion of exon 7. To do this, I designed a construct to tag FL SMN and SMNΔ7 with different fluorophores. SMN2 is comprised of 9 exons (Fig. 3.1A). The endogenous stop codon for FL SMN lies in exon 7 whereas the stop codon for SMNΔ7 is located in exon 8. I could not directly tag the FL SMN splice form by replacing the stop codon with a fluorophore as this would disrupt splicing recognition sites. Therefore, I planned to insert a single nucleotide just prior to the stop codon. This would allow the stop codon to be read through and would also shift the reading frame. Exon 8 would be truncated just prior to the endogenous stop codon for the SMNΔ7 splice form. GFP and mCherry would follow, each in the correct reading frame to tag only one of the splice forms (Fig. 3.1B). As a result, FL SMN would fluoresce green and SMNΔ7 would fluoresce red.

Once I determined how to tag the splice forms, I considered the complexity of using genomic SMN2 in my construct. SMN2 is approximately 28 kb long. A DNA fragment of this size would be difficult to manipulate and to incorporate into the zebrafish genome. In addition, while SMNΔ7 is the most commonly transcribed splice form, exon 5 can also be excluded. To eliminate this confounding factor and shorten the length of my construct, I decided to replace the genomic sequence from exon 1 through exon 6 with a cDNA cassette. By replacing the genomic sequence with the cDNA cassette, my modified SMN2 gene would be reduced to a manageable 3 kb. While researching
potential sources for the cDNA cassette, I found that a construct similar to ours had already been created \cite{13,90}. The only difference was that FL SMN was the only splice form tagged. I was able to obtain this construct from Dr. Eliot Androphy (University of Indiana).

Figure 3.1. The initial construct design showing fluorescently tagged splice forms.

A) Schematic of SMN2 with green and red dashed lines indicating FL SMN and SMNΔ7 splice forms respectively. B) The original construct design with GFP and mCherry positioned after exon 8. Green and red colored bars above and below exon 8 and the fluorophores indicate protein transcribed for FL and SMNΔ7 respectively.
Next, I had to consider the length of SMN2 promoter (SMNp) that I would use in my construct. Monani et al. examined the region upstream of SMN and determined that the minimal effective promoter is contained within the 550 bp preceding the gene sequence [91]. Promoter activity increased when the region was extended to 750 bp, suggesting the presence of enhancer or transcription factor binding sites in this region. Conversely, promoter activity was significantly reduced when the region was extended to 3.4 kb. In order to provide benefit, a therapeutic compound that increases transcription must overcome the negative regulatory regions upstream of SMN2. Therefore, I chose to build two transgene constructs, one containing the 3.4 kb promoter region examined by Monani et al., and a second containing a larger 5.7 kb region. This larger promoter was selected as it contains nearly the entire non-coding region of DNA preceding SMN2. Therefore, any unidentified regulatory factors within this region would also be present. These promoters were provided to us by Dr. Arthur Burghes (Ohio State University).

The SMNp vectors that were given to us contained GFP. This produced an alternative construct idea. If I created a dual promoter vector, one that promoted GFP and the other that promoted SMN2-mCherry, I would be able to identify and differentiate between compounds that affect splicing and those that either promote SMN2 transcription or stabilize SMN. A compound that affected splicing and enhanced inclusion of exon 7 would increase mCherry expression without impacting the level of GFP. A compound that increased both GFP and mCherry expression would be functioning by either promoting transcription or by stabilizing proteins. It would be unlikely for us to identify
a compound that increased GFP fluorescence without impacting mCherry as a compound that promotes transcription would cause SMN2 to produce more transcripts of FL SMN. Therefore, my model would not be able to differentiate between compounds that promote transcription and those that stabilize proteins, but would eliminate enhanced splicing as their MOA. As my constructs would contain both GFP and mCherry fluorophores, I referred to them as 3.4 NOEL and 5.7 NOEL (Fig. 3.2).

![Diagram](image)

**Figure 3.2.** The final construct design with dual promoters. Only FL SMN will be tagged with mCherry.

**Cloning techniques**

My next challenge was determining how to place both the SMNp:GFP and SMN2-mCherry constructs into the same vector. I looked at several different cloning techniques, starting with homologous recombination (referred to as recombineering) [92]. Recombineering is often the most efficient method of cloning as it requires little if any subcloning. It also avoids the use of endonucleases and ligations, which tend to be inefficient [93, 94]. To perform homologous arm recombineering, the desired DNA fragment for insertion is amplified by polymerase chain reaction (PCR). The primers for this reaction have about 50 bases that are homologous to the targeting area in the destination vector along with bases matching the 3’ or 5’ end of the DNA construct being
amplified. An example of homologous arm recombineering is shown in figure 3.3. In this example, the genomic sequence for *SMN2* exons 1-6 is replaced with a cDNA cassette. *Galactokinase* (*galK*) is used as a shuttle vector and to impart positive and negative selection depending on the plating medium used for bacterial growth \[92\]. While homologous recombineering has advantages over classical cloning, it lacks specificity. I would not be able to create an homologous arm primer that is specific for one location in my construct as *SMN2* and its promoter have a high number of repeat sequences. These endogenous homologous sequences would also be prone to recombining with each other, thus it was highly likely that my construct would have rearrangements or deletions.

