DEVELOPMENT AND ANALYSIS OF A ZEBRAFISH MODEL OF SPINAL MUSCULAR ATROPHY

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the
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By

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Spinal muscular atrophy (SMA) is an autosomal recessive disorder characterized by a loss of α-motoneurons in the spinal cord. SMA is caused by low levels of the ubiquitously expressed survival motor neuron (SMN) protein. Mouse models of SMA have been an extremely valuable tool for understanding the genetics of SMA. One of the drawbacks with the mouse system, however, is that it is not ideal for studying neural anatomy due to its neuromuscular complexity. Therefore, another vertebrate model system with more simplified and stereotypic neuromuscular organization, the zebrafish, will be used to model and further study SMA.

To mimic SMA in zebrafish, antisense morpholino oligonucleotides have been utilized to reduce Smn levels in the developing embryo. When Smn levels are reduced throughout the entire embryo, motor axon pathfinding defects are observed. These defects are specific to motor axons; other sensory and interneuron axons were unaffected by Smn knockdown. Reduction of Smn in individual motoneurons revealed that smn is acting cell-autonomously. These results show that Smn functions in motor axon development and suggest that these early developmental defects may lead to subsequent motoneuron loss.

Because SMN is a ubiquitously expressed protein, a paramount question in SMA research revolves around why reduced levels of SMN lead to a motoneuron-specific
It has been hypothesized that SMN may have a dual function: a well-characterized role in mediating snRNP assembly and a novel motor axonal-specific function. To begin to identify which function is important for axonal outgrowth and potentially SMA, non-Smn components in these separate pathways have been knocked-down by morpholino in zebrafish. Knockdown of Gemin2, a Smn interacting snRNP assembly component, does not yield motor axon defects. If Smn does have a role in motor axons, it is in the proper place to do so, as shown by hSMN-RFP localization to motor axons. While not conclusive evidence, these data suggest a non-snRNP assembly function for Smn in motor axon outgrowth and pathfinding.

Because morpholinos are a transient knockdown of protein and have an inherent variability in their phenotypes, a genetic model of SMA utilizing a zebrafish smn mutation can address questions that morpholinos alone cannot. Because targeted mutagenesis is unavailable in zebrafish, rapid high-throughput screens for mutations in a particular gene of interest are necessary to further study gene function. Screening methods have been developed to identify both ENU-induced point mutations and γ-induced deletion mutations in the zebrafish smn gene. Denaturing high performance liquid chromatography (dHPLC) has successfully been used to identify an intronic mutation and single nucleotide polymorphisms (SNPs) in the smn gene. Targeted Induced Lesion IN Genomes (TILLING) has identified a mutation in the smn coding region that may affect gene function. A mutation in the smn gene will allow for development of a genetic zebrafish model of SMA and further elucidation of the neuropathology and etiology of the disease. Elucidation of the mechanism by which reduced Smn levels result in SMA may aid in research into other motoneuron diseases.
Additionally, further understanding of the timing at which SMN is necessary for disease pathology will have implications for therapeutic strategies for SMA patients.
DEDICATION

Dedicated in memory of those youngest souls who have lost their battle against spinal muscular atrophy
ACKNOWLEDGMENTS

First and foremost, I would like to thank my graduate advisor, Dr. Christine Beattie. Without her guidance and expertise, none of this document would have been possible. I am eternally grateful to current and former members of the Beattie/Henion labs, who have made doing science fun and memorable. I finally found science nerds that are just like me! I would especially like to thank Dr. Michelle Gray who was a caring soul that tried to teach me everything she knew when I first started graduate school. Special thanks to Marsha Lucas, Erin Horan, Drs. Louise Rodino-Klapac, Brigitte Arduini, and Tessa Carrel for our intelligent and, more importantly, our non-intelligent conversations that made the bad days bearable. Thanks to all the fishroom staff who keep the fishroom running and the fish happy. I would like to thank each of my committee members for volunteering their time to make my graduate career and this document just that much better. Thanks to Dr. Vicki McGovern for being my go-between with King Arthur’s lab. I would especially like to thank my parents Vicki Seliskar and Jerry McCalmont and Pete and Sharon Seliskar who have made me what I am today. Their tireless support and encouragement is something I could never repay. Additional thanks to Dr. Carl Seliskar and Marie Seliskar for telling me at age 10 what a PhD degree was really all about. Last, but certainly not least, my caring husband, Brian. Although he has never quite
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<td>A</td>
<td>adenosine</td>
</tr>
<tr>
<td>α</td>
<td>alpha</td>
</tr>
<tr>
<td>Arg</td>
<td>arginine</td>
</tr>
<tr>
<td>β</td>
<td>beta</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>C</td>
<td>cytosine</td>
</tr>
<tr>
<td>CaP</td>
<td>caudal primary</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celcius</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>calcium chloride</td>
</tr>
<tr>
<td>dHPLC</td>
<td>denaturing high performance liquid chromatography</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotide</td>
</tr>
<tr>
<td>dpf</td>
<td>days post fertilization</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetra acetate</td>
</tr>
<tr>
<td>EMS</td>
<td>ethylmethane sulfonate</td>
</tr>
<tr>
<td>ENU</td>
<td>1-ethyl 1-nitrosurea</td>
</tr>
<tr>
<td>F₁</td>
<td>first familial</td>
</tr>
<tr>
<td>F₂</td>
<td>second familial</td>
</tr>
<tr>
<td>G</td>
<td>guanosine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>$G_0$</td>
<td>initial generation/parental</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>her1</td>
<td>hairy/enhancer of split-related</td>
</tr>
<tr>
<td>hpf</td>
<td>hours post fertilization</td>
</tr>
<tr>
<td>hn-RNP-Q</td>
<td>heteronuclear ribonuclear protein Q</td>
</tr>
<tr>
<td>hn-RNP-R</td>
<td>heteronuclear ribonuclear protein R</td>
</tr>
<tr>
<td>KCl</td>
<td>potassium chloride</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>potassium phosphate monobasic</td>
</tr>
<tr>
<td>$M$</td>
<td>molar</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MgCl$_2$</td>
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<tr>
<td>MgSO$_4$</td>
<td>magnesium sulfate</td>
</tr>
<tr>
<td>Met</td>
<td>methionine</td>
</tr>
<tr>
<td>Min</td>
<td>minute</td>
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<tr>
<td>ml</td>
<td>milliliter</td>
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<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>MO</td>
<td>morpholino</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>MUNE</td>
<td>motor unit number estimation</td>
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<td>sodium chloride</td>
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<tr>
<td>NaHCO$_3$</td>
<td>sodium bicarbonate</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>sodium phosphate dibasic</td>
</tr>
<tr>
<td>NAIP</td>
<td>neuronal inhibitor of apoptosis</td>
</tr>
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</table>
nm  nanometer
p44  subunit of basal transcription factor TFIIH
PCR  polymerase chain reaction
RNA  ribonucleic acid
RT-PCR reverse transcriptase PCR
Ser  serine
SMA  spinal muscular atrophy
SMN  human or mouse survival motor neuron protein
SMN  human or mouse survival motor neuron gene
Smn  zebrafish survival motor neuron protein
smn  zebrafish survival motor neuron gene
SNP  single nucleotide polymorphism
T  thymidine
TD PCR  touchdown PCR
TILLING targeted induced lesions in genomes
Tris  trizma base
γ  gamma
µg  microgram
µl  microliter
µM  micromolar
UV  ultraviolet
Val  valine
WT  wild type
CHAPTER 1

INTRODUCTION

Motoneuron diseases are commonly characterized as disorders which specifically result in motoneuron death (Kuncl, 2002; Cleveland and Rothstein, 2001). In general, these diseases affect only the motoneurons, leaving the rest of the nervous system unaffected (Cleveland and Rothstein, 2001). While these disorders usually result in loss of all or some motor function, the cognitive capabilities of the patient are generally unaffected leaving the patient of able-mind but not able-body (Kuncl, 2002). Although many different types of motoneuron disease occur, two of the most common disorders, amyotrophic lateral sclerosis (ALS; aka Lou Gehrig’s disease and motoneuron disease) and spinal muscular atrophy (SMA), have been the focus of intense scientific research over the past decade (Cleveland and Rothstein, 2001; Kuncl, 2002; Nicole et al., 2002). While studies of these diseases in mice have allowed researchers to understand more about the genetics of the diseases, the complex etiology and pathology of the diseases is still unclear (Cleveland and Rothstein, 2001; Kuncl, 2002; Nicole et al., 2002). Due to its relatively simple neuromuscular organization, the zebrafish, Danio rerio, can be used to further understand the neuropathology and etiology of motoneuron diseases such as ALS and SMA.
Classification of Spinal Muscular Atrophy

Historically, the term spinal muscular atrophy has been used to describe a class of muscle wasting diseases where lower spinal motoneurons are affected (Talbot et al., 2001). In the early 1890’s, Werdnig and Hoffmann described a childhood disorder that occurred within families that they termed “spinale muskelatrophie” (Werdnig, 1894; Hoffmann, 1900). Children were normal at birth, but then began to develop proximal weakness and muscle wasting, and ultimately died (Werdnig, 1894; Hoffmann, 1900). Then around 1950, Kugelberg and Welander published data about patients suffering from similar symptoms as those Werdnig and Hoffmann described, but who had retained the ability to walk and lived to adulthood (1956). Today, the term spinal muscular atrophy (SMA) refers to a specific inheritable disorder linked to chromosome 5q13 in humans (Talbot et al., 2001). Each of the patients described in Werdnig’s, Hoffmann’s, and Kugelberg and Welander’s studies would have been diagnosed with SMA by today’s standards, but with differing severities (Talbot et al., 2001).

The unifying feature of SMA is the symmetrical proximal muscle weakness associated with muscle atrophy that likely results from skeletal muscle denervation (Nicole et al., 2002). It is characterized by a loss of lower α motoneurons in the spinal cord (Crawford and Pardo, 1996; Melki, 1997). As a range of severities occur in this disease, the International SMA Consortium on Childhood SMA in 1991 divided SMA into 3 clinical groups (Munsat, 1991; See Table 1.1).
Table 1.1. Classifications of Childhood SMA

<table>
<thead>
<tr>
<th>Type</th>
<th>Onset (months)</th>
<th>Motor Milestones</th>
<th>Death (years)</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>&lt; 6</td>
<td>Never sit unaided</td>
<td>&lt; 2</td>
</tr>
<tr>
<td>II</td>
<td>&lt; 18</td>
<td>Never walk unaided</td>
<td>&gt; 2</td>
</tr>
<tr>
<td>III</td>
<td>&gt; 18</td>
<td>Stand and walk alone</td>
<td>Adult</td>
</tr>
</tbody>
</table>

Table 1.1. Three clinical groups of SMA were established in 1991 by the International SMA Consortium on Childhood SMA. These types are based on motor milestones reached and are summarized (Modified from Munsat, 1991).

In the most severe Type I cases, severe generalized muscle weakness and hypotonia occur at birth or within the 1st six months of life (Munsat, 1991). It has also been reported that mothers of Type I SMA babies occasionally feel a cessation of movement in the final trimester of pregnancy (Talbot et al., 2001). Many Type I babies never gain head control, must be intubated, and die of respiratory failure by the age of two (Munsat, 1991).

Type II (intermediate) SMA patients are at one time able to sit without support, but never gain the ability to walk (Munsat, 1991). SMA type II patients usually manifest with symptoms prior to 18 months of age and have variable overall prognoses which depend primarily on respiratory function (Talbot et al., 2001). In a clinical study, the survival rate for Type II SMA patients at 5 years was 98.5% while at 25 years, the survival rate of 68.5% (Zerres et al., 1997).

Type III (mild) SMA patients present with a continuous range of symptoms, and at one time were able to walk unassisted (Talbot et al., 2001). While many lose the ability to walk, others can be ambulatory with minor assistance (Talbot et al., 2001). There does not appear to be a decrease in life expectancy to adulthood (Talbot et al., 2001). Additionally, some researchers have identified and classified Type IV SMA
patients (Vitali et al., 1999). The type IV classification includes mild Type III patients that had adult onset of the disease (Vitali et al., 1999). One Type IV patient was asymptomatic until age 58 and walked with aid until her death (Vitali et al., 1999).

SMA is a relatively common genetic disease that is the leading hereditary cause of infant mortality (Roberts et al., 1970). It is inherited in an autosomal recessive fashion with an incidence of 1 in 6,000-10,000 live births (Pearn et al., 1973; Pearn, 1978; Burd et al., 1991; Ludvigsson et al., 1999). The approximate carrier frequency, or frequency in which any one individual harbors the mutation for SMA, is approximately 1 in 40-50 (Pearn, 1980).

**Identification of the genetics of SMA**

To determine the gene responsible for SMA, physical mapping was performed. It was found that the SMA locus lies within a highly complex genomic region with multicopy repetitive sequences, pseudogenes, and retrotransposon-like sequences (Melki et al., 1994; Francis et al., 1993). It is believed that this region of the genome is highly unstable due in part to the number of inherited and *de novo* deletions found in SMA patients (Melki et al., 1994). Refined mapping of the area revealed an inverted duplication of approximately 500 kb which is unique to *Homo sapiens* (Figure 1.1; Lefebrve et al., 1995).
Figure 1.1. Inverted duplication of SMA locus (5q13). Three genes are involved in this 500 kb inverted duplication that results in a gene duplication toward the centromere (Modified from Talbot et al., 2001).

Three genes are encoded in this inverted duplication, *NAIP* (neuronal inhibitor of apoptosis protein), *SMN* (survival motor neuron), and *p44* (subunit of basal transcription factor TFIIH), and each gene has a duplicate that is more centromeric (Lefebvre et al., 1995; Roy et al., 1995).

Identification of *SMN* as the SMA determining gene was confirmed by mutation analysis. Although there was initially some data that suggested *NAIP* played a role in SMA, it is now believed that the *SMN* genes are the disease-causing determinants in SMA (Roy et al., 1995; Nicole et al., 2002). Characterization of the telomeric copy of *SMN* (*SMN1*) and the centromeric *SMN* (*SMN2*) revealed an interesting genetic scenario in patients with SMA. The *SMN1* gene is deleted in 98% of patients while the other 2% of patients have missense, non-sense, or splice site mutations in *SMN1* (Lefebvre et al., 1995). All SMA patients have the *SMN* gene duplication (*SMN2*) while 95% of non-SMA patients have the gene duplication (Lefebvre et al., 1995). The *SMN2* gene is highly homologous to the *SMN1* gene in that over an approximate 32 kb region, the genes are greater than 99% identical with only 5 base pairs that differ between the two (Monani et al., 1999). None of these nucleotide differences change the amino acid sequence, but
one nucleotide change in exon 7 causes alternative splicing out of exon 7 (Figure 1.2; Lefebvre et al., 1995).

Figure 1.2. Genetics of SMA. Two SMN genes exist in humans, SMN1 and SMN2. The protein product from the SMN1 gene encodes for full length protein whereas the majority of the protein product from the SMN2 gene lacks exon 7. SMA is thought to result from low levels of full length SMN protein (Modified from Nicole et al., 2002; used with permission).

This nucleotide change is a C to T transition that occurs in the splice enhancer site of exon 7 which drastically reduces the efficiency of exon 7 inclusion (Lorson et al., 1999; Monani et al., 1999; Cartegni and Krainer, 2002). SMNΔ7 protein (lacking exon 7) does not oligomerize effectively and appears to be unstable and degraded (Lorson and Androphy, 1998; Lorson and Androphy, 2000). From a WT SMN1 gene, 80-90% of the transcripts encode for exon 7 to produce full-length WT protein (Lefebvre et al., 1995; Gennarelli et al., 1995; Parsons et al., 1996). Due to the base change in exon 7, 70-90%
of the transcript from the $SMN2$ gene lacks exon 7 resulting in a putative non-functional protein (Lefebvre et al., 1995; Gennarelli et al., 1995; Parsons et al., 1996). Although the majority of transcript from the $SMN2$ gene lacks exon 7, a small percentage (10-30%) of transcript does encode for full-length WT protein (Lefebvre et al., 1995; Gennarelli et al., 1995; Parsons et al., 1996). Therefore, only a small amount of functional WT protein (approximately 10%) comes from the $SMN2$ gene (Lefebvre et al., 1997). If the $SMN1$ gene is mutated (deleted in most cases), the only source of WT protein is from the $SMN2$ gene (Lefebvre et al., 1995; Gennarelli et al., 1995; Parsons et al., 1996). Ultimately, SMA is a disease that results from low levels of the SMN protein when $SMN1$ is mutated, but $SMN2$ is retained (Lefebvre et al., 1995; Coovert et al., 1997).

When the disease-causing genes were identified, the relationship between the amount of SMN protein produced and clinical severity of the disease was investigated. SMN protein studies in patients showed a tight inverse correlation between severity and amount of protein (Coovert et al., 1997; Lefebrve et al., 1997). In SMA patients, less SMN protein results in greater disease severity (Coovert et al., 1997; Lefebrve et al., 1997). This evidence was the first to provide a molecular basis for the clinical severity of SMA and suggested a dosage effect of $SMN$ (DiDonato et al., 1997; McAndrew et al., 1997; Nicole et al., 2002). To understand what was causing the variance in protein levels, the promoter regions of $SMN1$ and $SMN2$ were characterized and found to be virtually identical with respect to activity (Echaniz-Laguna et al., 1999; Monani et al., 1999). Therefore, it is unlikely that variance in protein levels is due to promoter activity. Genetic analysis in SMA patients revealed that protein levels vary due to copy number of $SMN2$ among individuals (Vitali et al., 1999; Harada et al., 2002; DiDonato et al., 1997;
Patients studied have as few as 1 \( SMN2 \) gene and as many as 8 \( SMN2 \) genes (Vitali et al., 1999). The typical SMA Type I patient has 1-2 copies of the \( SMN2 \) gene while SMA Type II and III patients have 3 or more copies on average (Vitali et al., 1999; Harada et al., 2002). Studies suggest that individual genetic variability can also modify the clinical phenotype. For example, a patient diagnosed with Type III SMA as a child was found to have 5 copies of the \( SMN2 \) gene (Prior et al., 2004). This patient’s sibling is homozygous mutant for \( SMN1 \) and has 5 copies of \( SMN2 \), suggesting he should also suffer from SMA Type III, but he is asymptomatic (Prior et al., 2004). For one sibling, 5 copies are sufficient to be asymptomatic while 5 copies for the other sibling led to childhood onset Type III. Additionally, a patient with 8 copies of \( SMN2 \) had adult onset SMA and was able to walk with aid (Vitali et al., 1999), further suggesting genetic variability as a differential characteristic of disease severity. These studies show that \( SMN2 \) is a modifier of SMA and that copy number and protein levels are important for determining clinical disease severity (Swoboda et al., 2005).

Clinical researchers have recently begun to perform electrophysiology studies on SMA patients. Motor unit number estimation (MUNE) is an estimate of motor units innervating distal muscle and is one way to functionally test the health of motoneurons (Swoboda et al., 2005). These studies have revealed that MUNE values in prenatally identified asymptomatic SMA patients show normal MUNE levels that decline as disease progression occurs suggesting that motoneuron loss correlates with clinical progression and occurs postnatal (Bromberg and Swoboda, 2002; Swoboda et al., 2005). MUNE values indicate the overall health of motoneurons and are complementary to functional outcome measures (i.e. sitting or walking) because MUNE values continue to decline.
even though functional outcomes may stabilize in patients (Swoboda et al., 2005).

Ultimately SMN2 copy number also has a modifying role on MUNE values (Swoboda et al., 2005).

**Animal models of SMA**

Once the SMN genes were found to be the SMA determining genes, researchers began to characterize SMN in the mouse. Upon cloning of the mouse homolog of SMN1, researchers found that no SMN2 orthologue existed in mouse (Schrank et al., 1997). Therefore, only 1 copy of the SMN gene exists in mouse, analogous to human SMN1. Mouse SMN is 83% identical to the human SMN protein, and there does not appear to be any alternatively spliced transcripts (lacking exon 7) as in humans (Schrank et al., 1997).

To understand the role of SMN in SMA, SMN knockout mice were created (Schrank et al., 1997). When SMN mutant heterozygotes (SMN+/-) were intercrossed, no SMN homozygous (SMN-/-) mice were born suggesting an early lethal phenotype (Schrank et al., 1997). Upon further characterization, SMN-/- embryos were shown to undergo developmental arrest and die prior to implantation (Schrank et al., 1997). Mutant embryo death coincides with depletion of maternal SMN suggesting that SMN is essential during development (Schrank et al., 1997). These data correlate with human studies as no SMA patients have presented without an SMN2 gene (Nicole et al., 2002).

To obtain a mouse model of SMA, the human SMN2 gene was used as a transgene in the mouse SMN knockout (Monani et al., 2000; Hsieh-Li et al., 2000). Mice homozygous for the SMN knockout allele and harboring 1-2 copies of the hSMN2 transgene (SMN-/-; SMN2) can survive until birth, unlike their knockout allele counterparts, suggesting that SMN2 rescues the embryonic lethal phenotype (Monani et
The low copy $SMN2$ transgenic mice appear phenotypically normal at birth until 48 h postnatal when they begin to exhibit SMA-like symptoms: decreased movement, diminished suckling, and labored breathing (Monani et al., 2000). By 72 h postnatal, SMA mice were considerably smaller in size than unaffected littermates, have limb tremors, and are unable to right themselves when placed on their sides (Monani et al., 2000). These SMA mice die 4-6 d postnatal and their levels of SMN protein are physiologically equivalent to what is observed in Type I patients (Monani et al., 2000). Consequently, these mice are termed severe Type I SMA mice.

When motoneurons were analyzed, WT controls and severe SMA mice had no difference in motoneuron cell numbers in the spinal cord at 1 d postnatal, but there was 35% loss of motoneurons in the spinal cord of SMA mice compared to controls at 5 d postnatal (Monani et al., 2000). The loss of motoneurons correlates with the onset of the SMA-like phenotypes and suggests that the motoneuron loss occurs in the later stages of the disease (Monani et al., 2000). Electrophysiology data from SMA patients also suggests that motoneuron denervation correlates with symptom onset (Bromberg and Swoboda, 2002).

In addition to the low copy (1-2) $SMN2$ transgenic mouse, there is a high copy transgenic mouse which harbors 8 copies of the $SMN2$ transgene (Monani et al., 2000). Mice homozygous for the $SMN$ knockout allele with 8 copies of $SMN2$ do not show any SMA-like phenotypes and do not exhibit motoneuron loss in the spinal cord (Monani et al., 2000). The SMN protein levels in these high copy mice are equivalent to control littermates suggesting that the $SMN2$ gene is capable of rescuing the SMA phenotype (Monani et al., 2000). Mouse model data suggests that $SMN2$ copy number modulates the SMA phenotype severity. This correlates with patient data as there are reports of
human individuals who are homozygous mutant for \( SMN \) but have at least 5 copies of \( SMN2 \) and are either unaffected or adult-onset, mildly affected (Prior et al., 2004).

Other mouse models of SMA have also been produced. The human \( SMN \) patient mutation A2G was used to generate a transgenic mouse that when crossed into the severe SMA mouse model modulates the severity (Parsons et al., 1998; Monani et al., 2003). While unable to rescue the embryonic lethality of the \( SMN \) knockout allele, the A2G transgene delays both the motoneuron loss in the spinal cord (loss at 3.5 months) and the SMA-like phenotype (symptoms at 3 weeks) resulting in a mild SMA mouse (Monani et al., 2003). These mice also often live past 1 year of age (Monani et al., 2003). It is believed that this mutant allele modulates the phenotype only in the presence of full-length SMN by increasing the overall levels of SMN protein, even though it is a mutant allele (Monani et al., 2003).

As one of the therapeutic possibilities for SMA patients is to increase expression from the \( SMN2 \) gene, the role of the \( SMN\Delta7 \) protein in SMA mice was studied. A cDNA of \( SMN \) lacking exon 7 was used to make a \( SMN\Delta7 \) transgenic mouse that was then crossed into the severe SMA mouse model. These transgenic \( SMN\Delta7 \) mice live approximately 1 week longer than the severe mice and have delayed onset of the SMA symptoms by about 1 week as well (Le et al., 2005). Therefore, there is no detrimental effect of \( SMN\Delta7 \) overexpression; in fact, there appears to be a decrease in phenotype severity (Le et al., 2005). Other mouse models have been developed that specifically excise exon 7 from the \( SMN \) gene using the Cre recombinase system to target the excision to muscle (\( \alpha \)-skeletal actin promoter) or neuronal (neuron-specific enolase promoter) tissues (Cifuentes-Diaz et al., 2002; Frugier et al., 2000; Cifuentes-Diaz et al., 2002; Cifuentes-Diaz et al., 2000).
In these models there is only SMN\(\Delta 7\) protein present in the muscle or neuronal tissues, not any full-length protein. It has previously been shown that cells lacking WT full-length SMN are not viable (Schrank et al., 1997). Therefore, it is difficult to understand the physiologic disease relevance of these models with respect to SMA as the disease results from low levels of full-length protein not expression of SMN\(\Delta 7\).

