Université du Québec
Institut National de recherche scientifique
Centre Institut Armand-Frappier Santé Biotechnologie
Laval, Québec

MODELLING SPINAL MUSCULAR ATROPHY (SMA) IN ZEBRAFISH

Marilou Préville

Master of science (M.Sc.) degree in Experimental Health Sciences

Examiners

Jury president and Internal examiner	**Dr. Stéphane Lefrançois**
External examiner	**Dr. Alex Parker**
Director of research	**Dr. Kessen Patten**

DEDICATIONS

It has been the most challenging, but yet wonderful past two years of my life. I never thought my Master's degree would end with a global pandemic, which added a level of difficulty in planning experiments. Nevertheless, I am glad to have experienced this adventure filled with extraordinary moments and people to guide me into this mesmerizing quest of knowledge. I would like to give my most sincere thanks to Dr. Kessen Patten who has been the greatest mentor. With his understanding and trust, I was able to fully dedicate myself into my work. I couldn't have asked for a better teacher and I will be forever thankful for this opportunity.

Of course, I could write a whole thesis on how wonderful my lab mates have been. I still would like to thank personally Zoé, for all your help when I arrived. You made it so easy for me to feel welcomed and I am thankful for your guidance. Also, Max, who taught me so much and helped me during my most struggling times. Priyanka, who became a source of inspiration throughout my studies and who helped me figuring out fundamental questions about our place in the science world. Gabrielle, your help was more than welcomed, especially at the end of my Master's, and I am glad to have known such a wonderful person. Finally, the great Charlotte, I would like to thank you for your practical and, most importantly, emotional support for those two years. I feel very lucky to have met such amazing scientists and I truly hope all the best for you.

Finally, I would like to thank all of my family for the emotional support. My parents who were always there for me and who had to listened to a graduate student's problems for two whole years. And last but not least, my beloved William, thank you for giving me the courage and strength to persevere.

ABSTRACT

Spinal Muscular Atrophy (SMA) is an autosomal recessive neurodegenerative disease. It is the leading genetic cause of infant mortality for which there is currently no effective cure. This rare disease is characterized by a degeneration of lower alpha motoneurons of the spinal cord leading to muscle weakness and atrophy. It is caused by the deletion of *SMN1* gene on chromosome 5q13. Although SMN is ubiquitously expressed, the mechanism underlying the defective synaptic connections between motoneurons and muscles and the ultimate degeneration of the motoneurons are still poorly understood. Morpholino oligonucleotides based knockdown are commonly used in zebrafish to study SMA. However, the specificity of this technique is still controversial. Thus, we aim to generate a more reliable and stable model to study SMA. Using the CRISPR/Cas9 system, we targeted the zebrafish *smn1* gene to create a knockout (KO) model. We successful identified a zebrafish *smn* KO mutant with a 7bp frameshift mutation. Smn protein levels were significantly reduced in our *smn* zebrafish mutants. These fish also displayed a significantly reduced locomotion compared to controls as well as NMJ defects and gross morphological abnormalities. With an established new SMA zebrafish model, we wish to pursue transcriptomic analysis and drug screenings. This could help finding new pathways for alternative therapeutic strategies and for the understanding of SMA pathogenesis as well as neurodegenerative disease in general.