![Diagram of homologous arm recombineering](image)

**Figure 3.3.** An example of homologous arm recombineering. Homologous primers are denoted as H1-4.

I then considered using the Gateway recombineering system, which was developed to improve specificity and fidelity of recombineering reactions \[93-95\]. This *in vitro* system utilizes the λ integrase family of recombinases, which recognize *att* recombination sites.
Hartley et al. developed *att* mutations that would only recombine with one other type of *att* site, thus increasing specificity and making it possible to control the orientation of inserted cassettes \[96\]. This also made it possible to simultaneously insert multiple DNA constructs into a destination vector (Fig. 3.4) \[93,94\]. While the Gateway system sounded promising, there are limits to its application. It has been used to recombine DNA constructs totaling 10 kb (N. Lawon, personal communication), but it loses efficiency with constructs larger than 3 kb \[95\]. My constructs exceed this limit by being approximately 13 kb and 17 kb in length.

![Gateway recombineering plan](image)

Figure 3.4. A Gateway recombineering plan to simultaneously insert our two DNA fragments into a destination vector. Adapted from Villefranc et al. 2007.

Due to size limits and the number of modifications that I wished to make, I decided to use classical ligation-mediated cloning to create my constructs. A detailed cloning plan is shown in Appendix A. Creating my construct by classical cloning would involve several
different steps, each requiring unique restriction sites. \textit{SMN} and its promoter can be cleaved by a vast number of endonucleases. This limited the number of unique restriction sites available in the regions that I would need to cleave. The few sites that were available would not be sufficient for the number of cloning steps involved. Therefore, I designed restriction site cassettes that were prepared by PCR and inserted into the appropriate locations. The PCR primers that were used to create these cassettes contained up to two unique restriction site sequences flanked by the sequence of a site already in the vectors.

I also modified my constructs by flanking them with I-SceI endonuclease recognition sites and adding polyadenylation (polyA) tails after each fluorophore. Co-injecting a construct with I-SceI into embryos improves germline transmission rate\textsuperscript{[97]}. The recognition sequence for I-SceI is very rare and is not found in the zebrafish genome, thus it will only act upon my injected construct\textsuperscript{[97]}. I-SceI is thought to assist in two ways. First, it cuts the construct out of the injected vector, providing short linear fragments that are easier to incorporate into the genome\textsuperscript{[97]}. Secondly, it counteracts an endogenous ligase, preventing large concatemers from forming\textsuperscript{[97]}. As large concatemers are known to induce gene-silencing in vertebrates, this helps to ensure transgenic expression. PolyA tails increased the likelihood that my construct will be expressed, as the polyA tail assists in exporting the mRNA to the cytosol and stabilizing it for translation.
My cloning process also required the use of more than one strain of *E. coli*. The sites in my restriction cassettes had to be unique to the vectors; therefore I was limited to the type that could be used. One of my sites was recognized by *Clai*. The activity of this endonuclease is blocked by methylation requiring the use of an *E. coli* strain that was *dam*<sup>−</sup>*/dcm*<sup>−</sup>. This mutation prevents DNA from being methylated. My constructs were also prone to recombination and deletions due to the high number of sequence repeats. To address this issue, I utilized a strain of *E. coli* that was *recA*<sup>−</sup> and therefore incapable of recombination<sup>[98]</sup>. Unfortunately, there was no strain of *E. coli* available that was both *dam*<sup>−</sup>*/dcm*<sup>−</sup> and *recA*<sup>−</sup>. Thus, recombination and deletion were common when my vectors were grown in the *dam*<sup>−</sup>*/dcm*<sup>−</sup> strain. To identify a vector that had not been subjected to recombination, I extracted vector DNA from several *E. coli* colonies and screened each one with restriction digests (Fig. 3.5). This method allowed us to confirm the size and integrity of each vector. It was necessary to perform multiple digests as several non-methylated vectors appeared to be the correct size with one digest but a secondary screen would reveal that restriction sites had been moved from their original location. To maintain the integrity of my identified vectors, each was transformed into the *recA*<sup>−</sup> cell line for cryogenic storage.