Modeling SMA in mice has been an extremely valuable tool for understanding the genetics of SMA. In fact, the analysis regarding SMN\(2\) copy number as a predictor of SMA severity was studied only after mouse models suggested a link (Monani et al., 2000). One of the drawbacks with the mouse system, however, is that it is not ideal for studying neural anatomy. Due to its neuromuscular complexity, it is difficult to study the etiology and pathology of SMA in mice. Therefore, another vertebrate model system with more simplified and stereotypic neuromuscular organization will aid in further study of SMA.

Recently, the single SMN gene in Drosophila melanogaster has been characterized and a mutant has been identified (Miquel-Aliaga et al., 2000). The homozygous SMN mutant animals die in late larval stages contrasting with early embryonic lethality seen in other animal models (Chan et al., 2003). Because of high maternal RNA contribution in Drosophila, SMN RNA is present through early larval stages explaining why early lethality does not occur (Chan et al., 2003). Analysis of the synaptic neuromuscular junctions (NMJ) in these mutants showed that glutamate receptors did not cluster properly in the post-synaptic muscle (Chan et al., 2003). Although this fly model does identify a potential role for SMN in NMJ formation and/or maintenance, the timing under which SMN levels are reduced is not physiologically
relevant to SMA. Since SMN levels are not reduced during embryonic stages of fly development, any potential developmental abnormality that may result from reduced SMN levels will not manifest. Additionally, *Drosophila* is an invertebrate with a glutamatergic neuromuscular system (Chan et al., 2003). An ideal SMA model organism is a vertebrate with a cholinergic neuromuscular system similar to humans. Although parallels can be made with this fly SMA model, the physiologic relevance of this late reduction of Smn protein and the presence of dissimilar neuromuscular architecture are caveats that must be considered when using this model organism.

**Function of SMN in snRNP assembly**

To determine the etiology of SMA, the function of SMN has been studied extensively. *SMN* is an essential gene in numerous divergent organisms such as human, mouse, *Caenorhabditis elegans*, and *Schizosaccharomyces pombe* (Shrank et al., 1997; Miquel-Aliaga et al., 1999; Hannus et al., 2000; Owen et al., 2000; Paushkin et al., 2000). The SMN protein is ubiquitously expressed in all tissues of metazoans (Lefebvre et al., 1995). Because of its ubiquitous expression, the fundamental question of why motoneurons are uniquely sensitive to low levels of SMN need to be addressed.

Considering its essential nature, SMN is believed to function in a process fundamental to all cells. On a cellular level, SMN localizes to both the cytoplasm and in the nucleus where it is concentrated in structures termed “gems” (Liu and Dreyfuss, 1996). SMN appears to function as a multiprotein complex (Paushkin et al., 2002). The SMN protein oligomerizes and forms a complex, termed the SMN complex, with a group of proteins termed the “gemins” (Gubitz et al., 2004; Figure 1.3).
The complex components include Gemin2, a novel protein formerly called SIP1 (Liu et al., 1997; Fischer et al., 1997); Gemin3, a DEAD-box RNA helicase (Charroux et al., 1999; Campbell et al., 2000); and Gemins4-7, also novel proteins (Charroux et al., 2000; Meister et al., 2001; Gubitz et al., 2002; Pellizzoni et al., 2002; Baccon et al., 2002). Gemin2, 3, 5, and 7 interact directly with SMN whereas Gemin4 and 6 require Gemin 3 and 7, respectively, for binding to the complex (Gubitz et al., 2004).

Further analysis of the SMN complex suggested that the complex may be involved in RNA metabolism, specifically assembly of snRNPs (small nuclear ribonucleoproteins). snRNPs are components of the spliceosome, pre-mRNA splicing machinery in eukaryotes, and consist of a snRNA (small nuclear ribonucleic acid) and a set of Sm proteins (Yong et al., 2004; Figure 1.4).
Figure 1.4. snRNP assembly. Schematic diagram shows formation of the Sm core by the SMN complex during snRNP assembly. The SMN complex plays an important role in regulating which RNA the Sm proteins bind. Sm proteins form 7-member core rings which bind to snRNAs (Reprinted with permission from Pellizzoni, L., Yong, J. and Dreyfuss, G. (2002) Essential role for the SMN complex in the specificity of snRNP assembly. Science 298, 1775-9. Copyright 2005 AAAS).

snRNAs, U1, U2, U5, U4/6, or U7, are transcribed and exported to the cytoplasm by export factor PHAX (Ohno et al., 2000). The Sm proteins, B or B’, D1, D2, D3, E, F, and G, or Lsm (Sm-like) proteins arrange into a 7-membered ring core and bind to the Sm site on snRNAs (Raker et al., 1996; Stark et al., 2001). The assembly of the Sm core to specific snRNAs, termed snRNP assembly, occurs in the cytoplasm and is necessary for nuclear import of the snRNPs for nuclear pre-mRNA splicing (Fischer et al., 1991; Fischer et al., 1993). The SMN complex has been shown to be essential in this ATP-dependent snRNP assembly of Sm proteins and snRNAs (Pellizzoni et al., 2002). The Sm proteins have a high affinity for RNAs and are believed to be promiscuous in that
without regulation of binding, the Sm proteins could bind deleteriously to other RNAs (Pellizzoni et al., 2002). SMN binds to snRNAs in a sequence specific manner to select the proper snRNA for Sm core assembly (Yong et al., 2002). SMN is a specificity factor for snRNP assembly by ensuring that Sm proteins only bind to the proper RNA (Yong et al., 2004; Yong et al., 2002). This function of SMN in mediating stringency control over snRNP assembly is believed to be an essential function required in every cell type and, by itself, does not explain why motoneurons are uniquely sensitive to reduced SMN levels (Gubitz et al., 2004).

Studies have attempted to understand the role of this snRNP assembly function of SMN with respect to SMA. snRNP assembly in patient fibroblast cells is perturbed, but not completely abolished, suggesting that a basal levels of assembly is occurring within patient cells (Wan et al., 2005). snRNP assembly has also been studied during spinal cord development of mice (Gabanella et al., 2005). snRNP assembly in the developing mouse spinal cord is highly up-regulated during embryonic and early post-natal stages and then down-regulated to basal levels that are maintained throughout adulthood (Gabanella et al., 2005). Although this data suggests a correlation between spinal cord development and snRNP assembly, assembly was also up-regulated during organogenesis of other non-neuronal tissues (Gabanella et al., 2005). Therefore, there may simply be a correlation between assembly activity and tissue development. SMA patients do not have clinical symptoms that suggest organs or tissues, other than motoneurons are affected (Kuncl, 2002).

To further test the role of snRNP assembly with respect to SMA, other components of the SMN complex have been functionally depleted. RNAi experiments in
cell culture showed that reduction of SMN, Gemin2, and Gemin6 leads to a decrease in snRNP assembly whereas reduction of Gemins3, 4, and 5 did not yield a significant decrease in assembly (Feng et al., 2005). These data suggest that other components of the SMN complex may be important in mediating assembly as well. However, no SMA patients have been found to have mutations in the gemin2 gene, and gemin2 is not believed to be a modifier of SMA (Helmken et al., 2000). gemin2 function in mice was studied by knockout analysis (Jablonka et al., 2002). Although gemin2 homozygous mutant mice are early embryonic lethal, when crossed into an SMN mutant background, a genetic interaction between the two genes has been identified (Jablonka et al., 2002). While gemin2 heterozygous mice show no decrease in motoneuron cell numbers, SMN and gemin2 double heterozygotes exhibit enhanced motoneuron loss in the spinal cord compared to SMN mutants alone (Jablonka et al., 2002). The relevance of this potential genetic interaction is unclear with respect to SMA. Since snRNP assembly is essential in all cells, it is possible that reduction of two different assembly components further perturbs assembly function. Since assembly is essential, this further perturbation may cause increased cell death throughout the animal. Other tissue types were not studied in these double heterozygous mutants. While there certainly may be a role of snRNP activity in SMA pathology, the ultimate molecular mechanism is still unknown.

Due to the essential housekeeping function of SMN in snRNP assembly, the pathophysiologic relevance of this defect with respect to clinical SMA is unclear because SMA is a motoneuron specific disease (Nicole et al., 2002). Although motoneurons may have a higher demand for snRNP assembly and, hence, may be more sensitive to a loss of assembly activity, there has been no conclusive evidence that snRNP assembly is the
molecular deficiency that leads to motoneuron degeneration and SMA (Gubitz et al., 2004). Alternatively, it has also been suggested that SMN has a motoneuron-specific function which is why they are uniquely sensitive to reduced levels.

**Potential SMN function in the motoneuron**

SMN may also have a motoneuron-specific function that is not related to snRNP assembly. It is well documented that SMN localizes to the nucleus and cytoplasm during snRNP assembly, but many of the cells studied are fibroblasts and lymphocytes (Liu and Dreyfuss, 1996). Recently, SMN localization has been studied in a physiologically relevant cell type, the neuron. Analysis shows that SMN localizes to branch points and growth cones in axons of primary cultured motoneurons from E14 mice embryos (Jablonka et al., 2001). Interestingly, Gemin2 does not co-enrich in these axonal branch points or growth cones suggesting a possible unique SMN function in the axons (Jablonka et al., 2001). Additionally, when P19 cells are differentiated with retinoic acid into neurons and glia, SMN is shown to localize to growth cone-like structures, which are the leading edge of axons (Fan and Simard, 2002). Specifically SMN co-localizes with GAP-43, an axonal growth cone marker (Fan and Simard, 2002). SMN has also been shown to localize with cytoskeletal elements and be actively transported down the axon (Zhang et al., 2003; Pagliardini et al., 2000). In addition to axons, SMN localizes to neuromuscular junctions and colocalizes with α-bungarotoxin (BTX) in muscle cells (Fan and Simard, 2002). Although SMN has been localized to axons, these studies have primarily been performed in cell culture so localization artifacts cannot be ruled out.

Researchers have noted that SMN protein and activity levels are upregulated during late
embryonic through early post-natal stages suggesting a developmental function for this protein (Fan and Simard, 2002; Jablonka et al., 2001; Gabanella et al., 2005).

While the function of SMN in axons remains largely unknown, some ideas have emerged. SMN may play a role in transporting RNAs into the axon (Rossoll et al., 2003). SMN interacts with hn-RNP-R and hn-RNP-Q which have previously been shown to be involved in RNA editing and mRNA transport (Rossoll et al., 2002). Specifically, hn-RNP-R binds to the 3’UTR of β-actin, a necessary growth cone component (Rossoll et al., 2003). Axonal RNA transport followed by localized protein translation is important for growth cone motility (Brittis et al., 2002). Another possibility for SMN is direct interaction with motor axon components. SMN has been shown to interact with profilin, a protein involved in regulation of actin polymerization and growth cone motility (Giesemann et al., 1999; Willis et al., 1999). Further characterization of SMN along with interacting components is necessary to elucidate whether SMN is involved in a motoneuron-specific function or if motoneurons have a greater requirement for snRNP assembly. The molecular mechanism by which either of these possibilities occurs, remains unknown.

**Zebrafish as a model system**

Zebrafish, *Danio rerio*, have a number of attributes that are amenable for developmental, genetic, and disease model studies. Zebrafish have high fecundity, meaning large clutch sizes, and these externally fertilized embryos are optically transparent during early development (Kimmel et al., 1995). Both forward and reverse genetic analysis can be performed using zebrafish (Eisen and Grunwald, 2002). Additionally, GFP transgenics
can be obtained to visualize a variety of cell types (Meng et al., 1997). Recently, zebrafish have also been used in disease model analysis (Dodd et al., 2000).

Zebrasfish were first utilized for their forward, or phenotypic based, genetic attributes (Grunwald and Eisen, 2002). Large forward genetic screens in both Germany and USA published in 1996 identified a vertebrate in which classic genetics was amenable (Grunwald and Eisen, 2002). These forward genetic screens have elucidated numerous mutations in a variety of developmental and cellular processes (Driever et al., 1996). Analysis of these mutants has facilitated not only understanding of basic vertebrate embryology such as pattern formation and germ layer generation, but also understanding of tissue specific development such as neural circuits and heart formation (Granato et al., 1996; Haffter et al., 1996).

Zebrasfish can also be used in a reverse genetic approach. One significant drawback, however, is that the ability to perform targeted mutagenesis, such as homologous recombination, has not been developed (Lekven et al., 2000). While reverse genetic knockout technology has not been successfully adapted to fish, there are numerous other genetic tools in zebrafish (Lekven et al., 2000). Due to external fertilization, zebrafish embryos can be micro-manipulated in a variety of ways (Detrich et al., 2004b). Genetic mosaics can be produced by transplanting blastula-stage cells from one embryo to another (Rodino-Klapac and Beattie, 2004). For gain-of-function studies, RNA can be microinjected into 2-4 cell stage embryos to overexpress the gene of interest (Roos et al., 1999). For loss-of-function analysis, chemically modified antisense oligonucleotide morpholinos (MOs) can be injected into 2-4 cell stage embryos to reduce gene function (Nasevicius and Ekker, 2000). Depending on the site targeted,
morpholinos can block translation if designed against the start site of the RNA, and then protein levels can be then assayed by western blot (Nasevicius and Ekker, 2000). Morpholinos can cause alterations in splicing if designed against a splice donor or acceptor site; these splice alterations can be detected with RT-PCR (Draper et al., 2001). Additionally, transgenic zebrafish can be obtained by injecting DNA into embryos (Meng et al., 1997; Higashijima et al., 2000). Due to optical transparency, transgenic zebrafish with GFP-driven promoters can be imaged live and analyzed over time (Meng et al., 1997; Higashijima et al., 2000). Thus, there are significant tools which can be utilized to study a gene-of-interest in zebrafish.

Biological processes and genetic pathways studied in zebrafish have been shown to be applicable to higher vertebrate organisms (Detrich et al., 2004a). Neuromuscular organization of zebrafish is similar to higher vertebrates, but is more simplified and highly stereotypic (Beattie, 2000). In zebrafish, there are approximately 30 reiterated chevron-shaped somites which will ultimately become muscle, bone, and skin (Kimmel et al., 1995; Gilbert, 2003). Spinal motoneurons along with other neuronal populations develop in 2 distinct waves (Kimmel and Westerfield, 1988). Each somite hemisegment has 3 primary motoneurons (Figure 1.5) which are born around 9-10 hpf (hours post fertilization; Eisen et al., 1986). Each primary motoneuron has a unique cell body position within the spinal cord hemisegment and has a very stereotypic axon projection (Eisen et al., 1986; Eisen and Pike, 1990).
Figure 1.5. **Primary and secondary motor axon pathfinding in the developing zebrafish embryo.** Lateral schematic views (anterior to the left; dorsal to the top) of primary motoneurons at 27 (A) and secondary motoneurons at 36 hpf (B). The first intermediate target is also known as the nascent horizontal myoseptum while the second intermediate target is also defined as the region adjacent to the ventral edge of the notochord. Secondary motor axons depicted in the diagram only represent the medial pathway. Reproduced from The Journal of Cell Biology, 2003, Vol 162, by copyright permission of The Rockefeller University Press.

The Caudal Primary (CaP) motoneuron extends its axon along the ventromedial myotome; the Middle Primary (MiP) extends its axon along the dorsomedial myotome; the Rostral Primary (RoP) extends its axon in the intermediate myotome (Eisen et al., 1986; Myers et al., 1986). The CaP, MiP and RoP axons begin to pathfind out of the spinal cord at approximately 18 hpf and travel along the common pathway (Myers et al., 1986). Along this common pathway, the axons extend ventrally along the medial surface of the myotome until the nascent horizontal myoseptum which separates dorsal from ventral myotome (Myers et al., 1986; Figure 1.5). It is believed that the horizontal myoseptum is an intermediate target for primary axons because the growth cones pause and then diverge along cell-specific pathways (Beattie et al., 2000). These intermediate targets are regions of the myotome where the axonal growth cones, it is believed, pause.
and assimilate guidance cues from the muscle environment to follow the correct pathway (Beattie et al., 2000).

**Figure 1.6. Cross-section of zebrafish.** Cross-sectional schematic (with dorsal to the top) of the trunk of 19 hpf zebrafish embryo shows CaP (purple), MiP (blue), and RoP (pink) axons extending outward toward their respective muscle targets. Medial is to the left and lateral is to the right (Modified from Lodish et al., 2000; Copyright 2000 by W.H. Freeman and Company. Used with permission).

The myotome adjacent to the ventral edge of the notochord is also suspected of being an intermediate target for the CaP axon (Beattie et al., 2000). Among other important cues, the anterior-posterior polarity within the somite is also essential for proper axonal outgrowth (Gray et al., 2001).

The second wave of motoneurons, termed the secondary motoneurons, are born at 14-15 hpf and do not extend their axons until 26 hpf (Myers et al., 1986; Pike et al., 1992). Approximately, 30 secondary motoneurons are present per hemisection (Myers et al., 1986; Pike et al., 1992). The secondary motor axons extend along similar pathways as primaries to their distinct myotomal regions, and it is believed incur the
same intermediate targets (Myers et al., 1986; Pike et al., 1992). Axons from primary and secondary motoneurons fasiculate to form dorsal and ventral motor nerves (Pike et al., 1992).

As zebrafish primary and secondary motor axons grow out of the spinal cord, they begin to form synapses on the developing myotome similar to higher vertebrates (Westerfield et al., 1990). These synapses of motor axon and muscle, termed neuromuscular junctions (NMJ), are cholinergic meaning that acetylcholine is the neurotransmitter (Grinnell, 1995; Behra et al., 2002). Acetylcholine (ACh) is secreted from the presynaptic terminal and binds to acetylcholine receptors (AChR) clustered at the postsynaptic membrane (Behra et al., 2002). In zebrafish, AChRs cluster on the muscle along the length of the motor axon as growth cones travel along the medial surface of the myotome (Westerfield et al., 1990). During pathfinding, these en passant synaptic contacts, which are used for axon and muscle communication, are important for proper axon growth (Lefebvre et al., 2004). These en passant synaptic contacts are often transient, but are functional in that shortly after AChR clustering, muscle fibers begin contracting (Liu and Westerfield, 1992; Melancon et al., 1997; Lefebvre et al., 2004). Zebrafish are ideal for analysis of this neuromuscular circuit primarily because in vivo imaging of the intact NMJ can be performed because of transparent skin (Ono et al., 2004). One architectural difference between zebrafish and mammals is that zebrafish myofibers are ultimately polyneuronally innervated whereas mammal myofibers are mononeuronally innervated (Myers, 1985).

Zebrafish are an excellent organism for studying human disease due mostly to well-characterized embryonic development and external fertilization (Dodd et al., 2000).
Several gene mutations in zebrafish have been identified that cause human diseases; a Holt-Oram syndrome model in fish has symptoms, heart and limb deformities, which mimic the human disorder remarkably well (Garrity et al., 2002). Along with gene mutation analysis, morpholino mediated protein knockdown technology, as also been shown to facilitate modeling of human diseases (Dodd et al., 2000). For instance, muscular dystrophy and cystic kidney disease have successfully been modeled in zebrafish using morpholinos (Parsons et al., 2002; Liu et al., 2002). Because of well-characterized embryology and simplified neuromuscular organization which is similar to higher vertebrates, a zebrafish model of SMA has the potential to elucidate any developmental abnormalities that may be associated with the disease and to dissect out the mechanism by which low levels, of ubiquitously expressed Smn, result in a motoneuron-specific disease.
CHAPTER 2

KNOCKDOWN OF THE SURVIVAL MOTOR NEURON PROTEIN (SMN) IN ZEBRAFISH CAUSES DEFECTS IN MOTOR AXON OUTGROWTH AND PATHFINDING¹

ABSTRACT

Spinal muscular atrophy (SMA) is an autosomal recessive disorder characterized by a loss of α-motoneurons in the spinal cord. SMA is caused by low levels of the ubiquitously expressed survival motor neuron (Smn) protein. As it is unclear how low levels of Smn specifically affect motoneurons, we have modeled SMA in zebrafish, a vertebrate model organism with well characterized motoneuron development. Using antisense morpholinos to reduce Smn levels throughout the entire embryo, we found motor axon specific pathfinding defects. Reduction of Smn in individual motoneurons revealed that smn is acting cell-autonomously. These results show for the first time, in vivo, that Smn functions in motor axon development and suggest that these early developmental defects may lead to subsequent motoneuron loss.

INTRODUCTION

Zebrafish is an excellent organism for studying human disease (Dodd et al., 2000) due to its well characterized embryonic development and the ability to perform both forward and reverse genetics (reviewed in Grunwald and Eisen, 2002). Protein knockdown technology has facilitated analysis of zebrafish forms of muscular dystrophy (Parsons et al., 2002) and cystic kidney disease (Liu et al., 2002). Due to its well-characterized nervous system and relatively simple neuromuscular organization, zebrafish are well suited for analysis of neuromuscular diseases. Taking advantage of these attributes, we have used protein knockdown in zebrafish to model spinal muscular atrophy (SMA).

SMA, an autosomal recessive disorder, is the leading hereditary cause of infant mortality (Roberts et al., 1970) and is characterized by loss of $\alpha$ motoneurons in the spinal cord (Crawford and Pardo, 1996; Melki, 1997). The disease results from low levels of the protein encoded by the Survival Motor Neuron (SMN) gene. Although SMN is expressed in all cell types, motoneurons are specifically affected in SMA, indicating their sensitivity to low SMN levels (Coovert et al., 1997; Lefebvre et al., 1997; Monani et al., 2000). Humans have two copies of the $SMN$ gene, $SMN1$ and $SMN2$, which differ in a single base change in a splice enhancer site for exon 7 of $SMN2$ (Lorson et al., 1999; Monani et al., 1999; Cartegni and Krainer, 2002). Thus, $SMN1$ produces a majority of full-length transcript whereas $SMN2$ generates mostly transcripts lacking exon 7, although some full length transcript is produced (Lefebvre et al., 1995). SMN protein lacking exon 7 does not oligomerize effectively (Lorson et al., 1998) and appears to be unstable and degraded (Lorson and Androphy, 2000). Thus, mutations in $SMN1$, but
retention of the \textit{SMN2} gene, results in reduced protein levels and ultimately SMA (Lefebvre et al., 1995; Coovert et al., 1997; Lefebvre et al., 1997).

The 38 kD SMN protein is ubiquitously expressed and localizes to both the cytoplasm and nucleus (Liu and Dreyfuss, 1996; Coovert et al., 1997; Lefebvre et al., 1997). In the nucleus, SMN localizes to structures termed gems, which overlap or are in close proximity to coiled bodies (Liu and Dreyfuss, 1996; Young et al., 2000a). It has been termed the master RNA assembler and, in particular, has been shown to be important in assembly of snRNP particles (reviewed in Terns and Terns, 2001). SMN also binds to hn-RNP-R (heteronuclear ribonucleoprotein R), which is involved in RNA editing and mRNA transport (Rossoll et al., 2002). Recent data shows that hn-RNP-R co-localizes with SMN in distal axons of embryonic motoneurons (Rossoll et al., 2002; Jablonka et al., 2001). SMN also has been shown to localize in the growth cones and branch points of developing neurons (Jablonka et al., 2001, Fan and Simard, 2002, Zhang et al., 2003). Ultimately, however, the function of SMN in relation to SMA pathology and etiology remains unclear.

To further analyze SMN function, animal models of SMA have been generated (Schrank et al., 1997; Hsieh-Li et al., 2000; Monani et al., 2000; Cifuentes-Diaz et al., 2002; Monani et al., 2003). In contrast to humans, mice have only one \textit{Smn} gene, which is equivalent to human \textit{SMN1} (Viollet et al., 1997; DiDonato et al., 1997). Complete loss of this gene results in an embryonic lethal phenotype (Schrank et al., 1997). Introduction of 1 or 2 copies of human \textit{SMN2} rescues the embryonic lethal phenotype and results in mice with severe SMA (Hsieh-Li et al., 2000; Monani et al., 2000) whereas 8-16 copies of \textit{SMN2} completely rescues the SMA phenotype (Monani et al., 2000). Although both
severe and mild SMA mice ultimately exhibit motoneuron cell body loss (Monani et al., 2000, 2003), no early morphological or biochemical abnormality of the motoneurons has been reported.