RESUMÉ

L'amyotrophie spinale (SMA) est une maladie neurodégénérative à transmission autosomale récessive. Elle représente la principal cause de décès infantile d'origine génétique et n'a présentement aucun traitement efficace. Cette maladie rare est caractérisé par une dégénérescence des neurones moteurs inférieurs de la moelle épinière qui mène à une faiblesse et une atrophie musculaire. Ceci est causé par une mutation dans le gène *SMN1* sur le chromosome 5q13. Bien que la protéine SMN soit exprimé de façon ubiquitaire, les mécanismes sous-jacent les connections synaptiques défectueuses entre les neurones moteurs et les muscles et ultimement la dégénérescence de ces neurones sont encore très peu détaillés. La réduction d'un gène avec des morpholinos a été utilisés abondamment chez le poisson-zèbre. Toutefois, cette technique demeure controversé due à sa spécificité. Nous souhaitons donc générer un modèle de poisson-zèbre plus fiable et stable pour étudier SMA. À l'aide de CRISPR/Cas9, nous avons cibler le gène *smn1* dans le poisson-zèbre pour créer un modèle knockout (KO). Nous avons identifié avec succès des mutants *smn* KO possédant une mutation causant un codon stop prématuré de 7 pb. Les niveaux de protéines Smn sont significativement réduite chez les mutants *smn.* De plus, les poissons mutants démontrent une activité locomotrice réduite comparé aux contrôles ainsi que des défauts au niveau des jonctions neuromusculaires et des défauts morphologiques grossiers. Avec ce nouveau modèle de poisson-zèbre SMA, nous souhaitons effectuer des analyses transcriptomiques ainsi que le criblage de médicaments. Cela pourrait permettre de trouver de nouvelles cibles thérapeutiques alternatives et aider à la compréhension de la pathogenèse de SMA ainsi que d'autres maladies neurodégénératives.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AAV9 : an Adeno-Associated virus 9 delivering system
AChR : Acetylcholine receptors
ALS : Amyotrophic Lateral Sclerosis
CHOP INTEND : Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders
CNS : Central nervous system
CPG15 : Cortical plasticity gene 15 / neuritin
CRISPRi : CRISPR interference
CRISPR/Cas9 : Clustered Regularly Interspaced Short Palindromic Repeats / CRISPR associated protein 9
crRNA : crispr RNA
CVS : chorionic villus sampling
eIF2a : eucaryotic translation initiation factor 2
ER : Endoplasmic reticulum
ESE : exon splice enhancer
FL-Smn : Full length Smn
FUS : Fused in sarcoma
Gap43 : Growth associated protein 43
hnRNP-R : heterogenous nuclear ribonucleoprotein R
HRM : High Resolution Melting
HuD : Elav like protein4
IMP1 : IGF2 mRNA-binding protein 1
iPSC: Induced pluripotent stem cells
LSm : Like Sm
mEPC : miniature end plate current
miRNA : micro RNA
MO: Morpholino antisense nucleotide
NHEJ : Non Homologous End Joining
NMJ : Neuromuscular junction
PAM: Protospace adjacent motif
PLS3 : Plastin 3
RBP : RNA Binding Protein
RNAi : RNA interference
ROCK : Rho associated protein kinase
SBMA : Spinal and Bulbar Muscular Atrophy
sgRNA : single-guide RNA
siRNA : small interfering RNA
SMA : Spinal muscular atrophy
Smn 1/2 : Survival motor neuron ½
snRNA: small nuclear RNA
snRNP : small nuclear ribonucleoproteins
TALEN : Transcription activator-like effector nuclease
TDP43 : TAR DNA binding protein 43
tracrRNA: trans-activating crRNA
UBA1: ubiquitin-like modifier activating enzyme 1
UPR : Unfolded protein response
ZFN : Zinc finger nuclea

1. INTRODUCTION

1.1. SPINAL MUSCULAR ATROPHY

1.1.1. CLINICAL FEATURES AND ONSET

Spinal muscular atrophy (SMA) refers to a group of neuromuscular disorders affecting spinal motor neurons. Various gene mutations can lead to different forms of SMA (M. A. Farrar *et al.*, 2015). The major form of SMA is proximal SMA (>95%) and is caused by mutations in the survival motor neuron 1 (*SMN1*) gene and leading to the degeneration of the anterior horn cell from the spinal cord resulting in muscle atrophy and weakness. SMA affects approximately one in 6000-10 000 live births and has a carrier frequency of one in 50 which makes it the first genetic cause of infant death (Ogino *et al.*, 2004). SMA is a clinically heterogenous disease with several types categorized from 0 to IV ranging from severe to mild and mainly based on clinical criteria and the age of onset (Table 1). Humans are the only species with two copies of *SMN*. A tandem chromosomal duplication event resulted in a highly homologous copy gene known as *SMN2* (M. E. Butchbach, 2016a, Cusin *et al.*, 2003). This gene differs from *SMN1* by lacking the exon 7, producing weaker levels of full-length functional protein although mutations in *SMN2* are not responsible for clinical phenotypes. However, several studies have demonstrated that *SMN2* copy number, ranging between 0 to 8 copies (Matthew E. R. Butchbach, 2016b), correlates inversely with the severity of the disease (Campbell *et al.*, 1997, Mailman *et al.*, 2002). Another study showed that some SMA patients with two *SMN2* copies display mild phenotypes, whereas patients with three copies have been classed as type I (Feldkötter *et al.*, 2002, Ogino *et al.*, 2003). Although the correlation it not perfect, measurement of *SMN2* copy number is still useful for SMA patient's prognosis and can help evaluate the probability of a young SMA patient to develop either type I, II or III. Furthermore, because *SMN2* are not functionally equivalent in all SMA patients, genetic background which influence splicing mechanism of SMN2 may also be relevant (Harada *et al.*, 2002).