Once my constructs were created and examined for accuracy, they were co-injected with I*-SceI* into embryos at the one-cell stage.
Figure 3.5. Examples of vectors that have been recombined. Lanes 1 and 2 show two preparations of the same vector whereas lanes 3 and 4 are examining a second vector. Samples 1 and 4 have undergone recombination as exhibited by the loss of some bands and presence of additional bands when compared to samples 2 and 3.

Results

*The 3.4 and 5.7 kb promoters are transiently functional in zebrafish.*

Prior to building my constructs, I needed to confirm that the 3.4 kb and 5.7 kb SMN2 promoters would be functional in zebrafish. To do this, I injected the SMNp:GFP constructs at the one-cell stage and examined the resulting larvae for GFP expression (Fig. 3.6). Only a few cells expressed a high level of GFP. This may have been due to my limited experience with micro-injections. However as expression was observed, I concluded that the promoters were functional in zebrafish.
The 3.4 NOEL and 5.7 NOEL constructs are expressed in microinjected zebrafish.

To increase the likelihood that the transgene would be inserted into the zebrafish genome, the highest tolerated concentration of DNA was injected. To determine this concentration, I microinjected early one-cell stage WT embryos with either 3.4 or 5.7 NOEL concentrations ranging from 25-75 ng/nl. Concentrations above 50 ng/nl resulted in severe morphological defects and embryonic death. At 50 ng/nl, approximately 80% of the embryos died or had severe morphologic defects. The remaining 20% survived. Of these, approximately 1% exhibited shorter tails, but as they were mobile and did not appear to be negatively impacted, they were considered acceptable for screening purposes. As 50 ng/nl DNA was survivable, it was used in all future microinjections.

For each NOEL, approximately 1,500 Long-fin (LF) wildtype embryos were co-injected with I-SceI at the early, one-cell stage. Injected embryos were examined at 4 dpf for expression of the fluorophores (Fig. 3.7). Of these, about 2% had robust expression of GFP in cells throughout their bodies and were maintained in the fish facility as F₀. In total, I maintained 34 and 24 F₀ for 3.4 and 5.7 NOEL respectively. SMNp:GFP was expressed at high levels that could be detected visually. However, SMNp:SMN2-mCherry required long 30-50 second exposures to view. This is not surprising as only about 10% of transcripts produced from SMN2 are FL SMN[16]. This low expression level may be beneficial in future drug screens as slight differences in expression will be easier to detect.
The 3.4 kb and 5.7 kb human SMN2 promoters are expressed transiently in zebrafish.

Screening F<sub>1</sub> for the development of the 3.4 and 5.7 NOEL lines.

Upon reaching maturity, the F<sub>0</sub> NOEL fish, which were on the LF background, were outcrossed with ABLF wildtype. The melanocytes in the adult LF appear in a spotted pattern. This pattern is easy to distinguish from the striped pattern of ABLF wildtype fish. To minimize stress, zebrafish are handled and bred only once a week. When outcrossing, one of the strains is fin clipped in order to distinguish it from its breeding partner. However, the fins regenerate in approximately a week. My pattern method simplified breeding efforts as it was possible to keep F<sub>0</sub> NOEL fish with their breeding
partners until a minimum of 150 progeny (F\(_1\)) had been screened for NOEL expression. This usually required two to three weeks.

As mCherry required long exposures to visualize in the F\(_0\), I screened the F\(_1\) for GFP only. To date, four 3.4 NOEL F\(_0\) and seven 5.7 NOEL F\(_0\) have been screened. Of all the embryos screened, only one positive 3.4 NOEL F\(_1\) has been identified. As SMN is ubiquitously expressed, I expected to see expression throughout the fish. However, only a few cells in the head and above the notochord were labeled (Fig. 3.8). This may be due to a chromosomal positioning effect where the construct was incorporated into a region that is silenced in most cells\(^{[99,100]}\). As the parent F\(_0\) must have the construct integrated into her germline, I bred her three more times and screened over 300 additional embryos. Unfortunately, no other F\(_1\) was identified.

Figure 3.7. NOEL is transiently expressed in zebrafish.
Discussion

Here I outline a strategy for generating a transgenic zebrafish that can be used to screen drug compounds that both affect transcription and SMN splicing. At this time, I have not identified a positive F\textsubscript{1} NOEL fish that can be used to establish a transgenic line. By using I-SceI in my microinjections, I hoped to integrate my construct into the genome at an early stage of development, thus increasing the probability of germline transmission. However, when I screened F\textsubscript{0} for expression of NOEL, I noticed a mosaic expression (Fig. 3.7). This suggests that the construct was not integrated early and was instead being distributed unevenly with each cell division\textsuperscript{[97]}. While integration could take place later in development, it is possible that this event occurred after gamete formation. As other transgenic fish were successfully created in our lab using the same lot of I-SceI, it is unlikely that the endonuclease was non-functional. Instead, it’s possible that the size of the NOEL construct hindered integration or that it was silenced within the genome. Alternatively, a positive F\textsubscript{1} might have not been identified due to very low expression of GFP. This may have been caused by a low transgene copy number or poor expression of

Figure 3.8. An F1 NOEL larvae with expression of SMNp:GFP affected by positional effects.
the human promoter in zebrafish\textsuperscript{78,101}. As a transgenic F\textsubscript{1} has been identified, albeit with positional effects, the transgene can be expressed at a visible level. Therefore, it is possible for us to identify F\textsubscript{1} that will be suitable for development of the NOEL lines.