A model of SMA in zebrafish has the potential to elucidate the effect of decreased Smn levels on motoneuron development in vivo. At 24 hpf, there are 3 well-characterized primary motoneurons per spinal cord hemisegment that innervate either the dorsal, rostral, or ventral region of each myotome (Eisen et al., 1986; see review Beattie, 2000). Over the next few days, each of the primary motor axons are joined by 20-30 secondary motor axons which form three distinct nerves that innervate the three myotome regions (Myers et al., 1986; Pike et al., 1992). As these axons extend into defined myotome regions, they can be followed in living embryos; thus perturbations in the organization of these neurons or their axons can be readily detected, followed during development, and quantitated (see review Beattie, 2000).

We have utilized antisense morpholino technology in zebrafish to model the effects of low levels of \textit{smn} in zebrafish. Reducing Smn protein levels in the developing embryo results in motor axon-specific truncations and branches, independent of motoneuron cell death. Moreover, by decreasing Smn levels in single motoneurons, we show that these defects are due to a cell-autonomous function of Smn in motoneurons. These are the first reported morphological abnormalities of motoneuron development, in response to low levels of Smn. These data reveal that one of the earliest consequences of Smn protein reduction is severely compromised motor axon outgrowth, indicative of an essential role for Smn in motoneuron development.
MATERIALS AND METHODS

Fish Maintenance

Adult zebrafish and embryos were maintained as described (Westerfield, 1995), allowed to develop at approximately 28.5°C, and were staged by hours (hpf) or days (dpf) post fertilization (Kimmel et al., 1995). All fish used in this study were on the *AB background unless otherwise specified.

Nomenclature

Consistent with guidelines for different species, the mouse, human or rat protein is “SMN” and the gene is “SMN” (human) or “Smn” (mouse). In fish the protein is “Smn” and the gene is “smn” (see http://zfin.org/zf_info/nomen.html).

Physical Mapping and Southern Blot Analysis

Zebrafish smn primers (5’-3’: GTGATGATTCTGACATTTGG, CCATCCTCACCTTTCAAAGC) were used to map the gene on the LN54 radiation hybrid panel (Hukriede et al., 1999). smn maps to Linkage Group 5, 3.25 cM from marker fb39c12. Southern blots were performed as previously described (Monani et al., 2000). The smn probe was generated by PCR with the following primer sequences: 5’-3’: GTGATGATTCTGACATTTGG and GTCTTCAGAGCATCTTCATCC. The zebrafish islet2 gene was used as a control.

Whole Mount in situ Hybridization

Zebrafish smn cDNA clone (accession numbers: Y17256; AA494875; AA494767; AF083557) was used to make sense and antisense digoxigenin labeled riboprobes of 1016 bp (Bertrand et al., 1999). The sense (Sp6) and antisense (T7) smn riboprobes were synthesized from plasmid linearized with XhoI and HindIII, respectively. Islet2 and
MyoD riboprobes were synthesized as described (Appel et al., 1995; Weinberg et al., 1996). Whole mount in situ hybridization protocol (Roche, 1277073) was performed as described (Thisse et al. 1993).

**Antisense Morpholino Oligonucleotide and Synthetic mRNA Injections**

An antisense morpholino oligonucleotide (MO) was designed against the 5’ start sequence of the *smn* gene (Gene Tools, Inc); 5’-3’: CGACATCTTCTGCACCATTGGC. An additional non-overlapping MO was also designed approximately 20 bp upstream of the original MO (designated 5’ UTR MO); 5’-3’:

TTTAAATATTTCCCAAGTCCAACGT. 2-4 cell *AB, gata2-GFP* (Meng et al., 1997), or *islet1-GFP* (Higashijima et al., 2000) embryos were injected with approximately 6 ng or 9 ng of MO in Danieau’s solution with phenol red dye according to protocol (Nasevicius and Ekker, 2000). A standard control (Gene Tools, Inc.) MO (5’-3’:

CCTCTTACCTCAGTTACAATTTATA-3’) was used at 6 ng or 9 ng for control injections. Synthetic capped human SMN mRNA were produced using mMESSAGE mMACHINE kit (Ambion, Inc.) according to manufacturer’s instructions. mRNA was produced from previously described plasmids (Le et al., 2000) linearized with Xho1. Approximately 300-500 pg of mRNA was co-injected with 9 ng of *smn* MO.

**Immunohistochemistry**

Immunohistochemistry and imaging was performed essentially as described (Beattie et al., 2000). The following mAb were used: znp1 (1:100; Melanacon et al., 1997), anti-acetylated tubulin (1:250; Sigma T-6793), anti-slow twitch myosin F59 (1:10; Crow and Stockdale, 1986; Devoto et al., 1996), anti-fast twitch myosin F310 (1:10; Crow and Stockdale, 1986; Zeller et al., 2002), 3A10 (1:10; Hatta, 1992), anti-neurolin zn-5 (1:75;
Fashena and Westerfield, 1999). FITC (IgG2A) and TRITC (IgG1) isotype specific conjugate secondary Ab (Southern Biotech, 1080-02 and 1070-03 respectively) were used for fluorescent detection of znp-1 (IgG2A) and F59/F310 (IgG1) mAb. Cross-sectional analysis was performed by embedding embryos in 1.5% agar/5% sucrose and sectioning on a cryostat at 16 µm. All immunofluorescent images were analyzed using a confocal microscope and photographed using digital imagery (Nikon Optiphot 2; BioRad MRC 1024) unless otherwise specified.

**Western Blot Analysis**

MO injected embryos were manually dechorionated and deyolked in a slurry of physiologic Ringers (Westerfield, 1995), Ringers ice chips, and protease inhibitors (Complete Mini, Roche 1836170). Deyolked embryo samples were prepared and western blots were performed as described (Monani et al., 2003). Smn (1:1000; MANSMA7 or MANSMA21; Young et al., 2000b), anti-synaptic vesicle (SV2) protein (1:200; Buckley and Kelly, 1985), and anti-Hu 16A11 (1:500, Molecular Probes, A-21271; Marusich et al., 1993) mAb were visualized using the ECL Detection Kit (Amersham Biotech, RPN 2109). Quantification of bands was performed using a densitometer (Shimadzu, Inc).

**Visualization of GFP transgenic Zebrafish**

Live *gata2*-GFP MO injected transgenic zebrafish (Meng et al., 1997) were anesthetized in tricaine (Sigma A-5040) at 36, 52, and 74 hpf and mounted on a glass coverslip. Individual motor nerves were visualized and imaged using a Photometrics SPOT camera. Images were compiled and edited using Adobe Photoshop software. MO injected *islet1*-GFP embryos (Higashijima et al., 2000) were fixed at 72 hpf in 4% paraformaldehyde for 2 h at room temperature.
**TUNEL Assay**

TUNEL assay was performed according to manufacturer’s protocol on staged embryos (Cole and Ross, 2001). Digoxigenin labeled dUTP was used to label fragmenting DNA ends (Terminal Transferase Assay, Roche 220582), and then embryos were sectioned on a cryostat at 16 µm.

**Detection of Neuromuscular Junctions**

MO-injected embryos were staged and fixed in 4% paraformaldehyde for 2 h at room temperature and water soaked for 3-6 h. After collagenase (Sigma C-9891) treatment, embryos were then incubated in Alexa 594 conjugated α-bungarotoxin (10 µg/ml; Molecular Probes, B13423) for 30 minutes, essentially as described (Ono et al., 2001). Embryos were then Ab stained using znp-1 and a FITC-conjugated secondary Ab.

**Single Cell Knockdown of smn**

Glass capillary microelectrodes were backfilled with a solution containing 2.5% rhodamine dextran (3,000 MW; Molecular Probes) and smn or control MO. 19.5-20.5 hpf embryos were anesthetized in tricaine and mounted in agar on a microslide as previously described (Eisen et al., 1989; Beattie et al., 2000). Individual CaP motoneurons or VeLD interneurons were impaled electrically by oscillating the electrode tip as described (Eisen et al., 1989). Solution was added to the cell by iontophoresis. Images were taken with Photometrics SPOT camera and were compiled and edited in Abode Photoshop.
RESULTS

Mapping and Expression of smn

The zebrafish *smn* gene was mapped to Chromosome 5 on the LN 54 radiation hybrid panel (Hukriede et al., 1999). Southern blot and radiation hybrid mapping (data not shown) indicated that there is only a single *smn* gene in zebrafish. RNA in situ hybridization showed that, like its mammalian counterpart, zebrafish *smn* appears to be expressed in all cell types based on diffuse and ubiquitous staining at 22, 27, 36, and 48 hours post fertilization (hpf; Figure 2.1).

![Figure 2.1](image-url)

**Figure 2.1. Smn is ubiquitously expressed during development.** Lateral view (anterior to the left; dorsal to the top) of 27 hpf (A and B) and 36 hpf (C and D) whole mount in situ hybridization of *smn* sense (A and C; n=10) and antisense (B and D; n=10) probes (purple). In situ hybridization was also performed at 22 and 48 hpf (data not shown). At all time points examined, the antisense *smn* probe shows ubiquitous staining, although the darker staining in the head region is most likely due to the dense nature of this region. Scale bar: 70 µm.

Knockdown of Smn Causes Spinal Motor Axon Defects

Morpholino antisense oligonucleotide “knockdown” technology was performed in zebrafish to decrease the levels of the Smm protein and mimic SMA. Morpholino
oligonucleotides (MO) inhibit the translation of their target mRNA, thereby causing reduced levels of target protein (Nasevicius and Ekker, 2000). Control or smn MO was injected into embryos at the 1-4 cell stage, then embryos were allowed to develop until the desired time. Western blot analysis showed that smn MO injected embryos exhibited a 61% decrease in Smn protein at 36 hpf (Figure 2.2 A, B).

Approximately 78% (n=580) of embryos injected with 6 ng of smn MO survived compared to 91% (n=139) survival when injected with the same concentration of control MO. Embryos died between late gastrulation and early somitogenesis, suggesting an
essential function of Smn during zebrafish development as well. *smn* MO injected embryos that survived did not exhibit gross abnormalities.

Initial examination focused on a subset of primary motoneurons. Cell bodies of CaP (Caudal Primary) and VaP (Variable Primary) motoneurons are located in the middle of each spinal cord hemisegment and express the LIM gene *islet2* (Appel et al., 1995). Using *islet2* RNA in situ hybridization we found that these motoneurons were present in both control MO (n=6) and *smn* MO injected embryos (n=42; data not shown). Using the znp1 monoclonal antibody (mAb), we examined CaP motor axons. At 27 hpf, CaP axon projections have a stereotyped morphology and project into ventral muscle, as shown in control MO injected embryo (Figure 2.2 C). *smn* MO injected embryos, however, showed motor axon defects that could be grouped into two categories based on axon length and axon branching. Approximately 35.3% of *smn* MO injected embryo sides had at least 1 truncated motor axon that did not fully project into the ventral muscle (Table 2.1, Figure 2.2 D, E). Affected sides in *smn* MO injected embryos had an average of 1.90 truncated axons (Table 2.2).
Table 2.1. Percentage of sides with at least one of the specified motor axon defects in embryos injected with smn MO. Znp1 antibody stained embryos were scored for truncation and branching defects (± 95% confidence interval); n=number of embryos and significance of control MO versus smn MO for each age, defect, and dose was determined by Student's t-test where * p<0.05; ** p<0.0005; ***p<0.0001

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<tr>
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<tr>
<td>smn MO</td>
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<tr>
<td>6 ng</td>
<td>35.3 ± 7.6**</td>
<td>61.5 ± 8.4***</td>
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<tr>
<td>n=156</td>
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<td>9 ng</td>
<td>13.6 ± 15.6</td>
<td>46.9 ± 12.4***</td>
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<td>n=81</td>
<td>n=128</td>
<td>n=81</td>
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Table 2.2. Average number of the specified motor axon/nerve defects per affected side of smn MO injected embryos. Znp1 antibody stained embryos were scored for truncation and branching defects (± 95% confidence interval); n=number of embryos and significance was determined by Student's t-test comparing each defect at 6 ng to the same defect at 9 ng where * p<0.01; ** p<0.0001

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<td>smn MO</td>
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<tr>
<td>6 ng</td>
<td>1.89 ± 0.32</td>
<td>2.81 ± 0.60</td>
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<td>n=156</td>
<td>n=130</td>
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<tr>
<td>9 ng</td>
<td>1.00 ± 0.00</td>
<td>2.47 ± 0.57</td>
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<tr>
<td>n=81</td>
<td>n=128</td>
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Axon branching was also observed in smn MO injected embryos. At 27 hpf, 14.3% of embryo sides had at least 1 branched axon (Table 2.1) and each affected side had an average of 2.31 branches (Table 2.2). These defects were typically found between hemisegments 5-17 corresponding to middle thru posterior trunk. It was rare that axon
defects where seen along the proximal portion of the axon, between the ventral root and the first intermediate target at the nascent horizontal myoseptum (white arrowhead in Figure 2.2).

By 36 hpf secondary motor axons also extend into the ventral muscle and fasciculate to form a nerve (Figure 2.2 F). At 36 hpf in smn MO injected embryos, 61.5% of the sides had at least 1 truncated axon (Table 2.1; Figure 2.2 G) compared to 35.3% at 27 hpf. Affected sides had an average of 2.81 truncated nerves which is consistent with what was observed at 27 hpf (Table 2.2). However, at 36 hpf, there were more sides with increased numbers of truncated nerves (Figure 2.2 G). 44.6% of the sides in smn MO injected embryos had at least 1 branched nerve in contrast to only 23.6% at 27 hpf. The average number of branches per side remained constant at 2.5 (Table 2.2).

**Further Knockdown of Smn Protein Results in Increased Motor Axon Branching**

It has been shown in human patients and mouse models that severity of SMA is dependent on the amount of SMN protein (Monani et al., 2000). To investigate if motor axon defects would become more severe when Smn protein was further reduced, 9 ng of MO was injected into embryos. Western blot analysis showed a 77% knockdown of protein (Figure 2.3 A, B). In contrast to 89% survival when control MO was injected (n=164), at this higher dose of smn MO, only 45% (n=433) of the embryos survived, further suggesting the essentiality of Smn in zebrafish development.
Figure 2.3. **Motor axons/nerves are abnormal in embryos injected with 9 ng of *smn* MO.** Western blot analysis of WT uninjected (lanes 1 and 3), control MO (lane 2), and *smn* MO (lane 4) injected (6 ng) embryos at 36 hpf. Hu-C, a neuronal marker, is shown as a loading control. Lateral views of whole-mount embryos labeled with znpl1 mAb at 27 hpf (C-E) and 36 hpf (F-H) in control MO (C and F) and *smn* MO (D, E, G, H) injected embryos. Truncated motor axons/nerves (D and G; black arrowheads) and branched motor axons/nerves (E and H; black arrows) occur when Smn protein levels are further reduced. Scale bars: (C-E), 25 \( \mu \text{m} \); (F-H), 30 \( \mu \text{m} \).

Injecting higher doses of *smn* MO resulted in a dramatic increase in motor axon branching. At 27 hpf, 77.3% of sides from *smn* MO embryos had at least 1 branched motor axon (Table 2.1; Figure 2.3 E). The average number of motor axon branches per side was also increased at higher doses of MO (Table 2.2). Branching defects were also significantly increased at 36 hpf; 76.6% of injected embryo sides had at least 1 branched motor nerve, compared to 44.6% of sides with the lower dose of *smn* MO (Table 2.1; Figure 2.3 H). These data indicate that the motor axon branching defect is more severe upon further knockdown of Smn.
Motor axon truncations were less evident at higher smn MO doses; in fact, at 27 hpf there is no significant difference when compared to control MO injections, where 5.6% of sides had truncated motor axons (Table 2.1, 2.2). Also, all of the smn MO defective sides only had 1 truncated motor axon (Table 2.2). At 36 h, a similar trend was apparent. 46.9% of 9 ng smn MO injected embryo sides had at least 1 truncated motor nerve, which is slightly reduced compared to lower doses of MO (Figure 2.3 G; Table 2.1). The average number of truncated motor nerves per side is 2.47, slightly less than at lower doses of MO (Table 2.2). These data suggest that further reduction of Smn protein levels result in more severe motor nerve branching defects and less severe truncation defects. To confirm that reduction in Smn results in motor axon/nerve defects, we used an additional, non-overlapping smn MO and observed similar defects (Figure 2.4), although this 5’ UTR MO was less efficient than the original MO described (Table 2.3).

**Figure 2.4.** Additional smn MO results in similar motor axon defects. Lateral views (anterior to the left: dorsal to the top) of 27 hpf whole mount embryos labeled with znp1 mAb in control MO (A) and 5’ UTR smn MO (B and C) injected (9 ng) embryos. Branched (B; arrows) and truncated (C; arrowheads) occur. Scale bar: 25 µm.
Table 2.3. Percentage of sides with at least one of the specified motor axon defects in embryos injected with 5' UTR smn MO. Znp1 antibody stained embryos were scored for truncation and branching defects (± 95% confidence interval); n=number of embryos and significance of control MO versus smn MO for each age, defect, and dose was determined by Student's t-test where * p<0.05 and **p<0.01.

Aberrant Motor Nerves Remain Defective Over Time

To determine the dynamics of the axon defects observed in smn MO injected embryos, we analyzed motor nerves over several days using gata2-GFP transgenic zebrafish (Meng et al., 1997). In this transgenic line, secondary motoneuron cell bodies and ventrally projecting axons express GFP starting at approximately 33 hpf. 9 ng of smn MO or control MO was injected into gata2-GFP embryos and ventral motor nerves were visualized from 36-74 hpf. smn MO was still effective in knocking-down Smn protein at later time points as evidenced by western blot analysis performed on 74 hpf, which shows a 88% reduction in Smn protein (Figure 2.5 A, B).
Figure 2.5. Time lapse imaging of defective motoneurons suggests that truncation defects precede branching defects. Western blot analysis of WT uninjected (lanes 1 and 3), control MO (lane 2), and smn MO (lane 4) injected (9 ng) embryos at 74 hpf. Synaptic vesicle protein 2, SV2, is shown as a loading control. Lateral views of the medial pathway of a GFP-expressing motor nerve of a transgenic gata2-GFP embryo injected with either control MO (C, E, G; n=10) or smn MO (D, F, H; n=13). Ventral projecting motoneurons are shown at 36 hpf (C and D), at 50 hpf (E and F) and 74 hpf (G and H). White lines demarcate the second intermediate target, the ventral aspect of the notochord. Only one GFP-expressing motor nerve was imaged, compiled, and placed on an artificial black background; the additional trunk nerves have been removed from the image. Scale bar: 50 µm.
Thirteen individual motor nerves were identified in \textit{smn} MO injected embryos (n=13) at 36 hpf, and were followed until 74 hpf. At 36 h, each of the 13 nerves was truncated at or below the ventral edge of the notochord (Figure 2.5 D), a second intermediate target for motor axons (Beattie et al., 2000). When the same motor nerves were examined at 52 hpf, 92% (12/13) of the nerves remained truncated, but had begun to branch (Figure 2.5 F). Upon examination at 76 hpf, 85% (11/13) of the nerves were truncated and branched (Figure 2.5 H). The remaining motor nerves (2/13) that did reach the ventral muscle projected into the preceding hemisegment, nearly touching the motor nerve in the adjacent hemisegment; while these motor nerves were not truncated or branched, their pathfinding was clearly erroneous. In contrast, all of the nerves in control MO injected embryos (n=10) exhibited the stereotypic ventral nerve projections (Figure 2.5 C, E, G). These data show that defective motor nerves are dynamic, first becoming truncated then branching at the site of truncation. Thus, nerve truncations are still occurring at higher doses of \textit{smn} MO, but the extensive branching obscures this phenotype.

\textbf{Knockdown of smn Also Causes Aberrant Dorsal Projecting Motor Nerves}

To determine whether other spinal motor axons were affected by decreased levels of Smn protein, we analyzed dorsal motor nerves in \textit{islet1-GFP} transgenic zebrafish. Among other neuronal types, this transgenic line has GFP-expressing secondary motoneuron cell bodies and dorsally projecting axons (Figure 2.6 A; Higashijima et al., 2000).
9 ng of smn MO or control MO were injected into islet1-GFP transgenic embryos and dorsal nerves were analyzed at approximately 74 hpf. Like ventral motor nerves, dorsally projecting motor nerves exhibited truncation and branching defects as a result of smn knockdown (Figure 2.6 B, C). Branching is also more prominent at higher doses of smn MO in these nerves. 69.1% (n=178) of sides of smn MO injected embryos had at least 1 branched dorsal projecting motor nerve, whereas control MO injected embryos had only 8.0% of sides with a branched dorsal nerve (n=50). In contrast, 32.6% (n=178) of sides of smn MO injected embryos had at least 1 truncated nerve, whereas control MO injected embryos had no truncated dorsal projecting nerves (n=50). These defects are consistent with those seen in ventral projecting motor nerves suggesting that dorsal projecting motoneurons are also affected by reduction of Smn protein.

**Human SMN mRNA Partially Rescues Motor Nerve Defects**

To confirm the specificity of the MO function, RNA rescue experiments were performed using human SMN mRNA. 1-4 cell gata2-GFP embryos were injected with a mixture of smn MO (9 ng) and hSMN mRNA. When compared to smn MO alone, co-injection of
hSMN and smn MO resulted in rescue of both the branching and truncation nerve defects (Table 2.4).

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<td>smn MO (9 ng)</td>
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<td>19.4 ± 5.4</td>
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<td>smn MO (9 ng) and hSMN RNA</td>
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<td>8.1 ± 7.0 *</td>
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<td>smn MO (9 ng) and hSMNΔ7 RNA</td>
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</table>

Table 2.4. Percentage of sides with at least one of the specified motor nerve defects in smn MO or smn MO and hSMN RNA injected embryos. GFP-expressing motoneurons were scored at 52 h (+95% confidence interval); n=number of embryos and significance of hSMN mRNA rescue versus smn MO alone for each defect was determined by Student's t-test where * p<0.02; **p<0.0001

The partial rescue observed was probably due to the mosaic nature of the mRNA and MO injections, as reported by others (McClintock et al., 2002). The specificity of the RNA rescue was confirmed by the lack of rescue when hSMNΔ7 mRNA, a common mutation and the major form of mRNA produced by SMN2, was co-injected with smn MO (Table 2.4; Lefebrve et al., 1995). The percentage of sides with branched motor nerves is slightly reduced compared to MO alone, likely due to the slight activity of the hSMNΔ7 protein (Cifuentes-Diaz et al., 2002, Le et al., 2005). These rescue experiments confirm that the motor nerve defects observed when smn MO is injected, are a result of the reduction of Smn protein and further validate this system as a model for SMA.

Motor Axon Defects are Not Caused by Cell Death

To determine whether the motor axon defects observed upon smn knockdown were caused by neuronal apoptosis, a TUNEL assay was performed. Unlike higher vertebrates,
naturally occurring motoneuron cell death in the zebrafish ventral spinal cord has not been reported (Cole and Ross, 2001). Embryos were injected with 9 ng control MO or smn MO then analyzed at 27 (n=10), 36 (n=10), and 50 hpf (n=15). None of the injected embryos had any TUNEL positive cells in the ventral spinal cord (Figure 2.7 A, B).
Figure 2.7. Decreased levels of Smn does not initially result in motoneuron cell death. Cross section view (dorsal to the top) of TUNEL stained 50 hpf control MO (A; n=3) and smn MO (B; n=15) injected (9 ng) embryos. TUNEL positive (purple) cells are present on the skin of both the smn MO and control MO injected embryos, which is not uncommon for this assay. Occasionally, (B) TUNEL positive cells (black arrowhead) present in the dorsal spinal cord, corresponding to the sensory Rohon-Beard neurons (entire spinal cord demarcated by black dashed lines). Cross section view of zn-5 mAb stained secondary motoneuron cell bodies (arrows) of 50 hpf control MO (C; n=6) and smn MO (D; n=41) injected (9 ng) embryos. (E) Motor pool diameter (µm) was measured on medial-lateral (M-L) and dorsal-ventral (D-V) axes (error bars indicate standard error). Anterior trunk was defined as hemisegments 1-5 while middle trunk was defined as hemisegments 6-14. T-test concluded that p>0.1 among motor pools of control MO and smn MO injected embryos. Scale bar: 25 µm.
TUNEL positive cells could occasionally be seen in the dorsal spinal cord corresponding to the Rohon-Beard sensory neurons (Figure 2.7 B; arrowhead), which have previously been shown to undergo apoptosis at this age (Cole and Ross, 2001). To further confirm that motoneuron numbers were not reduced by means other than apoptosis, the diameter of the spinal motor pool was measured. Embryos injected with control or smn MO were labeled with anti-neurolin mAb, which labels the cell surface of secondary motoneurons in the spinal cord (Fashena and Westerfield, 1999). To analyze the motor pool size, the diameter was measured both dorso-ventrally and medio-laterally on both sides of the midline (Figure 2.7 C, D). There was no significant difference in motor pool diameter on either axis between control MO and smn MO injected embryos (Figure 2.7 E). These data indicate that the motor axon defects observed upon Smn reduction are not caused by motoneuron death and further support that Smn is necessary for motor axon development.