Type 0 SMA is the most severe form affecting neonates. Patients present reduced fetal movements *in utero* and show severe weakness and asphyxia at birth (MacLeod *et al.*, 1999). SMA type 0 patients usually have a unique copy of *SMN2* and die within weeks after birth due to severe respiratory problems. SMA type I, also known as Werdnig-Hoffman disease, is the most common type of SMA accounting for approximately 60 % of patients (Verhaart IEC, 2017). It is also a very severe form of the disease diagnosed before the first 6 months of age and death occurring before two years of age. These patients have between two or three

copies of *SMN2* resulting in a large variety of phenotypes. Infants are not able to sit without support and show severe muscle weakness, mostly proximal, with poor or no deep tendon reflexes. Hypotonia and poor head control are also present though facial muscles are not affected. Intercostal muscles are weakened, but diaphragm is spared. This specific phenotype leads to paradoxical breathing and a bell-shaped chest. Finally, infants also have difficulty swallowing and feeding with tongue fasciculations due to bulbar weaknesses. These defects can often lead to impaired cough and recurrent pulmonary infections, which are the major causes of mortality in SMA (D'Amico *et al.*, 2011, Michelle A. Farrar *et al.*, 2017, Kolb *et al.*, 2015, Thomas *et al.*, 1994).

Intermediate SMA (Type II; Dubowitz form) patients have a 93% chance of surviving to 25 years old (M. A. Farrar *et al.*, 2013). The age of onset is around 6-18 months and approximately 80 % of patients have 3 copies of *SMN2* (Mercuri *et al.*, 2012). Patients will never walk independently, but they are able to sit on their own. Once again, infants show no deep tendon reflexes and experience muscle weakness especially in the legs. Intercostal muscles are still weakened combined with progressive scoliosis. These defects may contribute to lung diseases and respiratory impairments that require the need of monitored ventilation for some patients. Finally, they may also have difficulty swallowing and a weakness in the masticatory muscles that impairs their ability to chew (D'Amico *et al.*, 2011, Kolb *et al.*, 2015, Tangsrud *et al.*, 2001). SMA type III, also known as Kugelber-Welander disease, is considered a mild form of SMA. The age of onset is typically used to classify patients in either type IIIa (before 3 years of age) with a 73% probability of walking 10 years after diagnosis or type IIIb (after 3 years of age) with 97% probability (Zerres *et al.*, 1995). There is three or four copies of *SMN2* in 96% of these patients (Mercuri *et al.*, 2012). This mild form affects children as well as adults. Patients usually achieve the majority of motor milestones. Again, patients with SMA type III exhibit a large variety of phenotypes. Some can sit and walk and live through adult life with only minor muscular weaknesses. Some may need the help of a wheelchair considering the muscular weaknesses during their childhood. Those patients may have more complications due to poor mobility, like obesity and osteoporosis for instance (Khatri *et al.*, 2008, Kinali *et al.*, 2004, Shanmugarajan *et al.*, 2009). These patients have a normal life expectancy. The last type of SMA, type IV, represents the mildest form and onset is usually occurring during adulthood. These patients are similar to type III, but with less complications. They are also able to walk and represent only 5 % of all SMA patients (D'Amico *et al.*, 2011, Kolb *et al.*, 2015). SMA patients may have a wide range of phenotypes, though cognition is not affected in any types (von Gontard *et al.*, 2002).