While our lab has already developed a transgenic zebrafish model to screen potentially therapeutic compounds, the NOEL fish offers unique benefits\textsuperscript{78}. In the Tg(SMN2) model, treatment with a beneficial compound would extend survival and may postpone presynaptic defects. However, the drug would have to be provided at an effective dose and at the correct moment in development in order for an improvement in phenotype to be observed. It is also likely that multiple treatments would be required over the lifespan of the fish. In comparison, a drug compound could be screened at any point during development in the NOEL fish as they have endogenous \textit{smn} and would not need the compound in order to survive. Toxicity and effective dosages could also be determined quickly based on the effect of the compound on development, survivability and level of fluorophore expression. A drug screen using NOEL fish would be more efficient as it could be performed in 1-2 days instead of requiring two weeks. Another unique benefit of the NOEL model is that fluorescence could be measured in live animals without harm to the larvae. Therefore, the effect of compounds over time could also be examined. This may provide valuable insight to the decay rate and any additive effects caused by repeat dosing of a compound. Drugs identified through screening in NOEL could then be tested on the Tg(SMN2), morphant and mutant models to confirm benefit.
The Tg(SMN2) fish was created to identify drug compounds that promote the inclusion of exon 7 \cite{78}. In actuality, this model will identify drug compounds that impact splicing, activate the promoter or stabilize the protein. This model cannot distinguish between these MOA as they are all likely to increase lifespan and improve presynaptic defects. The NOEL model would be capable of differentiating between compounds that promote inclusion of exon 7 from those that either promote transcription or stabilize the protein. While any beneficial compound could lead to a potential therapeutic, compounds that enhance inclusion of exon 7 may provide more specific effects than compounds with other MOA. For example, drugs that function by promoting transcription may be doing so in a way that could also promote genes that are normally silenced. Likewise, compounds that stabilize SMN might also stabilize other proteins that are normally degraded. Depending on the changes induced, these compounds could cause serious deleterious effects.

While not yet complete, my transgenic NOEL fish are a promising tool for SMA drug screening. Their unique design and application may significantly increase the speed of identifying potential therapeutics in a vertebrate model. Additionally, the NOEL fish will be able to identify non-beneficial or toxic compounds that should be eliminated from further studies in mouse or pigs. As a result, the cost of drug development will also be reduced. Identification of a positive F1 gives me encouragement that my model can be created and will be useful in the future.
Chapter 4: Materials and Methods

Zebrfish maintenance

Standard protocols were followed for zebrafish maintenance[^102]. Unless otherwise noted, fish were housed at 27 to 29°C. Developmental staging was used to determine hours post-fertilization[^68]. Fish used in these studies were AB*LF, LF and SmnG264D^{+/—}[^75]. All fish were anesthetized in tricaine (Sigma-Aldrich) solution prior to sacrificing or mounting for microscopic examination.

Preparation of TSA

TSA was prepared in DMSO as it is insoluble in water. To prevent TSA from precipitating, all embryos were treated in fish water containing 1% DMSO.

TSA concentration experiments

To determine the minimum effective dose of TSA required to significantly increase H4ac, WT larvae were treated at 3 dpf with 0, 50, 100, 250 and 500 nM TSA in regular...
fish water containing 1% DMSO. The larvae were sacrificed 5 hours post-treatment, and nuclear protein was isolated. Lysates were quantified by BCA (Thermo Fisher Scientific) and analyzed by western blot.

To determine the minimum time required to significantly increase H4ac, 3 dpf WT larvae were treated with 0 or 100 nM TSA in regular fish water containing 1% DMSO. Larvae were sacrificed every hour post-treatment for five hours. Nuclear protein was isolated and quantified then analyzed by western blot.

To determine the minimum time and concentration of TSA necessary to elevate H4ac and Smn levels at 24 hours, 3 dpf WT larvae were treated with 0, 100, and 500 nM TSA. Larvae were sacrificed after 7 hours or 24 hours of treatment. Whole cell and nuclear proteins were extracted for Smn and H4ac respectively. Lysates were analyzed by western blot.