**Other Neuronal Cell Types and Axon Tracts are Not Affected by Smn Knockdown**

In human and mouse models of SMA, the affect of decreased levels of SMN is specific for motoneurons. To determine if the defects resulting from Smn knockdown were also motoneuron-specific, we examined populations of interneurons and sensory neurons using the acetylated tubulin mAb. Many zebrafish hindbrain interneurons have stereotyped axonal projections that extend into the spinal cord (Metcalf et al., 1986, Mendelson, 1986). We saw no defects in the medial longitudinal fascicle (MLF) or the lateral longitudinal fascicle (LLF), both of which contain hindbrain and midbrain axons descending into the spinal cord (Figure 2.8 A, B).
Figure 2.8. **Other neurons and axon tracts are unaffected upon knockdown of Smn protein.** Dorsal view (anterior to the top) of the hindbrain stained with acetylated tubulin mAb in 27 hpf control MO (A; n=40) and *smn* MO (B; n=39) injected (9 ng) embryos showing the medial longitudinal fascicle (black arrowheads) and the lateral longitudinal fascicle (black arrows). Dorsal view of the hindbrain Mauthner neuron cell body and axon (arrowhead) stained with 3A10 mAb in 34 hpf control Mo (C; n=61) and *smn* MO (D; n=32) injected embryos. Lateral views of (F-F) Rohon-Beard (black arrow) and (G-H) lateral line sensory neurons (white arrow) stained with acetylated tubulin mAb in 34 hpf control MO (E, G; n=40) and *smn* MO (F, H; n=39) injected embryos. Scale bar: (A and B), 50 µm; (C and D), 40 µm; (E-H), 50 µm.

To look for more subtle defects, we examined the single commissural axon of the Mauthner neuron present in hindbrain rhombomere 4. Using the 3A10 mAb, we found that in *smn* MO injected embryos, the Mauthner axon projected across the midline and down the spinal cord in a manner indistinguishable from control MO injected embryos (Figure 2.8 C, D). Rohon-Beard sensory neurons, present in the dorsal spinal cord (Lamborghini, 1980; Metcalfe et al., 1990), send axons into the peripheral muscle and skin. These cells and their axons were also unaffected by Smn knockdown (Figure 2.8 E, F). Lastly, we looked at the lateral line, a group of sensory neurons that send axons the length of the developing embryo. We observed no difference in length or morphology of the lateral line axons in control MO or *smn* MO injected embryos (Figure 2.8 H, G).
Thus, interneurons and sensory neurons are unaffected by decreased levels of Smn protein and support the finding that motoneurons are uniquely sensitive to decreased Smn levels.

**Smn Knockdown Does Not Affect Muscle Development**

To determine whether the motor axon defects observed were due to defects in muscle development, we examined the development and patterning of the myotomes. Overall myotome morphology was analyzed using live *gata2-GFP* transgenic embryos injected with either control MO or *smn* MO. Nomarski images were taken of muscle segments containing defective GFP-expressing nerves. No difference was seen in the muscle morphology between control MO and *smn* MO injected embryos (Figure 2.9 A, B). No defects were seen in early muscle patterning in *smn* MO injected embryos compared to control MO, as assayed by *myoD* expression, a gene expressed in the posterior region of the developing somite (Weinberg et al., 1996; Figure 2.9 C, D). Lastly, we asked whether muscle cells were correctly specified by characterizing slow and fast muscle in *smn* MO injected embryos. Using the slow muscle mAb F59 (Crow and Stockdale, 1986; Devoto et al., 1996) and the fast muscle mAb F310 (Crow and Stockdale, 1986; Zeller et al., 2002), we found no difference between muscle specification and organization upon depletion of Smn (Figure 2.9 E-H). These data suggest that low levels of Smn do not adversely affect muscle organization, specification, or development.
Figure 2.9. Muscle development is normal when Smn is decreased. Nomarski lateral views of mid-trunk muscle from 50 hpf gata2-GFP transgenic embryos injected with 9 ng of control MO (A; n=20) or smn MO (B; n=20) showing somitic boundaries (black arrows). Dorsal views of 22 hpf whole mount in situ hybridization of myoD (purple; black arrowhead) in control MO (C; n=31) and smn MO (D; n=18) injected embryos. Cross section of 27 hpf znpl (motor axon; arrows) and F59 (slow muscle; arrowheads) mAb stained embryos injected with control MO (E; n=40) or smn MO (F; n=65). Cross section of 27 hpf znpl (motor axon; arrows) and F310 (fast muscle; arrowheads) mAb stained embryos injected with control MO (G; n=20) or smn MO (H; n=41). Scale bar: (A-D), 75 µm; (E-H), 30 µm.
Since motor axons in \textit{smn} MO embryos displayed aberrant axon outgrowth, we asked whether these axons were still capable of forming synapses on the muscle fibers. To address this we analyzed acetylcholine receptors (AChR) clusters in \textit{smn} MO embryos at 3 dpf. Using rhodamine conjugated $\alpha$-bungarotoxin ($\alpha$-BTX), we found that all axons in \textit{smn} MO and control MO embryos co-localized with AChR clusters (Figure 2.10 A-F), suggesting that the axons are innervating muscle fibers, but lack the stereotyped pattern of innervation (Figure 2.10 D-F, arrows). Consistent with this observation, we found no overt movement defects or paralysis in these embryos up to 3 dpf (data not shown).

\textbf{Figure 2.10. AChR clustering is normal when Smn is decreased.} Lateral views of \textit{znp1} (A and D; motor axons, green) mAb, $\alpha$-BTX (B and E; AChR, red) stained, and merge (C and F; yellow) 74 hpf larvae injected with control MO (A-C; n=18) or \textit{smn} MO (D-F; n=49). Aberrant motor nerve (arrowhead) retains ability to cluster AChR. Scale bar: 50 $\mu$m.

\textbf{Smn is Acting Cell-Autonomously in Motoneurons}

Since Smn is expressed in all tissues, it is not clear where its function is required for normal motor axon development. To test whether Smn functions cell-autonomously with
respect to motoneurons, we reduced Smn levels in single motoneurons in otherwise WT embryos. Optical clarity of zebrafish embryos allows us to identify single primary motoneurons in living embryos and iontophorese these cells with vital fluorescent dyes (Eisen et al., 1989; Beattie et al., 2000). *smn* or control MO was iontophoresed with rhodamine dextran dye into CaP cell bodies at 19.5-20.5 hpf; CaP axons were then imaged at 43 h. 100 µM and 50 µM solutions were found to cause significant cell death when iontophoresed into motoneurons while a solution containing 1 µM of *smn* MO only caused 45.5% of the CaP motoneurons to die.

CaP motoneurons iontophoresed with 1 µM control MO exhibited no axon defects (n=10; Figure 2.11 A). In contrast, CaP motoneurons, iontophoresed with *smn* MO when their growth cones were at the first intermediate target, were defective 76.9% of the time (n=13; Figure 2.11 B, C, D). The defects observed, axon truncations and branches, were consistent with defects seen when the entire embryo was injected with *smn* MO. CaP motoneurons were also iontophoresed with *smn* MO when their growth cones were further ventral at the second intermediate target. Only 62.5% of these motor axons (n=8; Figure 2.11 D) were defective, suggesting that the longer the Smn protein is reduced during outgrowth, the more likely pathfinding errors, such as truncations and branches, will occur. The incomplete penetrance of these defects may suggest variability in the phenotype, as is seen when the entire embryo is injected with *smn* MO.
Figure 2.11. Smn is acting cell-autonomously in CaP motoneurons. Lateral views of live CaP motoneurons iontophoresed with rhodamine dextran and control MO (A; n=10) or smn MO (B and C; n=21) visualized and imaged at 43 hpf. CaP motoneurons ionotophoresed with smn MO exhibit axon branching (B) and truncation (C). (D) Percentage of smn MO injected CaP motoneurons with defective and normal axons quantitated (error bars represent standard error). HM, nasant horizontal myoseptum; VNC, myotome adjacent to the ventral edge of the notochord. Scale bar: 50 µm.

To show the specificity of these defects, smn MO and fluorescent rhodamine dextran were also iontophoresed into Ventral Longitudinal Descending (VeLD) neurons, spinal interneurons with axons that descend into the tail region by 43 hpf (Kuwada et al., 1990; Hale et al., 2001). All VeLD interneurons iontophoresed with smn MO (n=4) had axons that traveled into the tail region and showed no other defects, such as branching (data not shown). This further indicates that knockdown of Smn protein causes motoneuron specific defects. Taken together, these data show that Smn functions cell-autonomously in motoneurons and plays a critical and specific role in motor axon outgrowth and pathfinding.
DISCUSSION

Taking advantage of the relatively simple neuromuscular organization of the zebrafish embryo, we have shown that Smn has an essential function in motor axon development. The defects observed in motor axons upon knocking-down Smn are independent of cell death and are specific for motoneurons. The data we present indicate that the earliest defects in SMA are in the developing motor axons and suggest that these defects in axon outgrowth and pathfinding may eventually lead to motoneuron death and SMA.

The motor axon defect in smn knock-down embryos

Embryos with decreased Smn levels show defects in motor axon outgrowth and pathfinding during the first three days of development. Defects were rarely seen in the proximal axon extending from the spinal cord to the first intermediate target suggesting that initiation of axon outgrowth and pathfinding along the common pathway are unaffected by low Smn levels. Secondary motoneurons also displayed branching and truncation defects which continued up to 3 dpf. Since primary and secondary motor axons fasciculate to form nerves, we cannot be certain that the defects observed at 36-72 hpf are defasciculation of the nerve or true branching of the axons. Since branching in single CaP axons is seen, this suggests that true branching does occur. Although the motor axon projections are aberrant, these axons still co-localized with AChR indicating that functional synapses had formed.

The motor axon branching and truncation defects were initially discovered upon injection of 6 ng of smn MO into zebrafish embryos. When a higher dose of MO, 9 ng, was used, there was a significant increase in the amount of motor axon branching and an apparent decrease in truncations. Analysis of embryos with GFP-expressing motor
nerves, however, revealed that truncations were still occurring, but were followed by branching, thus obscuring the truncation phenotype. These data indicate that the less Smn protein translated within the neuron, the greater the likelihood that the axon will branch. Ventral nerve truncations were consistently occurring at the second intermediate target. It is believed that intermediate targets are important for growth cones to proceed to their final targets (Tessier-Lavigne and Goodman, 1996). It is possible that in the presence of low Smn, growth cones stall at these locations, suggesting that they still try to assimilate cues. At very low levels of Smn, the growth cone may not be even responding to the intermediate target and branches indiscriminately.

**Cell-Autonomy of Smn**

Due to the ubiquitous distribution of SMN protein, it has been difficult to conclusively determine whether the primary SMA defect resides in motoneurons or muscle. In smn MO injected zebrafish embryos, the motoneurons are defective, but muscle specification, early patterning, and muscle development are normal, indicating that decreasing Smn is critical in motoneurons. We specifically reduced Smn in single motoneurons without affecting Smn levels in other neurons or muscle. Exclusively decreasing Smn in motoneurons recapitulated the motor axon defect seen when Smn was decreased throughout the embryo, indicating that Smn functions cell–autonomously. We cannot, however, rule out the possibility that Smn also functions in muscle to affect motor axons.

**Smn and Motor Axons**

Recent mouse and human studies suggests that motor axons may be affected before motoneuron loss. Mice exhibiting neuronal depletion of full-length SMN (Smn^Fl/Smn^D7,
NSE-Cre+ exhibited a dramatic decrease in motor axons, but only a modest decrease in motoneuron cell bodies at postnatal day 15 (Cifuentes-Diaz et al., 2002). Due to the severe reduction in full-length SMN and the relatively late developmental timing of this reduction, however, it is difficult to determine the disease relevance of these defects. Severe SMA mice (Smn−/− with 1-2 copies of SMN2; Hsieh-Li et al., 2000; Monani et al., 2000), show moderate motoneuron cell body loss, while mild SMA mice (A2G missense) show no motoneuron cell body loss several days after birth; mild SMA mice do, however, exhibit a mild reduction in both motoneuron cell bodies and motor axon roots at 3.5 months (Monani et al., 2003). Motor Unit Number Estimation (MUNE) studies in humans show that presymptomatic individuals exhibit high motoneuron counts which dramatically decrease with onset of the disease (Bromberg and Swoboda, 2002). These results indicate that motoneuron cell body loss is a late feature of the disease. Our data are consistent with these findings and support the idea that axons are affected before cell death.

SMN has also been shown to localize in developing motoneurons. Rat SMN co-localizes with cytoskeletal elements in the spinal motor axons during nervous system maturation (Pagliardini et al., 2000). In primary motoneuron cultures from E14 mice embryos, SMN is enriched in branch points and growth cones (Jablonka et al., 2001). SMN has also been found in growth cones of P19 neurons (Fan and Simard, 2002). Interestingly, Gemin2, a protein which complexes with SMN in spliceosomal snRNP biogenesis (Fischer et al., 1997), does not co-enrich in axon branch points, indicating the possibility of a unique SMN complex and function at these locations (Jablonka et al., 2001). Taken together with our findings of abnormal motor axon branching when Smn is...
depleted, the data suggest that Smn plays an important function in motor axon development and maintenance.

While a mechanism by which SMN functions in motoneuron development remains unclear, recent data does give clues. SMN has been shown to interact with profilin (Giesemann et al., 1999), a protein involved in actin polymerization and growth cone motility (Gutsche-Perelroizen, 1999; Kim et al., 2001; Wills et al., 1999), processes important for axon outgrowth. Data has also implicated an interaction between SMN and motoneuron-enriched RNA processing molecules, hn-RNP-Q and hn-RNP-R (Rossoll et al., 2002). These proteins play a role in RNA transport, editing, and translation. RNA transport into the axon followed by localized protein translation has been shown to be important for growth cones to proceed past intermediate targets (Brittis et al., 2002). It is possible that SMN in axons and growth cones may be associated with RNP complex assembly associated with transport and/or translation of mRNA. Recent experiments have shown that SMN localizes with cytoskeletal filaments and is actively transported down axons (Zhang et al., 2003) further indicating that SMN may be involved in transport and the resulting translation of important axon guidance cues. It is possible that if SMN is reduced, motor axons cannot respond to cues at intermediate targets by turning on local translation which results in axon defects. Our finding that GFP-expressing motor nerves in smn MO injected embryos are truncated at the second intermediate target is consistent with this idea. The validity of this model, however, will depend on elucidating a direct relationship between Smn and localized protein translation machinery in motor axon growth cones.
ABSTRACT

Because SMN is a ubiquitously expressed protein, a paramount question in SMA research revolves around why reduced levels of SMN lead to a motoneuron-specific disease. It has been hypothesized that SMN may have a dual function: the well-characterized role in mediating snRNP assembly and a novel motor axonal-specific function. To begin to identify which function is important for axonal outgrowth and potentially SMA, non-Smn components in these separate pathways have been knocked-down by morpholino in zebrafish. Moderate knockdown of Gemin2, a Smn interacting snRNP assembly component, does not yield motor axon defects. In contrast, knockdown of Zbp1, a non-Smn interacting component involved in mRNA transport down axons, did result in motor axon defects similar to what was observed upon Smn reduction. If Smn does have a role in motor axons, it is in the proper place to do so, as shown by hSMN-RFP localization to motor axons. While not conclusive evidence, these data suggest a non-snRNP assembly function for Smn in motor axon outgrowth and pathfinding.

All experiments in this chapter were completed by ML McWhorter with the following exceptions: long term survival assays were completed in collaboration with Dr. Tessa Carrel (The Ohio State University); snRNP assembly assays were completed by Dr. Livio Pellizzoni (Dulbecco Telethon Institute at the Institute of Cell Biology; Rome, Italy); biochemical data suggesting an interaction between Smn and Zbp2 was provided by Dr. Gary Bassell (Albert Einstein College of Medicine, New York).
INTRODUCTION

As research into SMA and SMN continues to expand, two distinct groups have emerged: the “nucleocentric” group and the “axonocentric” group. Numerous research groups exist who believe that SMN’s function in nuclear processes, such as snRNP assembly, is directly linked to SMA pathology. Recent evidence has begun to challenge these ideas and has suggested a motor axon-specific function of SMN. To better understand which of these potential functions of SMN may be important for SMA pathology, functionality studies utilizing zebrafish have been performed. It is believed that SMN has at least a dual function in motoneurons, the well-characterized snRNP assembly function and a novel motor axon-specific function.

SMN has been termed the “master assembler” for its multiple interactions with different RNA processes (Terns and Terns, 2001). It is well known that in combination with Gemins2-7, SMN plays a role in RNA metabolism, specifically assembly of snRNP particles which are components of the eukaryotic spliceosome (Gubitz et al., 2004). Due to the essential housekeeping function of SMN in this process, the pathophysiologic relevance of this defect with respect to clinical SMA is unclear because it is motoneuron-specific disease (Nicole et al., 2002). Some have suggested that SMN is a specificity factor for snRNP assembly in that the promiscuous Sm particles would bind indiscriminately to other RNAs without SMN (Pellizzoni et al., 2002). Recent data suggests that snRNP assembly is reduced in SMA patient fibroblasts (Wan et al., 2005). However, no splicing abnormalities of any candidate mRNAs have been observed (Jablonka et al., 2000). While it is unclear how deficits in this essential cell process are causing motoneuron-specific defects, the possibility of defective snRNP assembly
causing SMA cannot be ignored. To date, reduction in snRNP assembly is the only biochemical abnormality attributable to SMA patients (Wan et al., 2005). Therefore, the role of snRNP assembly function in motor axon outgrowth will be examined.

SMN is known as the “master assembler” not only because of interactions with snRNPs and pre-mRNA splicing, but also for potential roles in ribosome production and transcription (reviewed in Terns and Terns, 2001). SMN has been shown to interact with GAR1 and fibrillarin which are important in biogenesis of ribosomes (Jones et al., 2001). During ribosome biogenesis, snoRNAs cleave and modify ribosomal rRNA, and GAR1 and fibrillarin are core components of snoRNPs (Pellizzoni et al., 2001a). Additional evidence suggests that SMN may be involved in transcription by associating with RNA helicase A and RNA polymerase II (Pellizzoni et al., 2001b). Although SMN may play a role in 3 different macromolecular RNA processes, its role in snRNP assembly will be the only one addressed here.

Analysis of the SMN protein has shown various functional domains (Figure 3.1).

**Figure 3.1. Diagram of SMN functional domains.** Diagram shows major functional domains in the SMN protein; not all known domains are listed (Kuncl, 2002).
The tudor domain of SMN mediates binding of proteins such as Sm, fibrillarin, and GAR1 (Jones et al., 2001). SMN also has a nucleic acid binding and oligomerization domain which has been shown to enhance Sm protein binding (reviewed in Briese et al., 2005; Wang and Dreyfuss, 2001; Sun et al., 2005). Interestingly point mutations in patients have been identified within the tudor and oligomerization domains (Wang et al., 2001; Pellizzoni et al., 1999; Sun et al., 2005). While these mutations affect both SMN’s binding to Sm, some Gemin proteins, and its self-oligomerization, there may be other proteins which bind to these regions or are influenced by these domains. The binding of these yet unidentified proteins could also be affected. Therefore, these analyses of patient mutations do not provide conclusive evidence for a link between SMA pathology and snRNP assembly especially considering that patient mutations also occur outside of the tudor and oligomerization domains (Sun et al., 2005).

Evidence which suggests an axonal role for SMN is mounting. Localization data suggests that SMN is present in motor axons and growth cones of primary cultured mice motoneurons and other cultured neurons (Jablonka et al., 2001; Fan and Simard, 2002; Zhang et al., 2003). Phenotypic studies suggest that when Smn levels are reduced, defects occur in the neuromuscular architecture: motor axon outgrowth defects in zebrafish embryos are observed when Smn is depleted (McWhorter et al., 2003; Chapter 2); primary motoneuron cultures from SMA (SMN2; SMN−/−) mice have short, branched axons (Rossoll et al., 2003); and neuromuscular junction defects are also observed in the Drosophila model of SMA (Chan et al., 2003). Finally, it has been shown that SMN complexes are transported down axons and into growth cones in cultured neurons (Zhang et al., 2003; Rossoll et al., 2003). The question has now become, what is SMN doing in
motor axons? It is possible that SMN forms a motor axon-specific complex that is transported down the axons and into growth cones; this complex may have an important function that is ultimately responsible for SMA pathology.

To begin to understand a possible motor axon specific role for SMN, it is necessary to understand the process by which motor axons pathfind to the proper target regions. The growth cone, the leading edge of the axon, needs to be able to respond to environmental cues as it journeys away from the cell body and into the muscle (reviewed in Goodman, 1996). Usually those environmental cues bind to receptors along the growth cone and begin a cascade of molecular dominos (Tessier-Lavigne and Goodman, 1996). Ultimately the cytoskeletal elements, specifically β-actin and microtubules, begin to polymerize and depolymerize to move the growth cone in response to the cue (Dent and Gertler, 2003). It is becoming increasingly evident that mRNA transport followed by localized protein translation occurs within growth cones (reviewed in Giuditta et al., 2002). Upon being transcribed, mRNAs are believed to complex with various proteins (RNP complexes) which are then translocated to their destination, anchored to the cytoskeleton, and locally translated into protein within the growth cone (Kiebler and DesGroseillers, 2000; Zhang et al., 2001). More recent data has implicated localized protein translation in the growth cone as critical for growth cone response to guidance cues (Campbell and Holt, 2001; Brittis et al., 2002; Condron, 2002). It has also previously been shown that if muscle targets are removed, motoneurons die (reviewed in de la Cruz et al., 1996). While there are numerous possibilities for SMN function in the motor axon, two examples will be presented in detail here: direct effects on actin polymerization and/or transport of mRNAs into the axon and growth cone.
Along with microtubules, the actin cytoskeleton is essential for axonal outgrowth to target regions (Dent and Gertler, 2003). The regulation of actin polymerization toward an environmental guidance cue and/or depolymerization away from another cue is essential during the pathfinding process (Dent and Gertler, 2003). The regulation of actin polymerization/depolymerization is achieved by numerous actin binding proteins (Wear et al., 2000). One of these regulatory proteins, profilin, binds to the proline-rich region of SMN spanning the end of exon 4 through the beginning of exon 6 (Giesemann et al., 1999). Profilin can regulate actin polymerization by sequestering actin monomers or by accelerating the ADP-ATP exchange on the actin monomers (Pantaloni et al., 1993; Witke, 2004). While profilin I is ubiquitous, SMN preferentially binds to profilin IIa, the dominant isoform in neuronal tissues (Lambrechts et al., 2000; Sharma et al., 2005). Recent data suggests that SMN may moderate the regulatory activity of profilin IIa on actin polymerization (Sharma et al., 2005). Reduction of profilin IIa results in stimulation of neurite outgrowth (DaSilva et al., 2003). Due to the necessity of actin polymerization and depolymerization during pathfinding, complex regulation undoubtedly occurs at many parts during the process (Dent and Gertler, 2003). While the role of SMN in actin polymerization during axon outgrowth is not completely known, the possibility exists that SMN may regulate this important process.

The second possible role for SMN in axons is transport of RNP particles (including mRNAs). Even before localized translation was shown to be important during axon guidance, many examples of selective localization of mRNAs into axons existed (Bassell and Kelic, 2004). One example of an mRNA which is localized to growth cones is β-actin (Kislauskis et al., 1997; Zhang et al., 2001). The enrichment of β-actin to sites
of dynamic actin polymerization aides in the motility of the cell and/or growth cone (Kislauskis et al., 1997; Zhang et al., 2001). The 3’ end of the β-actin mRNA encodes a 54 nt sequence, called the zipcode, which is necessary and sufficient for localization into growth cones and neurites (Kislauskis et al., 1994; Ross et al., 1997). Zipcode binding protein 1 (Zbp1) is an mRNA binding protein which also binds to the zipcode sequence on β-actin to localize the transcript to neurites (Ross et al., 1997). With its nuclear localization and export sequences, Zbp1 shuttles to and from the nucleus localizing mRNA and protein complexes, RNPs (Ross et al., 1997). If the interaction between Zbp1 and the β-actin zipcode is disrupted, β-actin mRNA is not enriched in cultured neuronal growth cones and the neurons have impaired growth (Zhang et al., 2001).