Table 1: Clinical features and onset specificity of the SMA spectrum

TYPE	***Age of onset***	***Clinical features***	**SMN2 *copy number***
0	prenatal	Severe muscle weakness, reduced fetal movements	1
1	< 6 months	Unable to sit without assistance	2-3
2	6-18 months	Can't walk independently	3-4
3 (a & b)	3 years old	Can sit and walk, or wheelchair assistance	3-4
4	Adult	Can sit and walk	4 +

Clinical features of SMA patients that allows the categorizing of every type (0-4) in function of the disease severity. SMN2 copy number are inversely proportional to the disease severity.

1.1.2. NEUROMUSCULAR DEFECTS

SMA is a neurodegenerative and also a neuromuscular disease. As mentioned before, mutation in the *SMN1* gene will cause degeneration of the alpha motor neurons. The SMN1 protein is ubiquitously expressed, though only motoneurons are majorly affected. Lower motor neurons (or alpha motor neurons) are located in the brainstem and the spinal cord, specifically in the ventral horn (Figure 1). They play a major part in the neuronal circuitry for the contraction of peripheric skeletal muscles. For their axon to develop and function adequately, it needs precise guidance, arborisation, and outgrowth. To innervate a muscle, the motor axons will also need make proper synaptic contacts, at the neuromuscular junctions (NMJs), with postsynaptic receptor on muscle fibers to promote contraction. Although the SMA pathogenesis is not yet exhaustive, it is known that these specific mechanisms are deficient, particularly at early stages of the disease.

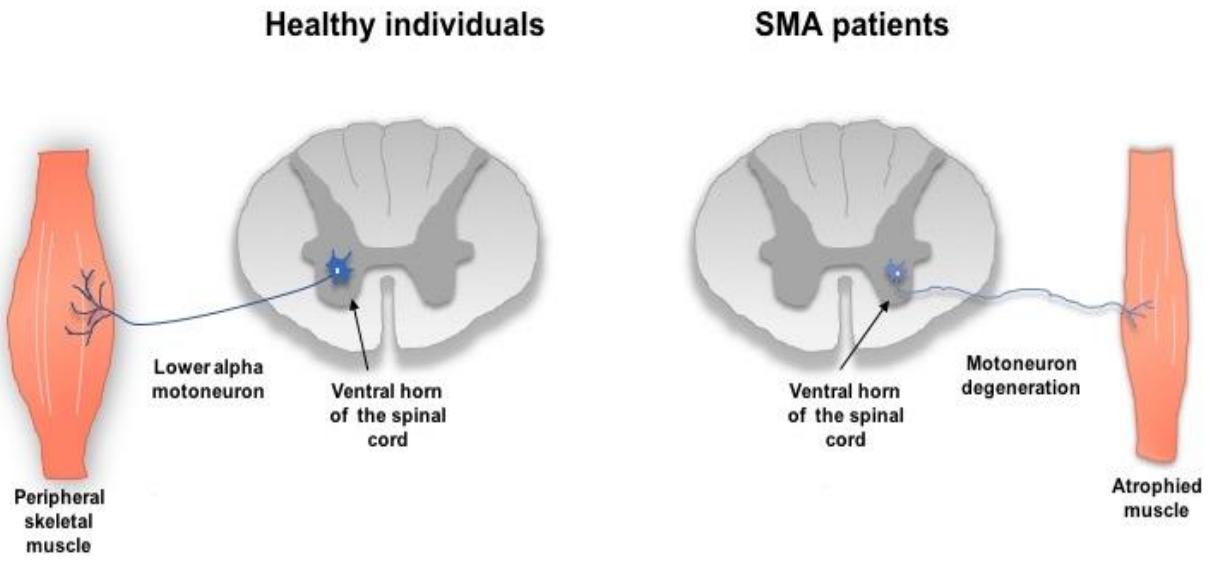

Figure 1 : Anatomy of the neuromuscular circuitry.
Alpha motor neuron are a type of lower motor neurons that have their cell bodies in the anterior or ventral horn of the spinal cord. Their axons will elongate through the body to innervate skeletal muscle and initiate muscle contraction. In SMA patients, low levels of SMN will cause the loss of motor neurons in the spinal cord. Without proper signals from the degenerate motor neurons, muscles will progressively weakened and atrophy.