**Protein extraction**

To isolate nuclear proteins, a nuclear extraction kit (Cayman Chemical) was utilized. Briefly, 40 larvae were homogenized in 200 μl 1X Hypotonic Buffer supplemented with 10 mM DTT and 10% NP-40 and incubated on ice for 15 minutes. Samples were centrifuged (4,000 x g) for 10 min at 4°C and the supernatant, which contained cytosolic proteins, was discarded. The pellet was suspended in 200 μl 1X Hypotonic Buffer and
incubated on ice for 15 minutes. Following incubation, 10% NP-40 was added, and the samples were centrifuged (4,000 x g) for 10 min at 4°C. The supernatant was discarded and the pellet was suspended in 50 μl Extraction Buffer. The samples were incubated on ice for 30 minutes, with periodic vortexing to ensure pellet suspension. Samples were centrifuged (14,000 x g) for 30 seconds at 4°C and the supernatant was collected and divided into aliquots. Samples were stored at -80°C until use. Protein was quantified by BCA.

To extract whole cell (total) protein, ten larvae were dissolved in 60 μl of blending buffer (62.6 mM Tris pH 6.8, 5 mM EDTA, 10% SDS and protease inhibitors) by boiling for 10 minutes. Samples were centrifuged and the supernatant was reserved. Lysates were divided into aliquots and stored at -80°C until use. Through previous experimentation, I have determined that protein concentration is consistent between samples at 24 μg/larvae. Therefore, individual samples were not quantified.

Western blotting

Either 25 μg of nuclear protein extracts or 6 μg of whole cell protein were resolved by electrophoresis in 12% polyacrylamide gels, and then transferred to polyvinylidene difluoride membrane (Hybond-P; Amersham Bioscience). Nuclear extracts were probed with mouse anti-TBP (1:1500; Millipore) and rabbit anti-H4ac antibodies (1:1000; Ab Serotec). Whole cell protein lysates were probed with mouse anti-SMN (1:500,
MANSMA7)\textsuperscript{[103]} and mouse anti-αTubulin (1:1000, Santa Cruz Biotechnology). Signal was detected using horseradish peroxide-conjugated goat anti-mouse or goat anti-rabbit antibody (1:5000; Jackson ImmunoResearch Laboratories, Inc.), ECL reagents (Thermo Fisher Scientific) and Amersham Hyperfilm ECL (Amersham Bioscience). Density analyses were performed on scanned film images.

**Western blot density analyses**

To identify a density analysis protocol that minimized user bias, protocols using UltraQuant 6.0 (UltraLum, Inc.), Photoshop (Adobe Systems, Inc.)\textsuperscript{[85]} and ImageJ (NIH)\textsuperscript{[86]} were compared. Three blots were analyzed three times for each program. Results for each program were averaged and standard deviations were calculated (data not shown). The ImageJ protocol provided consistent results with small standard deviations. Therefore, this protocol was used to obtain data for statistical analysis.

UltraQuant 6.0 protocol: For each sample, the region for the protein of interest (H4ac or Smn) was identified by free-hand tracing. The total gray level density and number of pixels were noted and the data were transferred into a Microsoft Excel file. Density per pixel was calculated for each sample. To normalize the data, the density per pixel for each sample was divided by the density per pixel of the control treatment sample (0 nM TSA). This gave a comparative ratio. This process was repeated to obtain the normalized density per pixel values for the loading control samples (TBP or αTubulin).
The comparative ratio was then adjusted for protein load by dividing the normalized values of the protein of interest by the normalized values of the loading control. Statistical analysis was then performed.

Photoshop protocol: Images were inverted prior to analysis as this resulted in a higher density value being correlated with a denser band. The region for the protein of interest (H4ac or Smn) was identified by free-hand tracing. From the histogram option, the mean density value and number of pixels were recorded. This information was transferred to an Excel file. Absolute intensity was calculated by multiplying the mean density by the pixel value. This value was then normalized to the control sample (0 nM TSA) to get the relative density. This process was repeated for loading control proteins (TBP and αTubulin). To adjust for loading variations, the relative density of the protein of interest was divided by the relative density of the loading control protein.

ImageJ protocol: Images were straightened using Photoshop Elements 5.0 (Adobe Systems, Inc.). In ImageJ, each sample lane was identified using the rectangular selection tool. A profile plot was generated for each lane, with protein bands appearing as peaks. In order to remove background signal, a straight line was drawn across the base of each peak. By using the label peaks command, the size of each peak was determined as a percentage of the total area of all peaks. These data was then transferred to an Excel file. The relative density was determined by dividing the percent value for each sample by the percent value of the control treatment (0 nM TSA). This process was repeated for the loading control proteins.
(TBP and αTubulin). To adjust for loading variation, the relative density of the samples were divided by the relative density of the loading control.