An additional zipcode binding protein (Zbp2) has been identified that also binds the β-actin zipcode sequence (Gu et al., 2002). Zbp2 is a predominantly nuclear protein which also localizes to growth cones and shuttles to and from the nucleus (Gu et al., 2002; Bassell and Kelic, 2004). Both of these zipcode binding proteins are important for the transport of mRNA, specifically β-actin, down axons into growth cones (Bassell and Kelic, 2004). Although currently unknown, other mRNAs may be transported in similar ways, as β-actin, down the axon. Zbp1 and Zbp2 may interact directly with microtubule motor proteins dynein and kinesin which are important for retrograde and anterograde transport, respectively (Bassell and Kelic, 2004).

Another potential important mRNA interacting protein is hn-RNP-R (Rossoll et al., 2002; Rossoll et al., 2003). Using yeast-2-hybrid analysis, two heterogeneous nuclear ribonuclear proteins (hn-RNP) Q and R were identified for their interaction with SMN (Rossoll et al., 2002). The expression of hn-RNP-R and hn-RNP-Q are developmentally
regulated in the spinal cord and hn-RNP-R specifically localizes to axons (Rossoll et al., 2002). hn-RNP-R binds to single stranded RNA, specifically to the 3’ end of β-actin mRNA (Rossoll et al., 2003). It is believed that hn-RNP-R is necessary for β-actin localization to axonal growth cones (Rossoll et al., 2003). These data suggest that SMN may play a role with hn-RNP-R to localize β-actin in cultured motoneurons (Rossoll et al., 2003). This is the first published data showing a specific mRNA transport role for SMN. To further understand this motor axonal transport role of SMN, other components of this potential transport pathway will be knocked-down in zebrafish.

Since SMN is a ubiquitously expressed gene, SMA researchers have pondered why motoneurons are so uniquely sensitive to reduced levels of this protein. The role of SMN in snRNP assembly is certainly one possible explanation. However, there is no data to suggest why motoneurons are sensitive to loss of snRNP assembly when this is an essential cell function. Alternatively, SMN may have a motor axon-specific function unrelated to snRNP assembly. Other researchers are studying the role SMN may have on β-actin polymerization thru interactions with regulatory proteins such as profilin (Sharma et al., 2005). Because localization of mRNAs and the resulting localized translation have been linked to axon guidance and growth cone motility (Campbell and Holt, 2001; Brittis et al., 2002; Zhang et al., 2001), SMN may be a component of complexes which transport mRNAs and resulting RNP particles into axons. When SMN levels are reduced, transportation and local translation of mRNAs (such as β-actin) to growth cones may be perturbed resulting in motor axon defects and ultimately SMA. The question of why motoneurons are sensitive to mutations in ubiquitously expressed proteins is not unique to SMA; other motoneurons diseases like ALS and SMA with respiratory distress
(SMARD) are also caused by mutations in ubiquitously expressed genes. Elucidation of the mechanism by which motoneurons are uniquely sensitive to low levels of SMN may also shed light on other motoneuron diseases. To understand the mechanism by which low levels of SMN lead to motor axon-specific pathfinding defects, a combination of MO knockdown experiments and axon localization data will be utilized in the developing zebrafish embryo.

**MATERIALS AND METHODS**

**Fish maintenance**

Adult zebrafish and embryos were maintained as previously described (Westerfield, 1995; see Chapter 2). All fish used in this study were in the *AB* background unless otherwise specified.

**Genbank/EMBL accession numbers**

The following is a list of Genbank or EMBL accession numbers used for the listed genes: 

- *smn* (Y17256, AA494875, AA494767, AF083557, ENSDARG00000018494), 
- *gemin2* (NP_001017608, ENSDARG00000015638), 
- *zbp2* (NP_9896087, AF533513, AF026527, BC064649; AF461020, ENSDARG00000026489), and 
- *zbp1* (AF026527, NP_006537, ENSDARG00000033836).

**Long term survival assay**

Individual larvae (WT or injected) were placed into separate housing tubes which were open at the top with mesh glued at the bottom. Housing tubes were placed within a larger fish tank that was placed on the main rack so that water would circulate within the tank and the individual housing tubes. Overall movement and survival were assayed twice
during weekdays and once per weekend day. Tublings were fed under the same regiment as other larval fish, twice during the weekday and once on the weekend days. Overall survival was calculated from 3-30 dpf.

**Antisense MO and synthetic mRNA injections**

Antisense MOs (GeneTools, LLC) were designed against the 5’ translation start site of the following genes (MO sequence is listed):  *smn* MO, 5’-

CGACATCTTCTGCACCATTGGC-3’; *gemin2* MO, 5’-

TATGCACGACCATCAAAAATAAATCA-3’; *gemin2* MO2, 5’-

CAACTCCTCGGCCTCTGACTTCAT-3’; *zbp2* MO, 5’-

TGTTGGATAAGTTGCGGTATCC-3’. Antisense MOs (GeneTools, LLC) were designed against the splice acceptor or donor site of exon 2 for the following genes (MO sequence is listed):  *smn* splice MO, 5’-CTAAGCATCCTCTGTTACCTTGAATG-3’;

*gemin2* splice MO 5’-CTAAGCATCCTCTGTTACCTTGAATG-3’; *gemin2* splice MO2 5’-GATGCTTCCCTCCTGTTACCTTGAATG-3’; *zbp2* splice MO 5’-

TGTTGGATAAGTTGCGGTATCC-3’. Injections of MO were performed according to previous protocol (McWhorter et al., 2003; Nasevicius and Ekker, 2000; see Chapter 2). A reticule was used to measure the bolus volume injected into each embryo. Unless otherwise stated, 9ng of MO was injected into each embryo.

Synthetic capped human RFP-tagged *SMN* mRNA (Zhang et al., 2003) was produced using an mMESSAGE mMACHINE kit (Ambion) according to manufacturer’s instructions. mRNA was subcloned into MCS1 of pCS2+ (gift from Dave Turner; http://sitemaker.umich.edu/dltturner.vectors) vector between BamHI and EcoRi sites. The
plasmid was linearized with Not1 enzyme prior to mRNA synthesis. Approximately, 400-600 pg of mRNA was injected into 2-4 cell stage embryos.

**Immunohistochemistry and TOTO-3 staining**

Immunohistochemistry was performed as previously described (McWhorter et al., 2003; see Chapter 2) on either whole embryos or cryostat sectioned embryos (16 µm sections). The following mAbs were used: znp1 (1:100; Melanacon et al., 1997); SMN (1:250; MANSMA 7, 12, & 21 (courtesy of Dr. Glenn Morris); Young et al., 2000). FITC (IgG1) and/or TRITC (IgG2A) isotype-specific conjugate secondary antibodies (1:300; Southern Biotechnology Associates, Inc.) were used for fluorescent detection of znp1 (IgG2A) and Smn (IgG1) mAb. TOTO-3 (Molecular Probes, T3604) was used as a nuclear dye/stain (2.4 nM working dilution). Immunofluorescent images were analyzed using a confocal microscope using digital imaging (Leica TCS SL Confocal Microscope).

**Western blot analysis**

Western blots were performed as previously described (McWhorter et al., 2003; see Chapter 2). Gemin2 mAb (1G14) was a gift from Dr. Livio Pellizzoni. This Gemin2 mAb was used at 1:10 dilution using the same secondary antibody and ECL protocol as previously described (McWhorter et al., 2003; see Chapter 2). Western blot quantitation was performed using the luminosity histogram function in Adobe Photoshop software.

**Visualization of GFP transgenic zebrafish**

Live *gata2-GFP* transgenic zebrafish (Meng et al., 1997) were anesthetized in tricaine (Sigma-Aldrich ; A-5040) between 48-52 hpf and mounted on a coverslip. Motor nerves were analyzed and images were taken with Zeiss AxioCam MRC5 camera using AxioVision software.
**Isolation of total RNA from zebrafish embryos**

The TRIzol (Invitrogen 15596-018) protocol was utilized essentially according to manufacturer’s instructions. Embryos were rinsed 3 times in PBS and homogenized in TRIzol (1 ml per 100 embryos) and incubated at RT for 5 min. 200 µl of chloroform:isoamyl alcohol was added and agitated for 15 sec followed by 3 min incubation at RT. Samples were centrifuged for 15 min at 12,000 x G at 4°C. Aqueous RNA layer was removed and 500 µl of isopropanol was added to the aqueous layer and incubated at RT for 10 min. After a 10 min centrifugation at 4°C at 12,000 x G, the supernatant was decanted and 1 ml of 75% EtOH was added to wash the RNA pellet. The RNA pellet was dried for 7 min before 50 µl of RNase-free water was added to the pellet. 15 µl of RNA solution was aliquoted per tube and stored at -80°C.

**Reverse transcriptase polymerase chain reaction (RT-PCR)**

Qiagen OneStep RT-PCR kit (210212) was used according to manufacturer’s protocol to perform RT-PCR on zebrafish embryos. After total RNA isolation from embryos, a 25 µl RT-PCR reaction was set-up using 0.6 µM primer concentration and approximately 1 µg of isolated RNA. Other reaction components were used as instructed. Reverse transcription occurred at 50°C for 10 min; After an initial 15 min activation incubation at 95°C, the following thermal cycler conditions were repeated 35 times: (3-step cycling) 1 min denaturing at 94°C, 1 min annealing at 57°C, 1 min extension at 72°C. Final extension at 72°C occurred for 10 min. The annealing temperature for each reaction was 57°C. The primer sequences for each gene are listed: smn (forward 5’-GTGCAGAAGATGTCGTATTTTGTC-3’; reverse 5’-TAATCTGTGTTAAGACCCCCACACAGG-3’); gemin2 (forward 5’-
CCTAGAAACCCTCAGGAATATCTCA-3'; reverse 5’-
ACAACCTGTCTGACTTCTGAAAATC-3’); zbp2 (forward 5’-
ACGGACAGCATGTCTGAGTACAG-3’; reverse 5’-
GATCTGAACTTTACAACCAGAGTCC-3’), and zbp1 (forward 5’-
TTCCATACTCTGGACAGTTTCTCAT-3’; reverse 5’-
GTTCACAGTTTCTCAACAGTTCCATA-3’). RT-PCR products were run on 2% agarose gels. RT-PCR product quantitation was performed using the luminosity histogram function in Adobe Photoshop software.

**snRNP assembly assay**

In vitro transcription of U1 and U1ΔSm snRNAs was carried out from linearized template DNAs in the presence of [³²P]UTP (3000 Ci/mmol) according to standard procedures (Yong et al., 2002). Fertilized zebrafish embryos at 1, 2, and 3 dpf were quick-frozen in liquid nitrogen prior to extract preparation. Extracts for snRNP assembly were prepared as previously described with minor modifications (Pellizzoni et al., 2002). Tissues were homogenized in ice-cold reconstitution buffer (20 mM Hepes-KOH pH 7.9, 50 mM KCl, 5 mM MgCl₂, 0.2 mM EDTA, 5% glycerol) containing 0.01% NP40 using approximately a volume to mass ratio of 5 µl of buffer per milligram of tissue. Extracts were passed five times through a 25G needle and centrifuged fifteen minutes at 10,000 rpm at 4°C. Supernatants were 5-10 mg/ml as determined by the Lowry method (Biorad). To minimize potential variations in SMN activity that may result from different extract preparations, extracts from either tissues at different ages or different tissues were prepared simultaneously for each set of experiments. Extracts were not subjected to more than two cycles of freeze and thaw. Unless otherwise stated, snRNP assembly reactions
were carried out for one hour at 30°C in a volume of 20 μl containing whole extracts, 10,000 cpm of in vitro transcribed [32P]UTP-labeled snRNAs, 2.5 mM ATP, and 10 μM E. coli tRNA. Following addition of heparin and urea to a final concentration of 5 mg/ml and 2 M, respectively, assembly reactions were analyzed by electrophoresis on 6% polyacrylamide native gels at 4°C and autoradiography as previously described (Raker et al., 1999).

RESULTS
Reduction of Smn protein persists and has long term consequences
Analysis of Smn reduction in zebrafish embryos reveals motor axon pathfinding defects (McWhorter et al., 2003; Chapter 2). Since SMA is a disease which usually manifests post-natally, the long term consequences of reduced Smn levels in larval zebrafish were examined. To assay for overall survival of injected zebrafish, individual larvae (injected or WT) were placed into a separate housing tube within a larger fish tank (Figure 3.2 A). Mesh glued to the bottom of each housing tube allowed for water circulation within each tube but kept each larvae isolated. The survival of the “tubling” fish was assayed from 3-30 dpf and is shown as a Kaplan-Meier survival curve (Figure 3.2 B). The survival of smn MO injected zebrafish is significantly diminished compared to WT and ctl MO injected larvae (Figure 3.2 B). Because MOs are considered a transient knockdown (Nasevicius and Ekker, 2000), western blot analysis of Smn protein levels was performed to determine when protein levels return to normal (Figure 3.2 C).
Figure 3.2. Long term consequences of smn knockdown. Housing tubes (A) in which larval WT and smn MO injected zebrafish were kept in isolation. Kaplan-Meier survival curve (B) of WT, control MO, and smn MO injected embryos. Western blot analysis (C) showing Smn protein levels at 3 (Lanes 1 & 2), 10 (Lanes 3 & 4), 14 (Lanes 5 & 6), 17 (Lanes 7 & 8), and 21 (Lanes 9 & 10) dpf in WT (Lanes 1, 3, 5, 7, 9) and smn MO (Lanes 2, 4, 6, 8, 10) injected embryos. β-actin is shown as a loading control. Smn protein is significantly reduced until 17 dpf and is completely restored to normal at 21 dpf.
According to this analysis, Smn levels are significantly reduced thru 14 dpf and return completely to normal levels by 21 dpf (Figure 3.2C). These western data correlate with the survival data in that when Smn levels return to normal, the survival of the smn MO injected fish levels off (Figure 3.2 B). Because Smn protein return to normal after survival has leveled off, it is difficult to speculate if the motor axon defects are involved in the diminished survival. The prediction would be that early pathfinding defects would result in premature death regardless if Smn levels returned to normal. It is very possible, however, that the larvae with compromised axons died prior to Smn levels returning to normal. Therefore, due to timing issues, these experiments cannot directly asses if motor axon defects lead to premature larval death. While more analysis needs to be performed to fully understand if the zebrafish model also suffers motoneuron degeneration which is observed in patients and mouse models (Swoboda et al., 2005; Monani et al., 2000), the decreased survival of larvae with reduced Smn levels further validates our zebrafish model of SMA.

**Motor axonal defects can be classified into categories**

Previously, motor axon defects observed upon reduction of Smn were scored using a motor axonal antibody, znpl (McWhorter et al., 2003; Chapter 2). To expedite the analysis of these axonal defects, the defects are currently being scored using the *gata2-GFP* transgenic zebrafish (Meng et al., 1997). Analysis of these GFP-labeled ventrally projecting motor nerves in live embryos alleviates the necessity for antibody staining and also shows finer detail in the axon projections. While these transgenic fish were initially used to understand the time course of motor axonal defects (McWhorter et al., 2003; Chapter 2), they can also be utilized for scoring of the defects. Because only the
secondary motor axons are labeled, the axon defects are scored at 50 hpf instead of 24 hpf and 36 hpf as with antibody staining. Similar defects are observed in the GFP-labeled nerves at 50 hpf that were seen with antibody labeling at 27 and 36 hpf. However, additional defects can also be observed (Table 3.1).

<table>
<thead>
<tr>
<th>Motor Axon Defect</th>
<th>Classification of Defect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Truncated above ventral muscle</td>
<td>severe</td>
</tr>
<tr>
<td>Excessively branched and truncated</td>
<td>severe</td>
</tr>
<tr>
<td>Excessively branched but not truncated</td>
<td>moderate</td>
</tr>
<tr>
<td>Innervation of neighboring myotome</td>
<td>moderate</td>
</tr>
<tr>
<td>Ectopic ventral branching but normal overall morphology</td>
<td>mild</td>
</tr>
<tr>
<td>Ectopic ventral spinal roots</td>
<td>mild</td>
</tr>
<tr>
<td>Ectopic RoP-like branches</td>
<td>mild</td>
</tr>
<tr>
<td>Lacking stereotypic morphology but no other defects</td>
<td>mild</td>
</tr>
</tbody>
</table>

Table 3.1. Classification of motor axon defects observed. The most common motor axon defects observed in the GFP-labeled ventrally projecting motor nerves at 50 hpf in the *gata2-GFP* transgenic zebrafish are listed. The assigned classification of each defect is listed in the column to the right.

Table 3.1 lists the most common axonal defects observed in the *gata2-GFP* transgenic line. To aid in the quantification of these axonal defects, they have been classified into categories based on severity (Table 3.1). Generally speaking any defect which results in the nerves not extending into the ventral-most muscle is considered a severe defect. Moderate defects are those defects in which the nerves extend into the ventral muscle but have other strikingly aberrant pathfinding defects, such as innervating the neighboring myotome. Finally, mild defects are ones in which the nerves have reached the ventral muscle with minor perturbations in nerve morphology.

Due to the complex and variable nature of the *smn* MO phenotype, we have attempted to classify the injected embryos into groups based on the prevalence and type of pathfinding defect (Table 3.2).
<table>
<thead>
<tr>
<th>Overall Fish Classification</th>
<th>Motor Axon Phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe</td>
<td>Fish with greater than 3 severe defects</td>
</tr>
<tr>
<td></td>
<td>Fish with greater than 7 moderate defects</td>
</tr>
<tr>
<td>Moderate</td>
<td>Fish with 2-3 severe defects</td>
</tr>
<tr>
<td></td>
<td>Fish with 4-7 moderate defects</td>
</tr>
<tr>
<td></td>
<td>Fish with greater than 7 mild defects</td>
</tr>
<tr>
<td>Mild</td>
<td>Fish with 1 severe defect</td>
</tr>
<tr>
<td></td>
<td>Fish with 1-3 moderate defects</td>
</tr>
<tr>
<td></td>
<td>Fish with 3-7 mild defects</td>
</tr>
<tr>
<td>No Defects</td>
<td>Fish with less than 3 mild defects</td>
</tr>
</tbody>
</table>

**Table 3.2. Overall fish classifications based on motor axon phenotypes.** Motor axon phenotypes are counted per embryo, and 20 GFP-labeled nerves are scored per embryo (10 per side). Depending on prevalence and type of motor axon defects observed, the entire embryo can be classified as severe, moderate, mild, or no defects with respect to motoneuron outgrowth.

The overall percentage of injected embryos that fall into these separate categories, severe, moderate, mild, or no defect, can be quantified and compared to other morpholinos. An example of each category of severity is shown (Figure 3.3 A-D). Ten nerves from each side of the embryo are scored (20 total per embryo) and used to classify the embryo.

When 9 ng of smn MO is injected into gata2-GFP embryos and this classification scheme is utilized, 22% of the embryos are severe; 32% are moderate; 22% are mild; and 24% have no defects. Due to the inherent variability of the axon phenotype and the potential mosaicism of the smn MO, western blot analysis was performed to insure that variance in protein levels was not the cause of this variability in phenotype (Figure 3.3 E). Smn protein levels in embryos categorized as severe, moderate, mild, or no defects have equivalent reduction in protein (Figure 3.3 E) suggesting that the overall levels of protein are not influencing the nerve phenotypes.
Figure 3.3. Motor axon defects classified into categories. Lateral views of the medial pathway in WT (A) and smn MO (B, C, D) injected gata2-GFP embryos. Based on criteria listed in Tables 3.1 and 3.2, examples of severe (B), moderate (C), and mild (D) embryos are shown. Western blot (E) analysis of Smn and β-actin protein levels in WT (Lane 1), no defects (Lane 2), mild (Lane 3), moderate (Lane 4), and severe (Lane 5) smn MO (9 ng) injected gata2-GFP embryos.
Identification, characterization, and knockdown of *gemin2* gene in zebrafish

Since Smn is a ubiquitously expressed protein, much research in the SMA field centers around why reduction in the protein causes motoneuron-specific defects. One well-characterized function of SMN is mediating stringency over snRNP assembly (for review see Chapter 1; Pellizzoni et al., 2002). To understand if the function of Smn in snRNP assembly is important for motor axon pathfinding in zebrafish, another component of the snRNP assembly complex, Gemin2, was knocked-down. The Gemin2 protein has been shown to be important in mediating snRNP assembly in cultured cells, and its reduction results in reduced snRNP assembly (Feng et al., 2005). Therefore by knocking-down *gemin2*, snRNP assembly should be reduced-independent of Smn. As a *gemin2* homolog had not been identified in zebrafish, 2 separate strategies were undertaken. Human and mouse protein sequences (from GenBank and EMBL, see methods for accession numbers) were used to BLAST the Welcome Trust Sanger Institute’s Ensembl Zebrafish Sequencing website (http://www.ensembl.org/Danio_rerio/index.html). Additionally, the orthologue predictions listed for the human and mouse Gemin2 proteins were utilized from Sanger’s website. In this case, both the BLAST results and the orthologue predictions identified the same gene as zebrafish *gemin2*. Fortunately, the sequence for the *gemin2* gene in zebrafish had been annotated, and sequence information for the 5’ UTR and exon/intron boundaries was available from the Ensembl website. Using the ClustalW program, a protein sequence alignment for human, mouse, and zebrafish was produced (Figure 3.4). The zebrafish Gemin2 protein is approximately 68% identical to its human and mouse counterparts and approximately 82% similar (Table 3.3).
Because a cross-reacting Gemin2 antibody was unavailable until recently, splice site MOs were designed against *gemin2*. Splice site MOs are designed against either the splice acceptor or donor sites of a particular exon in the RNA of interest (Draper et al., 2001). During the splicing of the RNA, blockage of the acceptor/donor site can result in
exon excision or intron inclusion in the mRNA transcript (Draper et al., 2001). RT-PCR of the mRNA can detect these insertions or deletions in the transcript alleviating the need for an antibody to determine efficacy (Draper et al., 2001). Two splice site MOs were designed against exon 2 of *gemin2* transcript, one against the splice donor site (*gemin2* splice MO) and one against the splice acceptor site (*gemin2* splice MO2). As a positive control, a splice donor site MO was also designed against exon 2 of the *smn* transcript. The survival rate for embryos injected with 9 ng of *gemin2* splice MO (n=233) was 58.0%; 9 ng of *gemin2* splice MO2 (n=167) was 47.3%; 9 ng of *smn* splice MO (n=100) was 78%.

After injection of 9 ng of the specified MO into WT embryos, total RNA was extracted from embryos at 30 hpf. RT-PCR with gene-specific primers was performed to determine efficacy of each splice MO; RT-PCR products are shown after agarose gel electrophoresis (Figure 3.5 A-C).
Using 2 different primer sets to amplify exons 1-4 of the zebrafish *smn* gene, RT-PCR shows that 100% of the 72 bp exon 2 has been excised from the transcript (Figure 3.5 A; Lanes 1-4). The exon 2 excision, 72 bp, removes 24 amino acids of the Smn protein, but the remainder of the transcript and protein are in-frame after the excision. Using 2 different primer sets to amplify exons 1-3 or 1-4 of the *gemin2* gene, RT-PCR shows that the 80 bp intron 2 was inserted into the transcript of the *gemin2* splice MO injected embryos (Figure 3.5 B; Lanes 5-8) while the 80 bp exon 2 was excised from the transcript of *gemin2* splice MO2 injected embryos (Figure 3.5 C; Lanes 9-12). The 80 bp
exon deletion or 80 bp intron insertion causes a frameshift in the resulting protein making it a theoretically non-functional protein. Sequencing confirmed each excision or insertion resulting from these splice MOs. As evident by the RT-PCR results, only 45% of the *gemin2* transcript has the 80 bp inclusion while only 43% of the *gemin2* transcripts had the excision. Doses higher than 9 ng result in greatly diminished survival of the embryos suggesting a secondary mistargeting defect of these splice MOs. These splice MOs are either highly inefficient or maternal RNA (unaffected by splice MOs because it has already been processed) remains in the 30 hpf embryo. RT-PCR of RNA extracted from 50 hpf embryos injected with *gemin2* splice MO still shows WT transcript present at high levels (data not shown) further suggesting possible inefficiency of the MO. Although RT-PCR results show that over 55% of the *gemin2* transcripts in the MO injected embryos are WT, there is still some decrease in *gemin2* function. Therefore, *gata2-GFP* embryos injected with either *gemin2* splice MO or *gemin2* splice MO2 were scored for potential motor axon defects (Figure 3.5 D). These *gemin2* splice MOs were compared to embryos injected with control MO and *smn* splice MO (Figure 3.5 D), and neither *gemin2* splice MO yielded axon defects significant from control MO. The caveat, of course, is that at least 55% of the transcript is correctly spliced. Motor axon defects could not be evident until gene/protein function is perturbed above 50%.