Hallmarks of SMA are defects at the NMJs and skeletal muscle atrophy. For clinical assessment of SMA patients, motor function is a key feature. Multiple tests have been used to asses motor function like the Gross Motor Function Measure (GMFM)(Nelson *et al.*, 2006), the Expanded Hammersmith Functional Motor Scale for SMA (ExpHFMS)(Glanzman *et al.*, 2011) or the Upper Limb Module for Nonambulant SMA Patients (Mazzone *et al.*, 2011). Furthermore, alterations at the NMJ have been found in patients and animal models of SMA. NMJ maturation defects seem to arise before symptoms onset. Histological evidence has shown early maturation alterations of the NMJ in type I patients during development. SMA fetal samples showed alterations in acetylcholine receptors (AChR) clustering, accumulation of synaptic vesicles and terminal nerve abnormalities (Martinez-Hernandez *et al.*, 2013). These findings were also found in mild-SMA mice that express a constant low level of SMN. Their analysis revealed NMJ defects, as well as neurofilament accumulation aggregates and poor terminal arborization (Kariya *et al.*, 2008). Indeed, SMN was shown to accumulate in growth cones and NMJs during neuronal differentiation and neuromuscular maturation putting forward its role in neuronal and muscular function (Fan *et al.*, 2002). Many researchers confirmed the presence of these defects in the mouse models of SMA, not only at the pre-synaptic levels, but also defects in muscle growth (Dachs *et al.*, 2011, Kariya *et al.*, 2008, Y. I. Lee *et al.*, 2011).

Neuromuscular defects were also confirmed in other animal models of SMA. For instance, in a zebrafish model of low levels of Smn, a group showed that Smn is needed for motoneuron development. Removing Smn very early leads to developmental defects in motoneurons like fewer and shorter dendrites as well as decreased axonal and dendritic network. These defects were rescued only by adding Smn back soon after motor axon outgrowth. These results suggest the important need for SMN during early stages of motoneuron development (Hao le *et al.*, 2013). Another study, using antisense morpholinos (MO) in zebrafish to lower levels of Smn, showed motor axon defects specifically in axon outgrowth and pathfinding (McWhorter *et al.*, 2003). In the *Drosophilia*, a team has shown abnormal motor behavior in a *smn* zygotic mutant. They have demonstrated neuromuscular defects by reduced excitatory post-synaptic currents, disorganized synaptic motor neuron boutons as well as a severe diminution in the clustering of neurotransmitter receptor subunits in the muscles (Chan *et al.*, 2003). Furthermore, *C. elegans smn-1* mutants were shown to display impaired neuromuscular functions through impaired locomotion, but also reduced pumping during feeding (Briese *et al.*, 2009). These nematodes possess a subset of pharyngeal muscles and neurons that allows them to pump their food. A team has shown that these defects were progressive and noted a decrease in their motor neuron function as well (Dimitriadi *et al.*, 2016).

1.1.3. OTHER CELL TYPES ASSOCIATED PHENOTYPES

SMA is considered to be a motoneuron disease, with its major site of pathology being motor neurons. As the SMN protein is expressed ubiquitously, recent studies suggests that multiple deficient tissues and cell types may contribute to overall SMA phenotypes (A. J. Lee *et al.*, 2012, Martinez *et al.*, 2012). Using the Cre-lox technology in mouse, a team (Park *et al.*, 2010) has shown that depleting SMN in motor neuron only induced minor phenotypic alterations compared to ubiquitous depletions. Indeed, it was enough to cause SMA-like phenotype, but these results suggest that other cell types may contribute to SMA pathology, especially in the most severe types of SMA. Another group (Hua *et al.*, 2015) increased SMN exclusively in peripheral tissues and saw complete rescue of necrosis in mild SMA mouse model. Survival of the severe mice model was also extended with improvements in motor function and vulnerable tissues. Thus, they suggest that SMA is not a cell-autonomous defect of motor neurons, in SMA mice, and that peripheral tissues would also be important. Another study focused on the roles of astrocytes in SMA. Glial inflammation is a key component in many neurodegenerative diseases (Glass *et al.*, 2010). As these cells play multiple roles, for instance as neuron's nutriment suppliers and most importantly as modulator of synaptic transmission, they hypothesized that they could contribute to SMA pathology. They restored SMN expression specifically in astrocytes in a ubiquitously low SMN level mouse model. Interestingly, results show increased lifespan, improvements in NMJs and partially normalized cytokine expression in that mouse

model. Indeed, they have also demonstrated that end-stage mouse and post-mortem SMA patient's spinal cord showed astrogliosis and elevated inflammatory cytokines. These data suggest that SMN deficiency in astrocytes could play a direct role in SMA pathogenesis (Rindt *et al.*, 2015).