Motoneuron axon outgrowth assay

A standard control and an antisense MO (Gene Tools, LLC) designed to block translation of *smn* were injected into AB*LF* embryos at the one- to two-cell stage as previously described [26, 104]. As the commercial formulation for MO production has changed, the optimal concentration for microinjections had to be determined for each new vial and ranged from 3.5 to 4.5 ng. The injected embryos were transferred to 10 cm plates with each plate containing no more than 20 embryos. Plates were incubated at 28°C until embryos reached the 50%-epiboly stage and then placed at room temperature overnight to slow development [68]. When the embryos reached 16 hpf, they were treated with 0 or 50 nM TSA and incubated at 28°C. At 36 hpf, embryos were dechorionated, sacrificed, and fixed in 4% paraformaldehyde overnight at 4°C. Motoneurons were labeled with mouse anti-znp1 (1:100; Iowa Hybridoma Bank) as previously described [105].

The morphology of the motor axons in hemisegments 7-15 was examined on both sides of the embryo and a total fish classification was given based on the number and severity of defects found [25]. Severe axon defects include missing axons, axons truncated above the ventral nerve cord, and axons that are excessively branched and truncated. Moderate defects include axons that are truncated at the ventral muscle, axons that are excessively
branched but not truncated, and axons that innervate neighboring muscle segments. Mild defects include ectopic ventral branching, ectopic ventral spinal roots, ectopic dorsal branches, and axons that lack stereotypic morphology but do not fall into other categories. Fish designated as severe had at least 2 severe or 4 moderate defects. Fish classified as moderate had 1 severe, 2-4 moderate or 4 mild defects. Fish designated as mild had 1 moderate or 2-4 mild defects.

Survivability assays

Embryos were obtained by in-crossing SmnG264D<sup>+/−</sup>. Embryos were sorted into 10 cm plates with no more than 20 embryos per plate. Plates were incubated at 28°C until embryos reached the 50%-epiboly stage and then placed at room temperature overnight to slow development<sup>[68]</sup>. When the embryos reached 16 hpf, they were treated with 0 or 50 nM TSA and incubated at 28°C. At 40 hpf, embryos were transferred to holding tanks containing PVC tublings and treated with 0 or 100 nM TSA. Tublings were transferred into new holding tanks with fresh preparations of TSA every 24 hours. Twice daily, larvae that had died or did not respond to touch were removed and date of death was noted. DNA was extracted using the REDExtract-N-Amp Tissue PCR kit (Sigma Aldrich). Genotyping was performed with derived cleaved amplified polymorphic sequence (dCAPS). Primers were designed to introduce a TfiI restriction site into amplified DNA containing the G264D mutation<sup>[75, 106]</sup>. Samples were digest with TfiI (New England Biolabs) and separated on 2% agarose gel by electrophoresis.
was determined by the resolved product sizes with 327 bp for WT, 295 bp for
\textit{smnG264D}^{-/-} and both product sizes for \textit{smnG264D}^{+/-}. The date of collection and
 genotype of each fish were used to create a Kaplan-Meier survivability plot.

Statistical analysis

Survival Kaplan-Meier plots and log-rank tests were prepared with SPSS version 19
(SPSS, Inc.). All other data were analyzed with InStat (GraphPad Software, Inc.).

Standard analyses of variance s were performed followed by Dunnett’s or Tukey’s post-
tests. Dunnette’s post-test was used when comparing treatments to a control and Tukey’s
was used when comparing all treatments to each other.

Cloning techniques

Figures outlying each cloning step for the 3.4 and 5.7 NOEL vectors can be found in
Appendix A. The pEGFP/3.4 kb SMNp and pEGFP/5.7 kb SMNp vectors were a gift
from Dr. Arthur Burghes (The Ohio State University). The pCEP4/SMN2-mCherry was a
gift from Dr. Eliot Androphy (University of Indiana). \textit{I-SceI} pBluescript was created by
Bruce Appel.

Classical cloning techniques using endonuclease digestion and ligations were utilized
throughout the construction of the NOEL vectors. Sequencing was used to verify the
location of I-Sce I sites in the pBluescript vector and to determine the directionality of inserted Dra III cassettes. The remaining vectors were confirmed by multiple endonuclease digests as the high number of sequence repeats prohibited sequencing.