Due to the presence of a recently obtained Gemin2 antibody that cross-reacts in zebrafish, the 5’ UTR sequence of zebrafish *gemin2* was utilized to design 2 non-overlapping translation blocking MOs. *Gata2-GFP* embryos were injected with 9 ng of *gemin2* MO and *gemin2* MO2. The survival rate for embryos injected with *gemin2* MO (n=149) was 47.0%, and over half of the embryos that survived had a C-shaped trunk,
indicative of a potential mistargeting of the MO because other *gemin2* MOs did not have this characteristic C-shape. The embryos with the C-shaped tails were not included in the analysis of motor axon defects. It has previously been shown that if body patterning is affected in the developing embryo, motor axons will pathfind aberrantly (Gray et al., 2003; van Eeden et al., 1996). Therefore, any axon defects observed in the C-shaped embryos could not be attributable directly to Gemin2 reduction. The survival rate for 9 ng of *gemin2* MO2 injected embryos (n=165) was 48.5% and of those embryos which survived approximately 40% were morphologically normal. Those which were morphologically abnormal had shortened trunk and tail regions. These survival rates are comparable with those seen in the embryos injected with 9 ng of *smn* MO (McWhorter et al., 2003; Chapter 2) suggesting that *gemin2* is also an essential gene in zebrafish as it is in other species (Jablonka et al., 2002). Since *smn* and *gemin2* are believed to be essential genes and function in snRNP assembly, this further suggests that snRNP assembly is also an essential function in cells (Schrank et al., 1997; Jablonka et al., 2002). A cross-reacting antibody has been obtained (a gracious gift from Dr. Livio Pellizzoni), and this antibody recognizes the approximately 32 kD Gemin2 protein in zebrafish (Figure 3.6 A).
Western blot analysis of morphologically normal embryos shows that, when corrected for total protein indicated by the β-actin control, there is a 30% reduction in Gemin2 protein upon injection of 9 ng of *gemin2* MO and 49% reduction of protein upon injection of 9 ng of *gemin2* MO2 (Figure 3.6 A). While these doses of MO do not reduce Gemin2 protein levels to what is observed when Smn is knocked-down, the MOs do cause a reduction in protein. Injection of higher doses of MO can result in further reduction, although higher doses will further compromise survival and morphology of injected embryos. Upon showing this moderate reduction in Gemin2 protein, 9 ng of each MO was injected into *gata2-GFP* embryos and motor axons were scored at 50 hpf (Figure 3.6 B). *smn* MO injected embryos scored for axon defects are also shown for comparison; compared to control (ctl) MO, neither *gemin2* MO nor *gemin2* MO2 showed significant motor axonal defects (Figure 3.6 B).

**Figure 3.6. Analysis of *gemin2* translational MOs.** Western blot (A) analysis of Gemin2 and β-actin protein levels in 50 hpf WT (1), 6 ng *gemin2* MO2 (2), 9 ng *gemin2* MO2 (3), and 9 ng *gemin2* MO (4) injected embryos. Motor axonal defect quantification (B) for 50 hpf *gata2-GFP* embryos injected with control (n=49), *smn* (n=55), *gemin2* (n=16), or *gemin2* (n=66) MO2. Error bars represent standard error. Mann-Whitney analysis shows that *gemin2* MO (p=0.668) and *gemin2* MO2 (p=0.283) injected embryos are not statistically significant compared to control MO injected embryos. When compared to control MO, *smn* MO injected embryos are significantly different (p<0.001).
To further reduce Gemin2 protein levels in the developing zebrafish embryo, *gata2-GFP* embryos were injected with 12 ng of *gemin2* MO2. The survival of these embryos injected with this higher dose of MO was, as would be expected, considerably lower at 38.0% (n=84). When this dose of MO was injected into WT embryos, 75% of these embryos are morphologically abnormal and developmentally delayed while only 25% have normal body morphology (Figure 3.7 A & B) at 50 hpf.

**Figure 3.7.** Morphological abnormalities of *gemin2* MO2 injected embryos. Lateral views of live morphologically normal (A) and abnormal (B) embryos at 50 hpf injected with 12 ng of *gemin2* MO2. Motor axonal defect quantification (C) for 50 hpf *gata2-GFP* embryos injected with control (n=49), *smn* (n=55), and *gemin2* MO with normal body morphology (n=66) or abnormal body morphology (n=40). Error bars represent standard error. Mann-Whitney analysis shows that *gemin2* MO2 injected embryos which are morphologically normal (p=0.642) are not statistically significant while *gemin2* MO2 injected embryos which are morphologically abnormal (p<0.001) are statistically significant when each is compared to control MO. Western blot (D) analysis of protein levels in 50 hpf WT (1), *gemin2* MO2 morphologically normal (2) and morphologically abnormal (3) injected embryos.
Morphologically abnormal embryos may have secondary motor axon defects (Gray et al., 2003; van Eeden et al., 1996), so to further address whether Gemin2 knockdown yields a primary defect in motor axon outgrowth, motor axon defects were assayed for in both morphologically normal and abnormal embryos. *gemin2* MO2 (12 ng) was injected into *gata2-GFP* embryos, and motor axons were scored (Figure 3.7 C). *smn* MO injected embryos were scored for axon defects are also shown for comparison (Figure 3.7 C). As shown, morphologically normal *gemin2* MO2 injected embryos do not have significantly increased motor axon defects when compared to control whereas morphologically abnormal *gemin2* MO injected embryos do. Importantly, western blot analysis (Figure 3.7 D) of morphologically normal and morphologically abnormal embryos injected with *gemin2* MO2 show that these embryos have the same degree of knockdown (57% for normal morphology and 60% for abnormal morphology) independent of their body morphology. Therefore, the only difference in these injected embryos is their overall trunk morphology suggesting that the motor axon defects that are observed may simply be secondary deficits of abnormal morphology not direct effects on motor axon guidance. These data indicate that when trunk morphology is taken into account, Gemin2 knockdown does not yield axon pathfinding defects suggesting that Gemin2, unlike Smn, does not have a specific role in axon outgrowth.

**snRNP assembly regulation during embryonic zebrafish development**

One of the target goals is to understand if snRNP assembly is important for the motor axon defects observed when Smn levels are reduced. Therefore, we have begun developing an assay, in collaboration with Dr. Livio Pellizzoni, that can quantify the potential for snRNP assembly in zebrafish embryos. Western blot analysis of increasing
concentrations of zebrafish extracts at 1, 2, and 3 dpf were probed with tubulin for a loading control, Smn for normalization, and PCNA to indicate cell proliferation (Figure 3.8 A).

As expected, all 3 proteins show an incremental increase as extract concentration increases; an increase in protein is also observed as well as the embryo continues to develop from 1-3 dpf. To test for snRNP assembly in zebrafish embryos, extracts were incubated with radioactive U1 or U1ΔSm (Sm binding site is mutated) snRNAs and ATP. snRNP assembly was analyzed by gel shift (Figure 3.8 B). Using the supershifted Sm core as an assay readout, very little snRNP assembly is occurring in 1 and 2 dpf embryos while at 3 dpf there is a considerable increase in snRNP assembly. Therefore, during the time periods when motor axon outgrowth defects are observed resulting from Smn.

Figure 3.8. snRNP assembly in embryonic zebrafish. Zebrafish extracts were prepared from 1, 2, and 3 dpf embryos. (A) Increasing amounts of extracts (10 µg, 20 µg and 30 µg of proteins) were analyzed by Western blot with anti-SMN, anti-PCNA and anti-tubulin antibodies as indicated. (B) snRNP assembly reactions with radioactive U1 snRNA were carried out using three increasing concentrations of each extract (10 mg, 20 mg and 30 mg of proteins) and were analyzed by electrophoresis on native gels. The position of U1 RNP complex containing the Sm core is indicated.
reduction, little snRNP assembly is occurring in WT embryos. While correlative, these data do suggest that the snRNP assembly function of Smn may not be involved in the axon pathfinding. More importantly, however, is that a snRNP assembly assay as been designed which can directly test the functional consequences of Gemin2 reduction.

**Identification and characterization of zbp2 gene in zebrafish**

Our data indicate that the snRNP assembly function of Smn may not play a role in axon pathfinding. Recently, we have identified a possible motor axonal-specific function for Smn through a collaborator, Dr. Gary Bassell. Bassell and colleagues have found that SMN interacts directly with Zbp2 and speculate that when interacting with Zbp2, SMN may be involved in mRNA (or RNP) transport down the axon. To investigate the function of this interaction, the zbp2 homolog in zebrafish was identified in the same manner as the gemin2 homolog described earlier with 2 exceptions: chick sequence data was also used to BLAST the zebrafish sequence database (Gu et al., 2002); and Genbank sequence (BC064649) regarding a cDNA sequence of the zbp2 homolog in zebrafish was not used. ClustalW protein alignment shows good homology among the 4 species especially in the RNA binding domains (zebrafish AA 184-258; 292-364; 433-465) (Figure 3.9); the zebrafish homolog of zbp2 is about 50% identical and 54% similar to the other species (Table 3.3).
Figure 3.9. Protein sequence alignment of Zbp2 homologs. Alignment of human, mouse, chick, and zebrafish Zbp2 proteins using the ClustalW software.
To determine if knockdown of Zbp2 resulted in similar pathfinding defects as knockdown of Smn, two MOs were designed against the zebrafish *zbp2*, 1 translational MO (*zbp2* MO) and 1 splice MO (*zbp2* splice MO) against the donor site of exon 2. The injection of either MO into WT embryos was lethal. The survival rate for 9 ng of *zbp2* MO was 3.9% (n=230); a 6 ng dose moderately increased survival to 5% (n=240); and a 3 ng dose (n=50) still resulted in only 10% survival. Those embryos that did survive were morphologically aberrant and unscorable. Due to the inability to find a cross-reacting antibody for Zbp2 in zebrafish, the efficacy of this MO was never determined.

A *zbp2* splice MO was obtained because a cross-reacting Zbp2 antibody was unavailable. Unfortunately, even when injecting as little as 3 ng of *zbp2* splice MO, survival of the embryos was virtually 0% (n=320) at 26 hpf. Since the embryos did not survive until 30 hpf, RNA was extracted to determine MO efficacy by RT-PCR at 4 and 6 hpf (Figure 3.10 A; Lanes 1-3). The goal was to simply determine if the MO was causing an aberrant splice product. Previous research has indicated that zygotic transcription can begin to occur between 2.75-3 hpf (Wagner et al., 2004).
Zbp2 splice MO injected embryos begin to degrade by 7-8 hpf, so 4-6 hpf analysis was chosen. As shown in Figure 3.9 A, there is no inclusion or excision in the zbp2 transcript at 4 or 6 hpf suggesting that the MO is not causing aberrant splice products. To show that a splice MO can cause aberrant splice products at these early time points, RNA was extracted from embryos injected with 9 ng of smn splice MO at 6 and 30 hpf (Figure 3.10 B; Lanes 4-5, respectively). A faint splice MO product can be seen at 6 hpf suggesting that zygotic transcription is occurring, and MOs can cause aberrant splice products at these early developmental stages. While it is possible that the MO begins to splice zygotic zbp2 RNA past 6 hpf, it is likely that this splice MO may be mistargeting and causing the high lethality. If the MO had been shown to be effective, single motoneuron injections could have been performed as was previously done with the smn MO (McWhorter et al., 2003: Chapter 2).
Identification, characterization, and knockdown of *zbp1* gene in zebrafish

While no direct interaction has been shown for SMN and Zbp1 (G. Bassell, personal communication), we have speculated that Smn may be involved in mRNA transport down the axon. Additional evidence for this potential transport mechanism has also been shown in cultured neurons (Rossoll et al., 2003). To understand the pathfinding consequences of aberrant transport down the axon, independent of Smn, zebrafish Zbp1 will be knocked-down. The *zbp1* homolog in zebrafish was identified as previously described for *zbp2*. Protein alignments for the homologs in human, mouse, chick, and zebrafish were performed in ClustalW (Figure 3.11); these proteins are highly homologous with approximately 77% identity and 86% similarity (Table 3.3).
Figure 3.11. Protein sequence alignment of Zbp1 homologs. Alignment of human, mouse, chick, and zebrafish Zbp1 proteins using the ClustalW software.
Once sequence was obtained for zebrafish zbp1, a splice MO was designed against the splice donor site of exon 2. When 9 ng of zbp1 splice MO was injected into WT embryos, survival was 58.4% (n=190). RT-PCR of RNA extracted from 30 hpf embryos injected with 9 ng of zbp1 splice MO shows that 100% of the transcript lacks the 61 bp exon 2 (Figure 3.12 A).

**Figure 3.12. Analysis of zbp1 splice MO.** RT-PCR products of exons 1-4 of zebrafish zbp1 mRNA (A) in WT (1) and zbp1 splice MO (2) injected embryos. The WT product is 255 bp while the splice MO product is 194 bp, indicating and excision of exon 2 which is 61 bp. Motor axonal defect quantification (B) for 50 hpf gata2-GFP embryos injected with control (n=49), smn splice (n=90), or zbp1 splice (n=46) MO (9 ng). Error bars represent standard error. Mann-Whitney analysis shows that smn splice MO and zbp1 splice MO (p=0.994) injected embryos are not statistically significant from each other. When compared to control MO, smn splice MO and zbp1 splice MO injected embryos are significantly different (p<0.001).

This 61 bp deletion results in a frameshifted and theoretically nonfunctional protein. Sequencing confirmed this exon 2 excision. When 9 ng of zbp1 splice MO was injected into gata2-GFP embryos, motor axon defects were observed at 50 hpf (Figure 3.12 B). While severe defects such as truncations were observed, the majority of the axons looked unhealthy and had abnormal morphology. The defects observed upon Zbp1 reduction are significantly different than control MO but not significantly different than smn splice
MO, suggesting that transport of mRNAs into the axon does affect axonal outgrowth, similar to Smn.

**Smn protein localizes to the motor axon**

SMN was originally localized to nuclear structures and cytoplasm of fibroblasts and other cultured cells (Liu and Dreyfuss, 1996). SMN has also recently been shown to localize to motor axons and growth cones of cultured neurons (Jablonka et al., 2001; Fan and Simard, 2002; Zhang et al., 2003). Due to the possibility that the localization is a culture artifact, the localization of Smn within an organism is preferred. To observe possible Smn localization within motor axons, *hSMN* (human) tagged with RFP was utilized. This approach was chosen over Smn antibody staining initially because of the enhanced fluorescent signal compared to antibody labeling. 400-600 pg of *hSMN-RFP* mRNA was injected into WT embryos which were then fixed at 2 dpf and antibody stained with a motor axonal marker, znp1. The medial pathway of the ventrally projecting motor nerves (Figure 3.14 A) was analyzed for co-localization with *hSMN-RFP* (Figure 3.13 B). hSMN-RFP was observed localizing in motor axons especially at intermediate targets such as the ventral edge of the notochord (arrow). Several caveats exist in this analysis, however; first, hSMN was considerably overexpressed in order to visualize it within the motor axons. It is possible, therefore, that the localization observed may be an artifact of the overexpression. Additionally, the RFP tag on the hSMN protein may impair the function of the protein (TL Carrel, personal communication). In this case, hSMN-RFP may be ectopically localizing to the axons, when it normally would not. Therefore, studies are currently ongoing to localize endogenous Smn to motor axons using antibodies against Smn.
DISCUSSION

To aid in analysis of the pathology and etiology of SMA, we have further characterized *smn* gene function during long term analysis of our zebrafish model; these data show that our fish model can accurately simulate the reduced life expectancy of mouse models and SMA patients, further validating the model. To begin to address the functional aspect of Smn which is responsible for motor axonal outgrowth, an additional component of the snRNP pathway, Gemin2, was knocked-down; when overall body morphology is considered, reduction of Gemin2 does not result in motor axon pathfinding defects. In this scenario snRNP assembly would be perturbed independent of Smn. Further, overall

Figure 3.13. hSMN-RFP localizes to zebrafish motor axons. Schematic of hemisegment in a 2 dpf zebrafish embryo (A) is shown for reference. Lateral view of the medial pathway of confocal imaged 48 hpf WT zebrafish embryo (B) stained with znpl motor axonal Ab (green) and injected with *hsMN*-RFP mRNA. Arrow denotes example of SMN co-localization with motor axonal marker. The localization is within the region designated as the ventral edge of the notochord which is a presumptive intermediate target for motor axons. Scale bar: 4 µm.
snRNP assembly in WT embryos is almost undetectable during motor axon pathfinding. Finally, in vivo analysis of Smn shows localization to both nuclear structures and motor axons.

**snRNP assembly and motor axon outgrowth**

Two distinct ideas regarding the function of Smn that is responsible for SMA pathology exist: a motoneuron-specific sensitivity to reduction in snRNP assembly or a novel motor axon-specific role for Smn, possibly in transport of mRNAs (Gubitz et al., 2004; Rossoll et al., 2003; G. Bassell, personal communication). Because motoneurons may be uniquely sensitive to diminished snRNP, we have attempted to address the role of snRNP assembly in motor axon pathfinding by perturbing snRNP function independent of Smn by knocking down Gemin2. Gemin2 has previously been shown to be important during snRNP assembly and, when knocked-down by RNAi in cultured cells, results in greatly diminished snRNP assembly (Feng et al., 2005). Therefore, reduction of Gemin2 in zebrafish should also affect snRNP assembly. Our data using 4 different non-overlapping MOs, 2 translation and 2 splice blocking, suggest that Gemin2 knockdown does not yield axon pathfinding defects (Figures 3.5 & 3.7). Additional analysis of Gemin2 reduction by *gemin2* MO2 (Figure 3.6) shows that only morphologically abnormal embryos exhibit motor axon defects even though protein levels are similarly knocked-down in morphologically normal embryos. The snRNP assembly assay that is being developed will also be useful in determining the degree of reduction in assembly activity when components like Gemin2 are knocked-down. Development of this snRNP assembly assay has also shown that overall snRNP assembly is quite low in the developing WT embryo up to 3 dpf (Figure 3.8B). Since snRNP assembly is so low during the
developmental time points when motor axon defects are observed, it is difficult to understand how this biological process is involved in motor axon pathfinding. While correlative, these data showing low levels of snRNP assembly during stages of axon guidance further suggest that the snRNP assembly function of Smn may not play a role in axonal outgrowth.

Recent data from another lab implicates a role for snRNP assembly in axon pathfinding (Winkler et al., 2005). Winkler and colleagues observe that 79.2% of zebrafish embryos injected with their gemin2 MO have axonal defects; however 57.1% of those embryos have malformations of the trunk/tail region (Winkler et al., 2005). If the 57.1% of embryos with trunk malformation are disregarded, that leaves only 22.1% of the embryos with axon defects, which is hardly significant considering that 21.4% of their control MO injected embryos have axonal defects. While it is certainly possible that when they reduce Gemin2 protein by 91% there are axonal defects, one cannot make the claim that the Gemin2 reduction is solely responsible. Gemin2 is also believed to be an essential gene (Jablonka et al., 2002), so if protein is reduced by 91% one might expect that cells within the embryo are not functioning properly hence leading to secondary deficits not directly related to motor axon guidance. It is paramount that overall patterning of embryos be taken into consideration during motor axon analysis. The evidence documented here shows that when Gemin2 protein levels are reduced below 50%, the only embryos which have motor axon defects are morphologically abnormal. Therefore, unless otherwise stated, no embryos with trunk malformations were analyzed in the experiments presented in Chapters 2 and 3.
Morpholino mistargeting

While MOs have been an invaluable tool for zebrafish researchers, they can also be problematic if the data is not interpreted properly. Since Spring of 2000 when MO efficacy was first published in zebrafish, over 300 papers have been published utilizing them according to PubMed. Morpholinos can be variable in their penetrance and overall severity of phenotype (Heasman, 2002). Therefore, this variability must be considered when interpreting the data. One of the biggest problems with MOs is the non-specific defects that can be observed especially when the effective dose (dose needed to exhibit the phenotype) is close to the lethal dose (Heasman, 2002). While the details regarding non-specific MO side effects have not been fully classified, they are generally caused by unexpected complementarity of the MO to other genes unknown to the researcher (Heasman, 2002). These non-specific defects can range from delayed development to anterior-posterior patterning defects to neuronal cell death (Nasevicius and Ekker, 2000; Heasman, 2002). The zebrafish community is now coming to a consensus that 2 separate non-overlapping MOs should be utilized to identify a common phenotype (Heasman, 2002). Overall MOs are a great tool for zebrafish researchers, but the data require careful interpretation. While it is unclear whether the 4 gemin2 MOs are causing non-specific defects or a true defect from reduction of gene function, conservative analysis means disregarding these malformed embryos so as to not portray axon defects as a primary deficit when they simply may be a secondary deficit. The trunk malformations that occur when Gemin2 is knocked-down greater than 50% may result from compromised and/or dying cells in the trunk. Interestingly, the gemin2 MO2 presented here and the gemin2 MO presented by Winkler and colleagues have virtually identical sequences. Therefore,
the only difference in our analyses is the amount of Gemin2 reduction and resulting morphology of the scored embryos. Further experiments with this *gemin2* MO2, such as single cell motoneuron injections, can further separate motor axon-specific defects from non-specific secondary defects on motor axon pathfinding.

**A potential motor axon-specific function for Smn**

Smn may have an additional motor axon-specific function that could be important during axonal outgrowth. Several recent experiments have suggested that SMN specifically may be involved in transport of mRNAs down the axon and into the growth cone (Rossoll et al., 2003; G. Bassell, personal communication). While MOs designed against one candidate interacting protein, Zbp2, resulted in almost 100% lethality, this interaction may be important, but due to obvious MO issues, this potential function of Smn could not be addressed here. Analysis of *zbp1* splice MO injected embryos suggests that proteins involved in mRNA transport into axons are involved in axon outgrowth. These data correlate well with published studies suggesting that blockage of the zipcode sequence causes reduced neurite outgrowth in cultured neurons (Zhang et al., 2001). Although there is no evidence for a direct interaction between Zbp1 and Smn, similar axon outgrowth defects are observed when both proteins are reduced; this could suggest similar functions in mRNA transport; however, no conclusive evidence exists to support this claim. If Smn does have a motor axon-specific role, Smn localization experiments (Figure 3.14) show that Smn would be in the proper location to be involved in an axon-specific function.
Conclusions

To dissect out the function of Smn that is important for motor axon outgrowth, analysis of other non-Smn components of these pathways has begun. To understand the role of snRNP assembly in axon outgrowth, an assembly assay has been developed in collaboration with Dr. Livio Pellizzoni that will allow snRNP activity to be directly measured. Using this assay, comparisons can be made between reduction in assembly and motor axon pathfinding defects. Additionally, this assay can alleviate the necessity for antibodies against candidate genes because it directly assesses function of the snRNP pathway. Now that the assay has been developed, it will facilitate studying other snRNP and/or axonal components which may interact with Smn.

The data presented here suggest that snRNP assembly is not involved directly in motor axon outgrowth. Additionally, snRNP assembly assays must show that this reduction in gemin2 does actually perturb assembly activity. Other MOs designed against essential snRNP assembly components, such as PHAX, Sm proteins, or other Gemins, can also be utilized if Gemin2 reduction does not yield decreased snRNP assembly. Due to MO lethality issues, the consequences of a potential Zbp2 and Smn interaction could not be addressed here. Other ways of teasing out this possible interaction exist, however. For example, if Zbp2 and/or specific mRNAs can be localized to axons/growth cones in the presence of Smn, but not in its absence, this would suggest a direct relationship. Additionally, as our collaborator Dr. Gary Bassell identifies new SMN interacting proteins, we can begin to address their functions with respect to SMN and motor axon pathfinding. The story pertaining to the function of Smn that is important during axon pathfinding and ultimately the etiology of SMA is far from clear;
hopefully the experiments described here begin to elucidate a potential mechanism for Smn function during motor axonal outgrowth.
CHAPTER 4

REVERSE GENETIC SCREENS TO IDENTIFY A MUTATION IN THE ZEBRAFISH smn GENE

ABSTRACT

The zebrafish has classically been used as a forward genetic model system. Recently, its potential as a reverse genetic model system has begun to be utilized. Because targeted mutagenesis is unavailable in zebrafish, rapid high-throughput screens for mutations in a particular gene of interest are necessary to further study gene function. We have developed two screening methods to identify ENU-induced point mutations and γ-induced deletion mutations in the survival motor neuron (smn) gene, the disease-causing agent of spinal muscular atrophy (SMA). Denaturing high performance liquid chromatography (dHPLC) has successfully been used to identify an intronic mutation and single nucleotide polymorphisms (SNPs) in the zebrafish smn gene. Additionally, multiplex quantitative PCR using fluorescently labeled primers has also been developed to identify a deletion mutation in the smn gene. TILLING has identified a mutation in the coding region of smn which may alter its function. A mutation in the smn gene will allow

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3 Zebrafish fin clips, DNA preparations, housing and maintenance was performed by ML McWhorter; ENU mutagenesis was done in collaboration with Dr. Paul Henion (The Ohio State University, Department of Neuroscience); dHPLC screening was performed in collaboration with Dr. Thomas Prior (The Ohio State University, Department of Pathology); TILLING was performed exclusively by Dr. Cecilia Moens (Howard Hughes Medical Institute, Fred Hutchinson Cancer Research Institute).
for development of a zebrafish model of SMA and further elucidation of the neuropathology and etiology of the disease.

INTRODUCTION

During the early 1990’s, zebrafish began to be recognized as an informative and valuable vertebrate model system (Grumwald and Eisen, 2002). In 1996, when the two “Big Screens” were published in an entire issue of Development, many researchers began to realize the amazing classical genetic approach that could be undertaken with this vertebrate (Grunwald and Eisen, 2002). Therefore, zebrafish has traditionally been recognized as a forward genetic model system, in which a phenotype based approach is used (Lekven et al., 2000). Analysis of various zebrafish mutants have allowed for a great understanding of the developmental processes that occur not only in zebrafish, but other higher vertebrates as well (Dodd et al., 2000). Because the embryology of the zebrafish is so well understood, many researchers have also begun to use zebrafish as a reverse genetic model system to study their gene of interest (Lekven et al., 2000). To aid in their endeavors, scientists have begun to develop a significant arsenal of genetic tools.

To characterize the function of various genes and promoter elements, techniques such as overexpression of genes, transgenesis of GFP-driven promoters, and/or inducible gene expression or reporter genes have all be modified to be used in zebrafish (Meng et al., 1997; Scheer and Campos-Ortega, 1999; Long et al., 1997).

One significant drawback to a reverse genetic approach in zebrafish is the lack of ability to perform targeted mutagenesis, such as homologous recombination (Lekven et al., 2000). While gain-of-function studies, by overexpression, are amenable in zebrafish,
loss-of-function analysis has not been developed (Grunwald and Eisen, 2002). Recent molecular techniques such as antisense morpholinos have been used to study loss-of-function phenotypes during development, but numerous drawbacks such as mosaicism and mistargeting make these techniques less than ideal (Nasevicius and Ekker, 2000). As the completion of the zebrafish sequencing project grows near, and as more researchers become interested in studying the function of their genes in this model system, techniques that allow for rapid screening of randomly mutagenized zebrafish are essential (Lekven et al., 2000). Since targeted mutagenesis is currently unavailable, the zebrafish community has begun to research different rapid screening methods and different mutagenic agents (Weinholds et al., 2002; Weinholds et al., 2003; Amsterdam et al., 1999; Draper et al., 2004).

Several ways exist to mutagenize zebrafish. While ethylmethane sulfonate (EMS) is the chemical mutagen of choice in Drosophila and C. elegans, EMS in not an effective mutagen in zebrafish (Mullins et al., 1994; Westerfield, 1995; Knapik, 2000). Instead, 1-ethyl-1-nitrosourea (ENU) is used to induce small intragenic lesions across the entire genome (Knapik, 2000; Westerfield, 1995; Shibuya and Morimoto, 1993). ENU is an alkylating agent meaning that the ethyl group can be transferred to guanine and thymine (Shibuya and Morimoto, 1993) causing miscoding of the DNA which results predominantly in single base pair changes. Mutagenic parameters such as ENU concentration, time and number of exposures, and water pH are essential for maximum mutagenesis and minimum toxicity and mortality (Knapik, 2000; Driever et al., 1996). While many of the ENU-induced mutations can lead to null alleles, the majority of mutations will result in hypomorphic alleles of the gene (Knapik, 2000; Weinholds et al.,
In order to induce mutations, adult zebrafish males are soaked in an ENU solution which allows premeiotic spermatogonia to be mutagenized (Driever et al., 1996; Mullins et al., 1994). After initial matings to use up mosaic mutagenized sperm, the F₁ generation is propagated with WT females to give rise to non-mosaic mutant offspring (Knapik, 2000). Additional protocols have been created that increase the induction of mutations within zebrafish, but these protocols involve mutagenesis of post-mitotic spermatogonia where mutagenized males are immediately crossed with WT females (Riley and Grunwald, 1995). It is believed that because these F₁ offspring will be mosaic for a particular mutation but will be able to tolerate larger numbers of mutations that non-mosaic heterozygous F₁ offspring (Riley and Grunwald, 1995).

In order to increase the chances of finding a null allele, researchers can perform a different type of mutagenesis; γ-ray mutagenesis causes large, often multigenic, lesions such as deletions and translocations (Fritz et al., 1996; Westerfield, 1995). γ-irradiated mutations are often more difficult to isolate and maintain due to the complex nature of the lesion, but these large lesions are more likely to be null alleles (Knapik, 2000; Fritz et al., 1996). In order to induce deletions and translocations, in vitro collected sperm is γ-irradiated and then used to in vitro fertilize WT eggs (Westerfield, 1995). The resulting F₁ generation should be heterozygous for the lesion. Zebrafish eggs and blastulae can also be irradiated, but damage to large amounts of non-chromosomal material in the eggs and embryos results in increased death (Westerfield, 1995; Chakrabarti et al., 1983; Walker et al., 1983; Walker et al., 1999). A previous multiplex PCR screen was developed to identify γ-induced lesions in zebrafish, but the protocol relied only on haploid embryos from adult F₁ females (Fritz et al., 1996). To identify deletion allele
carriers in humans, multiplex quantitative PCR using fluorescently labeled primers has been used (Nazarenko et al., 2002; Mansfield et al., 1993; Morgan et al., 1999; Barrois et al., 2004). This method has correctly identified dystrophin gene deletions in both Duchenne and Becker muscular dystrophy carriers and BRCA1 lesions in breast and ovarian cancer families (Mansfield et al., 1993; Barrois et al., 2004; Morgan et al., 1999). In these scenarios, fluorescently labeled primers are designed against the gene of interest along with a control gene (such as tubulin or GADPH) and quantitative PCR allows for visualization of the gene of interest compared to the control (Nazarenko et al., 2002). This gene dosage analysis allows detection of deletion carriers in humans (Mansfield et al., 1993; Barrois et al., 2004; Morgan et al., 1999), and while this method has not been published using zebrafish, the technique should allow for identification of heterozygous γ-deletion fish.

One solution to the targeted mutagenesis problem is to use insertional mutagenesis. Instead of using traditional chemical mutagenesis or irradiation, retroviruses are injected into zebrafish embryos (Amsterdam et al., 1999). These retroviruses integrate randomly into the genome giving rise to insertions within genes (Amsterdam and Hopkins, 1999). Retrovirus-mediated insertional mutagenesis has advantages over traditional mutagenesis in that the gene harboring the insertion can be identified rapidly by PCR due to identification of proviral insert sequence (Golling et al., 2002). One drawback, however, is that this procedure is usually used to screen for a particular phenotype (Amsterdam, 2003). If a reverse genetic approach is desired, all insertional mutations would need to be cloned to find a mutation in the gene of interest. While there are some advantages to insertional mutagenesis, unless a forward genetic
approach is desired, cloning individual mutations to find a mutation in a gene of interest would be cumbersome and ill-advised.

For lack of targeted mutagenesis, researchers have modified several rapid screening methods to identify mutations in specific zebrafish genes. In all cases, zebrafish are randomly chemically mutagenized which is then followed by screening for mutations in target genes (Weinholds et al., 2003). TILLING which stands for Targeting Induced Local Lesions IN Genomes utilizes the potential heteroduplex nature of heterozygous mutant zebrafish (Figure 4.1; Stemple, 2004).

![Diagram of TILLING procedure](http://www.nature.com)

**Figure 4.1. Targeting Induced Local Lesions IN Genomes.** Schematic shows an overview of the TILLING procedure and how it can be used to identify ENU-induced mutations in the gene of interest. Heteroduplex DNA is cleaved with Cel1 nuclease and products are analyzed by gel electrophoresis (Reprinted from Stemple, 2004 with permission from Nature Publishing Group; http://www.nature.com).
After random chemical mutagenesis, males with mutated spermatogonia are crossed to WT females, and the resulting F₁ generation will be heterozygous for any particular mutation (Draper et al., 2004). Genomic DNA is isolated from individual F₁ males and sperm is frozen for future recovery of mutations (Stemple, 2004; Draper et al., 2004). Gene specific primers are used to PCR amplify the gene of interest; PCR products are then heated and cooled slowly to cause heteroduplexes to form in the reannealed product (Figure 4.2; Weinholds et al., 2003).

![Figure 4.2: Heteroduplex formation in heterozygous zebrafish.](image)

Upon heating and slow cooling of PCR products, hetero- and homoduplexes form in the gene of interest. Because hetero- and heteroduplexes have differing chemical properties, heteroduplexes can be identified.

Treatment of the heterduplexed PCR product with Cel1, an endonuclease that cleaves mismatches at heteroduplexed DNA, results in digestion of the product and therefore visualization of two bands by gel electrophoresis (Figure 4.1; Stemple, 2004; McCallum et al., 2000). When a mutation in the gene of interest is found, frozen sperm with that mutation is used to fertilize WT eggs, resulting in the F₂ generation of heterozygous carriers for the mutation (Draper et al., 2004). TILLING was successfully developed in *Arabidopsis thaliana* and is now being adapted to zebrafish (McCallum et al., 2000;
Weinholds et al., 2003; Draper et al., 2004). One of the drawbacks to the TILLING method used by many researchers is that the testes of F1 males are cryopreserved, and the animal is sacrificed (Draper et al., 2004). One worry is that if sperm is improperly frozen, and is unable to fertilize, mutations may be lost (Weinholds et al., 2003). Additionally, frozen sperm is a limited supply, so only a certain number of eggs can be fertilized. Alternatively, F1 fish can be kept alive, as a living library, but drawbacks also exist with this housing method. With a live library, a facility needs to be large enough to house a large number of fish to be screened; another drawback is that fish can become diseased and ultimately die before they can be out-crossed to propagate the mutant line.

Denaturing high performance liquid chromatography (dHPLC) is an alternative way to screen for mutations in a gene of interest. dHPLC was originally developed to detect DNA variations, such as single nucleotide polymorphisms (SNPs; Cremonesi et al., 2003) in humans, and the procedure has been adapted to screen for DNA variations in zebrafish. In this protocol, genomic DNA from F1 progeny of mutagenized males is used to PCR amplify the gene of interest, and the products are cooled slowly to allow heteroduplex formation. If there is DNA variation in the sample (SNPs or mutations), the denatured and reannealed DNA will form heteroduplexes (Xiao and Oefner, 2001). Homo- and heteroduplexes result from random association of DNA in a heterozygous sample after denaturation and reannealing (Xiao and Oefner, 2001). The PCR products are then loaded onto the dHPLC column, a reverse-phase column near the melting temperature of the DNA (Cremonesi et al., 2003). Column retention results from the negatively charged phosphate groups of the DNA binding to the positively charged cartridge beads, and as buffer concentration increases, DNA begins to elute off the
cartridge (Kuklin et al., 1997). The mismatch sequence of heteroduplexes causes a reduction in column retention time compared to homoduplexed DNA (Fasano et al., 2005). Heteroduplexed DNA has abnormal elution patterns compared to WT homoduplexed DNA (Cremonesi et al., 2003) that can be detected by computer software (Figure 4.3).

If sequence analysis of the sample confirms a mutation in the gene of interest, the F₁ fish corresponding to that mutation is out-crossed to start the F₂ generation. dHPLC has proven to be a powerful tool for identifying mutations in many human genes such as \textit{BRCA1}, \textit{BRCA2}, and \textit{p53} (Cobb et al., 2002; Wagner et al., 1999; Fasano et al., 2005; Leonard et al., 2002). One of the drawbacks is that dHPLC only identifies the presence of a mutation (Cremonesi et al., 2003). Resequencing needs to be performed to confirm the lesion (Xiao and Oefner, 2001). Screening for mutations in zebrafish by dHPLC has many potential advantages. The automation of the system should result in a cost-

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure4.3}
\caption{Retention of DNA on dHPLC column. Schematic shows an example of column retention time of homo- and heteroduplexed DNA in the WAVE dHPLC column. Due to reduced retention time, heteroduplexes elute before homoduplexes (Figure copyright of Transgenomics, Inc).}
\end{figure}
effective screening method (Kulkin et al., 1999; Fasano et al., 2005). Another cost-saving advantage is the presence of a collaborator, Dr. Thomas Prior, at The Ohio State University. Dr. Prior owns and operates a dHPLC machine used in other projects in his lab, and is collaborating with our lab in this endeavor. Finally, F₁ progeny can be kept alive as a live library during the screening procedure such that if a mutation is identified, there is a live animal to propagate the mutant line.

One final way in which zebrafish researchers are screening for mutations in their genes of interest is resequencing. During this process, mutagenized F₁ offspring are screened for mutations in a particular gene by sequencing of the gene (Weinholds et al., 2002; Berghmans et al., 2005). Usually the most conserved regions or the most important functional regions of the gene are amplified and then sequenced (Berghmans et al., 2005). Several zebrafish mutations have been identified using this approach including zebrafish homolog of \(tp53\), a known oncogene (Weinholds et al., 2002; Berghmans et al., 2005). This method of screening by resequencing is undoubtedly the most direct way to find a mutation in the gene of interest, but still requires significant automation and other personnel resources.

In order to understand the genetics and etiology of SMA, researchers have developed several mouse models (Schrank et al., 1997; Hsieh-Li et al., 2000; Monani et al., 2000; Monani et al., 2003; Cifuentes-Diaz et al., 2002; Cifuentes-Diaz et al., 2001). One of the limitations with these mouse models is the difficulty of embryologic study. It is becoming increasingly evident that SMN may be playing a role during development (McWhorter et al., 2003; Rossoll et al., 2003) and a model system with well characterized embryology, especially nervous system development, is essential to understanding, not
only the etiology of SMA, but the molecular mechanism by which SMN functions. While we have shown that morpholinos designed against smn are valid for modeling SMA in zebrafish (McWhorter et al., 2003), morpholinos have several drawbacks that hamper study of SMA in zebrafish. First, morpholinos are a transient knockdown of protein; protein levels eventually return to WT levels making analysis in later stages of development (past 21 dpf) impossible. Second, morpholinos are mosaic in that each cell within the embryo can harbor slightly different levels of morpholino and protein (McWhorter et al., 2003). A genetic mutation in the zebrafish smn gene will alleviate the problems with MO knockdown analysis. With a mutation, gene/protein function will be perturbed throughout the life of the organism, unlike transient knockdown observed with MOs. Previous work in other models systems suggest that homozygous SMN mutant animals do not survive past gastrulation (Schrank et al., 1997); there is no reason to believe that zebrafish will be any different. Therefore, a hSMN2 transgenic zebrafish project is also ongoing such that a SMA model can be made in a similar fashion to the SMA mouse model (Monani et al., 2000). With this genetic model of SMA in zebrafish (smn<sup>-/-</sup>; hSMN2), analysis of motoneurons survival and neuromuscular junction activity can be performed. Therefore, the goal is to identify a mutation in the zebrafish smn gene (Figure 4.4). This zebrafish model of SMA will allow for further elucidation of disease etiology and pathology.
ATG GCCA ATTG TG CAGA AG ATGT CG TA TTTTG TG TGGGACTG GTCAAAGT GATG GT CT 
GACAT TTTGGG ACTGTA CAGCTT TG ATTAAACCA TA AGAT TA AAGGTG TGGCTCA TATCAAG 
ATGCTTG AA AGTG AAGG AAGG CAGCCCA ACA AAAGAACGACA ACCAGGGAA GA 
AGGAAAA AACA AA AA AGAATG CAGGAA AGGATGCAA GCGACGACA 
AGAGTG CGACGG TGAAGTTCA CTTTGTTAT CGTG TGAAGTGG CAAACTG C 
TGCCACCA TATACCTACGG ACAAGGAGAGG GCCACCTTG TGGTTTTTACACAGAATTA 
TGAAATG GAAGGAGCAAACCTCAGTGACCTTCTGACTGACCTTCCACAGACATGAGT 
AGAATG CTTGAAGCAAGAAGAAAAGAAGAGTCTCTCCACAGAGAAGAGAGAT 
CCTCTTTCCA AACCACAAGAAAGTCCG CTAATGCA AAGCA AAAATCT AAAAGCAATTT 
CCTATGG ACCCCCA TCAATT TG TTCCCA AATGCTGACC CGCCACACCACGACC 
TAAAAAGATGATGAGCAGAAGGAGAAGAGTCCTTGCTC TTCTTTCTTGATGSGCCT 
CCATGTACTCCACTCGTCCACCAGATGATCCCA CACCCAACTTACCTGAGATCC 
GAGGAGCAGAGAGCTTTGGGCAGTA TGCTGATTCTCTTGTA AATGAGC CGGCTAICAC 
ACTGGATAC TATAT TGTTTAA AGACAGGCGT TAAAGAGGCTGCTCGCATTCCA AAGAAATC 
GCCAT CGAATAAAAAACCACACGACTGGCATATGAAACGGGATCTGCTGAGACAATA 
ATGGTTTTTGT AATGCTT TGGTTAAAT AAAGAATGTA AATGTTCTCTTTACA

Figure 4.4. WT exonic sequence of zebrafish smn gene. Sequence from all 8 exons is shown. Exon boundaries are shown in alternating colors. Sequence information was obtained from Genbank Accession # Y17256, AA494875, AA494767, AF083557 (McWhorter et al., 2003).

MATERIALS AND METHODS

Fish maintenance

Adult zebrafish and embryos were maintained as previously described (Westerfield, 1995). All fish used in this study were in the *AB background unless otherwise specified.

ENU mutagenesis

Adult male zebrafish were mutagenized according to published protocols (Driever et al., 1996; Westerfield, 1995). One gram isopacks of ENU (Sigma catalog # N-3385) were used to make a 3mM mutagenesis solution to which adult males were allowed to swim for 1 hour at 21°C. After rinses in fresh water, fish were returned to circulation. Fish were treated once per week for 4 weeks. After 6 weeks, mutagenized males were crossed with WT females to propagate an F1 generation. The adult F1 generation was screened for mutations.

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γ Ray Irradiation

WT adult male zebrafish were sacrificed with a lethal dose of anesthetic (Tricaine; Sigma catalog # A-5040), and their testes were harvested. Testes were placed in Hank’s saline solution (0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 1.3 mM CaCl₂, 1.0 mM MgSO₄, 4.2 mM NaHCO₃; Westerfield, 1995), ground with a pestle, and placed on ice. Mutagenesis of sperm was performed according to published protocol (Westerfield, 1995; Walker et al., 1983; Chakrabarti et al., 1983). Sperm were placed on a watch glass on ice and exposed to approximately 250-300 rads in a Gammacell 40 Cesium¹³⁷, sealed-source irradiator. After irradiation, sperm were used to in vitro fertilize WT eggs according to protocol (Westerfield, 1995). The resulting adult F₁ generation was screened for mutations.

Adult fin clips

To isolate genomic DNA from ENU mutagenized and γ irradiated F₁ generation adults, fin clips were performed. Adults were anesthetized in a Tricaine solution for approximately 1-2 minutes. Using small scissors (World Precision Instruments # 14393), the anal and caudal fins were cut approximately half way between the body and the end of the fin (Westerfield, 1995). Genomic DNA was obtained from individual fin tissue. Each fish was kept in an individual container until the completion of DNA analysis.

Genomic DNA isolation

Genomic DNA extraction for either ENU mutagenized or γ irradiated fish was performed in 1 of 2 ways. Either the protocol was modified using reagents from Gentra Systems (Minneapolis, MN; Kit # D-7000A) or a Qiagen DNeasy Tissue Kit (catalog # 69506) was used exactly according to manufacturer’s instructions. The following modifications
were made to the Gentra kit protocol: after the protein precipitation step, a phenol:chloroform extraction followed by a chloroform only extraction was performed; and DNA precipitation with 100% isopropanol (instead of ethanol) and carrier DNA (empty plasmid vector) was preformed overnight at -20°C. Other than these modifications, Gentra’s manufactures’ protocol was followed.

ENU sample analysis

PCR and heteroduplexing

PCR reaction mixture contained 1 unit Taq polymerase, 0.5 mM dNTPs, 1X ammonium persulfate buffer (67 mM Tris, 10 mM β-mercaptoethanol, 16.6 mM ammonium persulfate, and 6.7 mM EDTA), and varying concentrations of MgCl₂. A standard PCR cycle consisted of a 95°C denaturation, fragment specific annealing temperature, and an extension step at 72°C. If traditional PCR did not yield highly concentrated product, touchdown PCR (TD PCR) was used. Prior to traditional PCR cycling, 16 cycles were inserted. The first cycle was set at 8°C above the desired annealing temperature; each subsequent cycle lowered the annealing temperature half of a degree until the final annealing temperature was reached. For specific primer sequences, MgCl₂ concentrations, and annealing temperatures see Hejmanowski (2004).

WAVE injection

Denaturing high performance liquid chromatography (dHPLC) was performed on a WAVE DNA fragment analysis system (Transgenomic, Inc.) Heteroduplexed PCR samples were loaded onto a reverse phase DnaSep column (Transgenomic, Inc.). Hetero/homoduplex analysis was carried out using elution Buffers A (0.1 M triethylammonium acetate) and B (0.1 M triethylammonium acetate, 25% acetonitrile) at
a constant flow rate of 1.5 ml/min. Eluted DNA fragments were detected at 260 nm with a UV-C detector. Optimum elution gradients and oven temperature were calculated using WAVEmaker or Navigator software. For specific conditions, see Hejmanowski (2004).

**Sequencing**

When heteroduplexed DNA fragments were detected, samples were sequenced. Prior to sequencing, PCR products were purified using Sephacryl S300 or S400 spin columns. Sequencing was performed on an ABI Prism machine using Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Biosystems) following manufacturer’s protocol. A forward and reverse sequencing reaction was performed for each sample; sequencing products were purified using Sephadex G50 Fine spin columns before being loaded onto the sequencing gel. Sequences for WT and F1 samples were compared to determine presence or absence of mutations. For further specifics regarding the sequence reaction procedure see Hejmanowski (2004).

**γ Deletion sample analysis**

Quantitative multiplex PCR using fluorescently labeled primers was performed on a Ericomp 9600. Using zebrafish β-actin (Accession # BC067566) as a control, fluorescent dosage of *smn* was performed using ABI Prism 377 according to manufacturer’s protocol. Bands were analyzed using GeneScan software. For specific primer sequences, MgCl₂ concentrations, and annealing temperatures see Hejmanowski (2004).
RESULTS

DNA isolation and fish isolation housing

Because DNA quality is essential for these screening processes, the DNA isolation protocol needed to be optimized. Initially, Gentra Systems’ PureGene DNA Isolation Kit was used to extract DNA from clipped fins. This method did not seem to yield high-quality DNA as 20-30% of the PCR reactions failed, and the integrity of the dHPLC column was reduced because of unclean DNA. To better optimize the Gentra protocol, a phenol:chloroform and chloroform extraction were added following the protein precipitation step just prior to the DNA precipitation step. While this extraction step did decrease PCR failures, the DNA was still causing the dHPLC column to lose resolution prematurely. In simplest terms, the DNA was still “dirty” and was clogging the column. These DNA isolation protocols used glycogen as a “carrier” for DNA precipitation. Instead of glycogen, we tried tRNA and empty plasmid DNA as “carriers” for DNA precipitation, but neither changed the resolution of the dHPLC column. On the suggestion of another zebrafish lab, we abandoned the Gentra protocol and switched to Qiagen DNeasy Tissue Kits. While expensive, these kits allow for efficient PCR reactions that do not cause decreased resolution on the dHPLC column. Screening for mutations has been much more efficient since the Qiagen Kits have been utilized.