To create the restriction site cassettes, complimentary primers were designed to contain one to two unique restriction sites flanked by a restriction site contained within the vector. To anneal the primers, 200 ng of each was suspended in 100 μl of distilled water and placed into a thermocycler. The solution was heated to 95°C for five minutes and then allowed to slowly cool to room temperature. The cassettes were then digested with the endonuclease that recognizes the flanking sites. The vector requiring the insertion was also digested, and the cassette was ligated into place. XL1 Blue E. coli were transformed with the ligated solution, and plated on LB containing the appropriate selection antibiotic for the vector. Minis were inoculated from resulting colonies and DNA was extracted using a plasmid mini kit (Qiagen). To identify a vector containing the insert, restriction digest were performed using two endonucleases, one that recognized an inserted restriction site and a second that recognized a unique site within the vector. Digests were analyzed by electrophoresis on 0.7% agarose gels. For the Dra III cassette, vectors that cut appropriately were sequenced to determine the directionality of the insertion. The Dra III cassette contained Xho I and Cla I sites and was inserted into the pEGFP/3.4 kb SMNp and pEGFP/5.7 kb SMNp vectors. The Acc65I cassette contained an Aat II site and was inserted into the I-SceI pBluescript vector.
The parent vectors pEGFP/3.4 and 5.7 kb SMNp, pCEP4/SMN2-mCherry, and I-SceI pBluescript were grown in XL1 Blue (Stratagene). The endonuclease Cla I is blocked by methylation, thus vectors were grown in dam\textsuperscript{-}/dcm\textsuperscript{-} INV110 (Invitrogen) prior to digest with Cla I. Vectors that contained a high number of sequence repeats were grown in Stbl2 (Invitrogen) to prevent recombination and deletions. All vectors were transformed into Stbl2 for long-term cryogenic storage.

**Micro-injections for transgenesis**

Injection solutions for transgenesis consisted of 50 ng/nl freshly prepared DNA, 1/10 volume I-SceI buffer (New England Biolabs), 5 units of I-SceI, and 0.1% phenol red. This solution was injected into early one-cell stage LF embryos to no more than 10% of the cell volume (approximately 1 nl). Injected embryos were placed in standard fish media containing penicillin and streptomycin (1:100; Invitrogen) and incubated at 28°C overnight. Live embryos were transferred to fresh fish media daily. Four days post-injection, larvae were screened. Those exhibiting strong GFP expression in the majority of their cells were transferred to our nursery as F0s. Once matured, F0s were out-crossed with AB*LF. The resulting embryos were screened at 4 dpf for expression of GFP. Once developed, the transgenic lines will be designated as Tg(--3.4SMN2::GFP;--3.4SMN2::SMN2-mCherry) and Tg(--5.7SMN2::GFP;--5.7SMN2::SMN2-mCherry), and referred to as 3.4 and 5.7 NOEL respectively.
Bibliography


Appendix A: Detailed cloning plans for the 3.4 and 5.7 NOEL vectors

Cloning 3.4 NOEL

Figure A.1. 3.4 NOEL cloning step 1. A PCR-created DraIII cassette, containing XhoI and ClaI sites, was inserted into the pEGFP/3.4 kb SMNp vector. This provided unique restriction sites for future cloning steps.
Figure A.2. 3.4 NOEL cloning step 2. A) The 3.4 kb SMNp:GFP cassette was removed from pEGFP/3.4 kb SMNp and placed into the I-SceI pBluescript between the Acc65I and XhoI restriction sites. This flanked the cassette with I-SceI sites. B) The resulting I-SceI 3.4 kb SMNp:GFP vector. This vector was grown in dam'/dcm' cells in order to produce non-methylated DNA.
Figure A.3. 3.4 NOEL cloning step 3. A) The SMN2-mCherry cassette was removed from the pCEP4/SMN2-mCherry vector and ligated into the pEGFP/3.4 kb SMNp with DraIII cassette vector between the SacII and NolI restriction sites. This ensured that the 3.4 kb promoters would be exactly the same in our final construct. In addition, this provided a polyA signal following mCherry to ensure translation. B) The resulting 3.4 kb SMNp:SMN2-mCherry vector. This vector was grown in dam/dcm cells to produce non-methylated DNA.
Figure A.4. 3.4 NOEL cloning step 4. A) The 3.4 kb SMNp:SMN2-mCherry cassette was removed and placed into the I-SceI 3.4 kb SMNp:GFP vector between the SalI and ClaI restriction sites. ClaI is sensitive to methylation, thus non-methylate DNA was used. Note that each fluorophore was followed by a polyA signal and the I-SceI sites flank both the 3.4 kb SMNp:SMN2-mCherry and 3.4 kb SMNp:GFP cassettes. B) The resulting 3.4 NOEL vector.
Figure A.5. 5.7 NOEL cloning step 1. A PCR-created Acc65I cassette, containing an AatII site, was inserted into the I-SceI pBluescript vector.
Figure A.6. 5.7 NOEL cloning step 2. A PCR-created DraIII cassette, containing XhoI and ClaI sites, was inserted into the pEGFP/5.7 kb SMNp vector.
Figure A.7. 5.7 NOEL cloning step 3. A) The 5.7 kb SMNp:GFP cassette was removed from pEGFP/5.7 kb SMNp with DraIII cassette and placed into the I-SceI pBluescript with Acc65I cassette at the XhoI restriction site. Vectors were sequenced to identify one with the cassette in proper orientation. This step resulted in I-SceI sites flanking the cassette. B) The resulting I-SceI 5.7 kb SMNp:GFP vector. This vector was grown in dam'/dcm' cells in order to produce non-methylated DNA.
Figure A.8. 5.7 NOEL cloning step 4. A PCR-created EcoRI cassette, containing a SalI site, was inserted into the pEGFP/5.7 kb SMNp with DraIII vector.
A) The SMN2-mCherry cassette was removed from the pCEP4/SMN2-mCherry vector and ligated into the pEGFP/5.7 kb SMNp with Dual cassettes vector between the SacII and NotI restriction sites. This attached the 5.7 kb promoter and provided a polyA signal. B) The resulting 5.7 kb SMNp:SMN2-mCherry vector. This vector was grown in dam/dcm- cells to produce non-methylated DNA.
Figure A.10. 5.7 NOEL cloning step 6. A) The 5.7 kb $SMNp$:$SMN2$-mCherry cassette was removed and placed into the I-SceI 5.7 kb $SMNp$:GFP vector between the $SalI$ and $ClaI$ restriction sites. $ClaI$ is sensitive to methylation, thus non-methylate DNA was used. Note that each fluorophore was followed by a polyA signal and the I-SceI sites flank both the 5.7 kb $SMNp$:SMN2-mCherry and 5.7 kb $SMNp$:GFP cassettes. B) The resulting 5.7 NOEL vector.
### Appendix B: Definitions of Abbreviations