To keep the fish isolated, each fish was kept in a small 0.5-1 qt container. Since these fish were removed from main water circulation, they were fed a small amount of food every 2 days and the water was siphoned weekly. This schedule was optimal because it minimized fish maintenance but allowed for high percentage survival.
Screen for a deletion in the zebrafish *smn* gene

Multiplex quantitative PCR was used to screen for a deletion in the zebrafish *smn* gene. Fluorescently labeled primers designed against exons 1 and 7 were used in this fluorescent dosage analysis (Hejmanowski, 2004; Figure 4.5).

![Figure 4.5. Genetics of γ-ray induced deletion screen.](image)

All eight exons of the *smn* gene are shown. Fluorescently labeled primers amplify exons 1 and 7 of *smn* gene. Quantitative multiplex PCR using these primers will identify a deletion.

The 3’ end of the zebrafish *β*-actin2 gene was used as a control. By varying primer concentrations in the multiplex PCR reaction, the intensity of the fluorescent bands was made roughly equivalent in WT fish (Barrois et al., 2004; Nazarenko et al., 2002). Therefore, if exons 1 and/or 7 were deleted, the resulting fluorescent product would be cut in half relative to *β*-actin2. Because of the essential nature of the *smn* gene, we would expect any deletion to manifest as a heterozygote (Schrank et al., 1997).

Before screening for an unknown *smn* mutation, the fluorescent dosage analysis procedure was used to identify a gene deletion in a known zebrafish deletion mutant, *Df(LG05)her1b567*. In this mutant line, the her1 gene is deleted (Henry et al., 2002). Using fluorescent primers for *her1* and blinded DNA samples, this dosage analysis identified heterozygous *her1* deletion fish in 100% of the samples. This test confirms that the dosage analysis can correctly identify a carrier deletion fish.

As γ-irradiation induces deletions, the F1 progeny resulting from γ-irradiated sperm and WT eggs were used to screen for a deletion in the *smn* gene (Figure 4.6).
The adult F₁ progeny were fin clipped and genomic DNA was isolated. Fluorescent dosage analysis using \( smn \) and \( \beta\text{-actin} \) genes was performed on these samples. In total, 134 F₁ progeny were screened for deletions in exons 1 and 7 of the zebrafish \( smn \) gene. While no mutation was found, this technique has correctly identified deletions in known mutations. Therefore, further screening of additional F₁ progeny should ultimately yield a \( smn \) deletion allele.

**Screen for ENU-induced \( smn \) mutation**

dHPLC was used to screen for an ENU-induced point mutation in the zebrafish \( smn \) gene. Intronic primers were used to amplify exons 1, 2, 3, 4, 6, and 7 of the \( smn \) gene (Figure 4.7).
Figure 4.7. **Genetics of ENU induced point mutation screen.** All eight exons of the *snm* gene are shown. Primers amplify exons 1, 2, 3, 4, 6, and 7 of *snm* gene. Heteroduplex formation due to base pair changes will be identified by dHPLC analysis.

Exon 5 was not included in this screen because the flanking introns 4 and 5 had not been fully characterized at the time of screening (Hejmanowski, 2004) and exon 8 is non-coding (Lefebvre et al., 1995). F₁ progeny from ENU mutagenized males and WT females were used to screen for a point mutation in the *snm* gene (Figure 4.8).

The adult F₁ progeny were fin clipped and genomic DNA was isolated. PCR amplified exons were run through the dHPLC column and abnormal peaks were identified (Figure 4.9). Sequencing of abnormal dHPLC peaks confirmed any base pair changes (Figure 4.9 B, C, E, F, H, I).
Figure 4.9. dHPLC and sequence analysis of *smn* gene. SNP analysis from exons 2 (A-C), 3 (D-F), and 4 (G-I) are shown. dHPLC elution curves (A, D, G) with time on X axis and buffer concentration on Y axis showing heteroduplexed products; WT elution curves are shown in black (A, D) while SNP elution curves are shown in blue (A,D); confirmation sequence analysis of WT (B, E, H) and SNP (C, F, I) are also shown. Base pair changes are indicated with arrows (B, C, E, F, H, I).
Upon screening 910 F_1 progeny, no mutations were found in the coding regions of exons 1, 2, 3, 4, 6, or 7. Several single nucleotide polymorphisms (SNPs) were found in the exons of interest and are summarized in Table 4.1. Examples from elution curves from SNPs are shown (Figure 4.9 A, D, G).

<table>
<thead>
<tr>
<th>Exon/Intron</th>
<th>Base Change (bp position)</th>
<th>Codon</th>
<th>Predicted Amino Acid (#) Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intron 1*</td>
<td>C to T (14 upstream of I/E)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exon 2</td>
<td>A to C (181)</td>
<td>AGG to CGG</td>
<td>None-Arg(61)</td>
</tr>
<tr>
<td>Intron 2</td>
<td>C to T (8 upstream of I/E)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exon 3</td>
<td>A to G (409)</td>
<td>ATG to GTG</td>
<td>Met(137) to Val(137)</td>
</tr>
<tr>
<td>Exon 4</td>
<td>C to A (498)</td>
<td>TCC to TCA</td>
<td>None-Ser(166)</td>
</tr>
<tr>
<td>Intron 7</td>
<td>inserted T (39 upstream of I/E)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1. Summary of base changes identified in zebrafish *smn* gene. SNPs were seen in multiple fish and occurred as homozygotes. I/E symbolizes the intron/exon border. *Only one F_1 zebrafish, a heterozygote, was seen with this genotype.

It is believed that these base changes within the exons are SNPs and not ENU-induced mutations for several reasons. First, these base pair changes were seen in multiple fish. It is unlikely that the same mutation would occur in multiple fish. Second, many of these SNPs are homozygous, and it is believed that any homozygous mutation in *smn* would be embryonic lethal (Schrank et al., 1997). Finally, while several of these SNPs do change the codon and the resulting amino acid, the other SNPs do not change either. Therefore, these base pair changes are likely SNPs, not ENU-induced mutations, and are likely strain differences in the zebrafish.

In addition, several base changes were identified in the introns flanking the exons of interest. While introns were not specifically screened, primer sequences were designed against intronic sequence, so portions of each intron were consequently screened. The changes in introns 2 and 7 are very likely SNPs as they have been seen in
multiple samples and occur homozygously. The base change in intron 1, however, may be an ENU-induced mutation. It is unique in 910 samples screened and occurred as a heterozygote. While it is likely that, if a mutation, this change would be silent and not have an effect on gene function, there is a possibility that this base change may occur in a regulatory region of *smn* or in a (cryptic) splice site. While regulatory regions within intron 1 have not been described, they could exist (Monani et al., 1999b; Echaniz-Laguna et al., 1999). Because of this possible mutation, fish F1 682 could have been out-crossed to propagate an F2 generation. Traditional RT-PCR to detect potential splice variants resulting from the mutation and real time RT-PCR to detect amounts of transcript due to regulatory changes in the gene could have been be performed on the heterozygous F2 progeny. Unfortunately, this fish was diseased and died before out-crossing.

**TILLING for *smn* mutation**

To increase the odds of finding a mutation in the *smn* gene, we have begun collaborating with Dr. Cecilia Moens at the Fred Hutchinson Cancer Research Institute. Dr. Moens’ lab is currently TILLING for gene mutations (Draper et al., 2004) and has graciously placed *smn* in the queue of genes to be screened (C. Moens personal communication). Upon screening over 4,000 genomes, TILLING has identified 3 mutations in the *smn* gene. Two of these mutations are silent in that the codon remains unchanged in the mutant. One mutation, however, changes a conserved serine to alanine at amino acid position 38 of the Smn protein. This is a very conserved region of the Smn protein in exon 2 (Figure 4.10) suggesting that there is an important functional aspect to this amino acid. Further analysis of this mutation will determine if gene function is perturbed.
Screening two independent sets of mutagenized F1 fish and utilizing two different screening methods should ultimately yield a mutation that will affect *smn* gene function.

**DISCUSSION**

Zebrafish is an ideal model system for a classic forward genetic approach because of its well characterized embryology and external fertilization (Dodd et al., 2000). Recently, researchers have also begun to utilize the reverse genetic attributes of this model system (Lekven et al., 2000). Zebrafish are now also being used as a disease model. Due to its simplified neuromuscular organization, we believe that a genetic zebrafish model of

<table>
<thead>
<tr>
<th>Species</th>
<th>Protein Sequence</th>
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<tbody>
<tr>
<td>Human</td>
<td>MANSGGGGSGGGVEQEDSVLERRGTCGQSPDSDIVDOTALYDKAAVASEKHALKNGIDC 60</td>
</tr>
<tr>
<td>Dog</td>
<td>------HSQGGGGLEFEDSVLERRGTCGQSPDSDIVDOTALYDKAAVASEKHALKNGIDC 55</td>
</tr>
<tr>
<td>Mouse</td>
<td>------MANSGGGGGEQEDTVLERSTCGQSPDSDIVDOTALYDKAAVASEKHALKNGIDC 57</td>
</tr>
<tr>
<td>Zebrafish</td>
<td>------MANC------ADEVFRCRGRMQTCGQSPDSDIVDOTALYDKAAVASEKHALKNGIDC 49</td>
</tr>
</tbody>
</table>

**Zebrafish SMN Protein Alignment**

**Figure 4.10. Protein sequence alignment of SMN homologs.** Alignment of human, dog, mouse, rat, and zebrafish Smn proteins using the ClustalW software. Arrow indicates the position of the conserved serine mutation identified by TILLING.
SMA has the potential to elucidate the neuropathology of the disease. While morpholinos allow for reduction of protein during early development, a \textit{smn} mutant is necessary to alleviate the inherent mosaicism of MOs and for study of SMA pathology after 21 dpf. Since targeted mutagenesis techniques are not available in zebrafish, development of rapid screening methods is essential (Weinholds et al., 2003). Rapid screening methods for mutations in the gene of interest not only impact specific human diseases, but the zebrafish community as a whole. These reverse genetic screens will add another technique to the genetic arsenal of zebrafish researchers.

\textbf{\textit{γ} deletion screen for \textit{smn} mutants}

We have shown that fluorescent dosage analysis can correctly identify \textit{γ}-induced deletions in zebrafish. This quantitative multiplex PCR identified 100\% of \textit{Df(LG05)her1}^{b567} deletion heterozygotes, showing that the technique works for deletion screening in \textit{γ}-irradiated F\textsubscript{1} progeny. While no deletion mutation was identified at the \textit{smn} locus, the same F\textsubscript{1} progeny have been phenotypically screened successfully (Rodino-Klapac, 2005) indicating that the fish do harbor mutations, just not in the \textit{smn} gene.

Several research groups have attempted to estimate the specific locus mutation rate of \textit{γ}-irradiated F\textsubscript{1} progeny. In a phenotype-based non-complementation analysis of 3 independent pigment loci (\textit{golden}, \textit{brass}, and \textit{albino}), the locus specific mutation rate was approximately 0.01, for sperm irradiated with comparable \textit{γ} rays used in our experiments (Chakrabarti et al., 1983). This means that approximately 1 in 100 screened fish will have a lesion in the gene of interest. The loci used in these complementation tests, however, are believed to be in mutational “hot spots” of the genome (Fritz et al., 1996). Therefore, the estimate of 0.01 may be inaccurate when screening the entire
genome. In a similar multiplex quantitative PCR-based screen (without fluorescent primers), the locus specific mutation rate was determined to be 0.0022 to 0.0043 (Fritz et al., 1996). Therefore, in order to find a γ-induced mutation (deletion or translocation) in your gene of interest, you would need to screen approximately 230-460 fish (Fritz et al., 1996). This is a more conservative and accurate estimate because 13 different loci were used in this calculation (Fritz et al., 1996). The frequency of mutations generated by γ-irradiation varies considerably among different chromosomal regions (Fritz et al., 1996; Walker, 1999). Our fluorescent dosage analysis was designed specifically to screen for deletions, not translocations, as many translocation lesions are difficult to propagate and do not segregate in a Mendelian fashion (Fritz et al., 1996). Therefore, loci-specific mutation rates using our screening method would likely decrease, meaning we would need to screen more than 230-460 fish in order to find a deletion allele of smn. In our analysis, we screened 134 F1 progeny, clearly under the published estimates for finding a lesion in the average gene (Fritz et al., 1996). If more F1 progeny were screened, we are confident that our screening method would be effective in detecting a deletion allele of smn. Our focus has shifted, however, away from a deletion allele of smn predominantly because most γ-induced deletions affect more than a single gene (Fritz et al., 1996). Loss of genes other than smn may make analysis of a zebrafish SMA model more difficult.

**dHPLC screen for smn mutants**

We have shown that a dHPLC-mediated screen for ENU-induced mutations successfully identifies base pair changes in zebrafish. Several SNPs have been identified in exons 1, 2, 3, 4, 6, and 7 of the smn gene (Table 4.1). These SNPs were found in multiple fish and are likely strain differences among the species. Our dHPLC screening method also
identified a potential ENU-induced mutation in intron 1. This base change is unique and manifested as a heterozygote which is expected for an ENU-induced mutation. As this mutation occurs in non-coding sequence, it would likely not affect smn gene function, but this lesion could have been analyzed further to determine if regulatory or splice regions were perturbed. The F1 progeny that were screened for smn mutations were also used in a classic forward genetic screen in which many mutants have been identified (P Henion, personal communication). Identification of an intronic smn mutant and phenotypic mutants from a neighboring forward genetic screen indicate that these F1 progeny have been effectively mutagenized and harbor many random heterozygous mutation in their genomes. Several research groups have estimated the mutation frequencies of fish exposed to similar doses of ENU. Using their estimate of mutation frequency (2.1 x 10^{-6} per base pair) and the size of the smn exons screened (approximately 1800 bp), we should find one smn mutation in approximately 588 F1 progeny screened (Weinholds et al., 2002). These estimates agree with earlier estimates that similar doses of ENU induced a mutation frequency of approximately 1 in 650 progeny (Driever et al., 1996). The intronic smn mutation that was identified occurred in F1 682 which correlates well with published frequencies. Since this mutation likely did not affect gene function, dHPLC screening will continue in order to find mutations in the coding region of the gene.

As the human and zebrafish genomes continue to be sequenced and annotated, there will be more of a need to develop an efficient, high-throughput screening method to identify mutations in genes of interest, specifically human disease-causing genes. This target-selected mutagenesis is necessary to fully understand the functions of numerous genes. We believe that our dHPLC-mediated ENU screening method and fluorescent
dosage-mediated deletion screening method are effective for identifying mutations in genes of interest. Upon finding a mutation in the smn gene, a model of SMA in zebrafish will help elucidate the neuropathology and etiology of the disease.
CHAPTER 5

DISCUSSION

For years, SMA research has focused on the function of SMN in fibroblasts and other cultured cells. To better understand the neuropathology of the disease, however, animal models were needed. While several mouse models have been produced and are invaluable for studying many aspects of SMA, an animal model was needed that would allow for better characterization of the neuromuscular phenotypes that might be associated with the disease. Because of its stereotypic and relatively simple neuromuscular organization, zebrafish were used to model SMA (McWhorter et al., 2003; Chapter 2). The motor axon defects observed are motoneuron-specific and are cell-autonomous with respect to motoneurons (McWhorter et al., 2003; Chapter 2). While long term consequences of the developmental pathfinding defects with respect to SMA pathology remain unclear, reduced Smn levels in zebrafish larvae do lead to premature death of the organism, and further validate the system as a model for SMA. Analysis of the function of Smn which is important for motor axon outgrowth suggests that its well-characterized role in snRNP assembly may not be involved and further indicate a novel motor axon-specific role in mRNA transport. Two separate screens for point mutations in the smn gene have yielded several lesions in the gene with one potentially affecting gene function; this S38A smn mutation is a good candidate to affect
gene function because the lesion lies within a conserved region on exon 2. While the dHPLC method was somewhat effective, the TILLING method is a higher-throughput approach for finding mutations in the gene of interest.

**A motoneuron-specific SMN-mRNA complex?**

Both nucleocentric and axonocentric researchers have failed to show why reduced Smn levels specifically affect motoneurons. The answer may reside in what makes a motoneuron different from other cells and neurons. It is not just the presence of a long axon, because other neurons (such as interneurons) also have long axons. Since Smn has been shown to localize to axons of other neurons, not just motoneurons, the possibility exists that the connections made with muscle cells are important for Smn function. To address Smn specificity for motoneurons, experiments that test how motoneurons are different from other neurons could be performed. For example, expression profiling of SMA motoneurons may indicate if particular mRNAs are abundant or lacking compared to WT motoneurons. Recently these types of experiments have been performed in dorsal root ganglion neurons (Willis et al., 2005) and should be applicable to motoneurons. Laser capture microdissection, in which a laser is used to excise a small piece of tissue (Nazarian et al., 2004), could be used to remove the motor growth cones of WT and smn MO injected zebrafish embryos. The mRNA profiles from each could be compared. These types of experiments may directly assess mRNA components that are either too abundant or lacking in Smn diminished growth cones; such analysis may identify mRNA components that may lead to motoneurons being uniquely sensitive to reduction of Smn protein.
If indeed a motoneuron-specific Smn mRNA complex exists, this may be a low affinity complex in which Smn binding to the complex only occurs when Smn protein is abundant (WT). Smn binding to this low affinity complex may be affected if protein levels are significantly reduced (SMA) whereas high affinity complexes to which Smn binds, such as the Gemins, may only be moderately affected by reduction of Smn.

**Consequences for SMA therapeutics**

Data presented here showing motor axon pathfinding defects during zebrafish development (McWhorter et al., 2003; Chapter 2) along with data suggesting that primary motoneuron cultures from SMA embryonic mice have reduced axonal outgrowth (Rossoll et al., 2003) indicate a potential role for Smn during motoneuron development. Until these reports, reduction of SMN was believed to have its degenerative effect post-natally (Nicole et al., 2002). Since SMA usually did not manifest itself until after birth, researchers and clinicians thought SMN’s role was in maturation of neuromuscular architecture (Nicole et al., 2002). Therefore therapeutic strategies have focused on agents which would simply increase levels of full-length SMN from the SMN2 gene (Mercuri et al., 2004; Andreassi et al., 2001; Lunn et al., 2004). To this end, assays that measure full-length versus SMNΔ7 transcript and overall full-length SMN protein levels have been designed (Andreassi et al., 2001; Lunn et al., 2004); previously approved FDA therapeutics and recently designed therapeutics have been shown to increase SMN levels (Andreassi et al., 2001; Lunn et al., 2004), but none have had more than mild functional effects on SMA patients or mouse models (Mercuri et al., 2004; M. Butchbach, personal communication). Several other studies, although limited, have tested the effects of a class of drugs known as neuroprotectants such as riluzole (Haddad et al., 2003). These
drugs have had moderate success, but they were administered post-symptomatically (Haddad et al., 2003; Russman et al., 2003). If, in fact, SMN does have a motoneuron development function that relates to SMA pathology, these therapeutic agents may need to be administered significantly earlier, and/or as pre-symptomatically as possible. The finding that SMN may have a developmental role, not only has consequences for experiments which address its function, but also for research into therapeutic agents for SMA. Therefore, it is possible that a post-natal increase in full-length SMN may not have the desired outcome. Alternatively, drug discovery of more neuroprotectants may ultimately be the best strategy.

**SMA in the context of other motoneuron diseases**

SMA is just one of many in a class of motoneuron diseases. Spinal muscular atrophy with respiratory distress (SMARD) was once mistakenly misdiagnosed as SMA (Grohmann et al., 2003). SMARD is also an autosomal recessive disorder which results in loss of α-motoneurons in the spinal cord but unlike SMA, distal lower limbs are affected (Grohmann et al., 2003). One of the main differences between these disorders is that paralysis of the diaphragm and the associated respiratory ailments are the prominent symptom in SMARD patients (Grohmann et al., 2003). Mutations in the *Ighmbp2* gene (immunoglobulin µ-binding protein 2) cause SMARD (Grohmann et al., 2001). The cellular function of Ighmbp2 is unclear, but like SMN, *Ighmbp2* is also ubiquitously expressed and may associate with RNA and DNA (Grohmann et al., 2004). Specifically, Ighmbp2 has helicase activity that some believe may be important for transcription, nuclear export, and splicing (Grohmann et al., 2004). Additionally, Ighmbp2 also localizes to axons (Grohmann et al., 2004). Although differences in pathology of the
diseases exist, there are striking similarities between these diseases and especially the genes which cause them. Therefore, research on SMA/SMN certainly may aid in research on SMARD/Lghmbp2.

The most well-known motoneuron disease is undoubtedly ALS (aka Lou Gehrig’s disease and motoneuron disease). ALS unlike SMA and SMARD, occurs later in life and affects both the upper and lower motoneurons (reviewed in Cleveland and Rothstein, 2001). In over 90% of ALS patients there is no genetic linkage, called sporadic ALS; only 10% of ALS cases are inherited, called familial ALS (Cleveland and Rothstein, 2001). The only gene that has been shown to cause ALS is superoxide dismutase (SOD1) which converts superoxide to water and hydrogen peroxide (Rosen et al., 1993). SOD1, like SMN, is ubiquitously expressed leading researchers to ponder the specificity of the disease for motoneurons. Given that SOD1 is the only known disease causing agent, oxidative damage is one obvious hypothesis for the causality of ALS (Cleveland and Rothstein, 2001). Other hypotheses have also emerged; neurofilament accumulation in cell bodies and axons is one of these alternative hypotheses (Julien, 2001). In the SOD1 ALS mouse model, there is a decrease in slow axonal transport and a disorganization and accumulation of neurofilament (Collard et al., 1995; Zhang et al.1997; Williamson and Cleveland, 1999). While research has progressed on the SOD1 gene as a disease causing gene, about 98% of ALS patients have no mutation in SOD1 gene. With so much unknown about other disease causing genes, there is still much to learn about pathway(s) and interactions that specifically affect motoneurons in ALS.

Not all motoneuron diseases have disease causing genes which are cryptic with respect to motoneuron function. Recently, several groups have found that disruption of
components of the retrograde transport machinery, specifically dynein and dynactin, lead to progressive motoneuron degeneration (LaMonte et al., 2002; Puls et al., 2003; Hafezparast et al., 2003). Dynein is a minus end directed molecular motor responsible for retrograde transport of protein complexes and molecules, such as trophic factors, back to the cell body (LaMonte et al., 2002). Dynactin is a multiprotein complex that associates with dynein (LaMonte et al., 2002). Several pieces of evidence indicate dynein/dynactin in motoneuron disease; mice with mutations in cytoplasmic dynein heavy chain or disruption of the dynein/dynactin complexes suffer from progressive motoneuron disease (LaMonte et al., 2002; Hafezparast et al., 2003). Additionally human patients with a mutation in the dynactin gene also exhibit signs of motoneuron degeneration and disease (Puls et al., 2003). While dynein also has an essential housekeeping function in all cells (Harada et al., 1998), there appears to be a specificity for motoneurons—much like SMA, SMARD, and ALS.

**Conclusions**

While researchers have made substantial progress towards understanding the genetic mechanism which underlies motoneuron disease, there is still no clear consensus as to why mutations in so many ubiquitously expressed genes ultimately lead to motoneuron-specific diseases. If these diseases were caused by mutations in motoneuron-specific genes, analysis would certainly be much simpler, but this is not the case. Especially when all the sporadic mutations that cause ALS are considered, there are a grand number of genes and potential pathways that may be involved in motoneuron disease etiology. Several possible hypotheses exist for how these disease causing genes relate to each other. There may be a common mechanism(s) that many of these genes are involved in
that is currently not well understood or has not yet been fully discovered. For example, axonal transport may be a common theme. SMN may be involved in transport of mRNAs into axons (Rossoll et al., 2003; Zhang et al., 2003); SOD1 ALS mice have neurofilament accumulation and diminished axonal transport (Collard et al., 1995; Zhang et al., 1997; Williamson and Cleveland, 1999); transport motors like dynein, when mutated, also lead to motoneuron disease (Hafezparast et al., 2003; Puls et al., 2003; LaMonte et al., 2002). It is possible that all of these disease causing agents have a ubiquitous cell function, but also have a specific role with respect to neuromuscular architecture. Alternatively, motoneurons may simply be more sensitive to reductions in essential cell processes than other cells. If true, research would simply have shown that numerous ways exists to kill a motoneuron. Ultimately, learning about the mechanism of SMN function and how reduced levels result in SMA has more far-reaching effects than SMA alone. Research into SMN and SMA may yield insights into the other motoneuron diseases as well.
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