Table 1. Definitions of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABLF</td>
<td>A strain of wildtype zebrafish derived from the *AB and LF lines.</td>
</tr>
<tr>
<td>dCAPS</td>
<td>Derived cleaved amplified polymorphic sequence; a method of determining genotype.</td>
</tr>
<tr>
<td>dpf</td>
<td>Days post-fertilization; notation used for zebrafish</td>
</tr>
<tr>
<td>FL SMN</td>
<td>Full-length SMN protein</td>
</tr>
<tr>
<td>galK</td>
<td>Galactokinase; an enzyme that converts galactose to bio-available glucose and 2-deoxy-galactose to a toxic, non-metabolic form. <em>galK</em> is used in homologous arm recombineering for selection purposes.</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>H4ac</td>
<td>Histone 4 acetylation; acetylated H4</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone acetyltransferases; enzymes that acetylates specific lysines on histone proteins, causing chromatin structure to relax.</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylases; enzymes that remove acetylation from specific lysines on histone proteins, causing chromatin structure to condense.</td>
</tr>
<tr>
<td>HDACi</td>
<td>Histone deacetylase inhibitor(s)</td>
</tr>
<tr>
<td>hpf</td>
<td>Hours post-fertilization; notation used for zebrafish</td>
</tr>
<tr>
<td>HTS</td>
<td>High through-put screening; performed in cell culture to identify potentially therapeutic drug compounds.</td>
</tr>
<tr>
<td>iPSCs</td>
<td>Induced pluripotent stem cells; cell that are reverted to a stem cell-like morphology.</td>
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<tr>
<td>LF</td>
<td>Long-fin; a strain of wildtype zebrafish.</td>
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<tr>
<td>MO</td>
<td>Morpholino; a synthesized oligomer.</td>
</tr>
<tr>
<td>MOA</td>
<td>Mechanism/mode of action; pertains to drug compounds.</td>
</tr>
<tr>
<td>NMJ</td>
<td>Neuromuscular junction; located between motoneurons and muscle cells.</td>
</tr>
<tr>
<td>PBA</td>
<td>Phenylbutyrate; an HDACi</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction; used to amplify specific pieces of DNA.</td>
</tr>
<tr>
<td>polyA</td>
<td>Polyadenylation; a 3’ signal found on transcriptions in eukaryotes that ensures maturation of messenger RNA for translation.</td>
</tr>
<tr>
<td>SMA</td>
<td>Spinal muscular atrophy</td>
</tr>
<tr>
<td>SMN</td>
<td>Survival Motor Neuron Gene</td>
</tr>
<tr>
<td>SMN1</td>
<td>Telomeric copy of SMN</td>
</tr>
<tr>
<td>SMN2</td>
<td>Centromeric copy of SMN; present only in humans</td>
</tr>
<tr>
<td>SMNp</td>
<td>SMN promoter</td>
</tr>
<tr>
<td>SMNΔ7</td>
<td>SMN lacking exon 7; the most common splice form produced by SMN2</td>
</tr>
<tr>
<td>TSA</td>
<td>Trichostatin A; an HDACi</td>
</tr>
<tr>
<td>VPA</td>
<td>Valproic acid; an HDACi</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
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