Heart defects have also been investigated in SMA pathology. Atrial septal defects, dilated right ventricle and ventricular septal defects have all been recognized as congenital heart defects in most severe types of SMA patients. One of the most abundant defects is hypoplastic left heart syndrome that arise at birth causing abnormal blood flow through the heart. Rudnik-Schonebron and al. revealed that approximately three out of four patients with a single copy of SMN2 had congenital septal defects whereas healthy individual's frequency is around 1 in 50 million (Rudnik-Schoneborn *et al.*, 2008). Another group focused on the requirement for SMN in cardiogenesis and its impact on heart abnormalities at multiple developmental stages in a very severe (*Smn−/−, SMN2+/+*) and severe (*Smn−/−, SMN2+/+; SMNΔ7+/+*) SMA mouse model. The results show cardiac remodeling at embryonic stages, even before any quantifiable motoneuron alterations. They also observed structural defects post-birth in the heart of severe mice and increased levels of interstitial fibrosis through aging. The authors suggest that this particular phenotype could be caused by oxidative stress. These results provide evidence that SMN deficiency leads to abnormal cardiogenesis in severe mouse model (Shababi *et al.*, 2010).

Various other cell types have been studied in their contribution to SMA pathogenesis. For instance, muscles, that are directly connected to the motoneurons, have also been studied. A study in severely affected prenatal patients showed that their myotubes appeared smaller in skeletal muscles. This suggests a delay in growth and maturation in SMA muscles (Martinez-Hernandez *et al.*, 2009). Other neuronal populations have also been investigated, like interneurons and sensory neurons to unravel neuromuscular circuitry (Jablonka *et al.*, 2006, Ling *et al.*, 2010, Mentis *et al.*, 2011) as well as Schwann cells (Desarnaud *et al.*, 1998, Hunter *et al.*, 2014). The contribution of SMN protein in the brain has been less studied (Ito *et al.*, 2004, Shishikura *et al.*, 1983), because it is not usually seen in SMA patients. Furthermore, other groups have focused on multiples different peripheral tissues like bones, liver, lungs, pancreas and intestine(Bowerman *et al.*, 2012b, Schreml *et al.*, 2013, Shanmugarajan *et al.*, 2007, Vitte *et al.*, 2004). Taken together, all of these findings suggest that SMN could be important for functioning in multiple cell types. Investigation of SMN deficiency in other tissues could be essential for an optimal treatment for SMA.

1.1.4. CURRENT THERAPIES

1.1.4.1. SMN dependent therapies

Since SMA is a neurodegenerative disease that arises very early in development, drug treatment that allows complete cure of the disease is very complicated to manage. Indeed, if both parents are known carriers, prenatal screening is available. They can undergo a chorionic villus sampling (CVS) at 10 to 14 weeks or amniocentesis at 16 to 20 weeks of pregnancy. The best strategy, especially for more severe types, would be to administrate treatment very early in development, before any motor neuron loss or other phenotypes. But, there is currently no routine screening newborn program for SMA. The longer it is to diagnose a patient, the less benefit they will receive from treatment, because motoneuron degeneration cannot be reversed, only the severity can be reduced. There is currently no effective cure for SMA, but a few treatments have been approved to improve their quality of life and delay neurodegeneration. Since SMA is very heterogenous, there is still a significant amount of patients that do not respond to these treatments.

The first treatment, Spinzara or Nusinursen, was approved in 2016 for all types of SMA. It is an antisense oligonucleotide drug that will elevate SMN levels through restoring *SMN2* pre-mRNA splicing. Since this drug does not cross the blood-brain-barrier, it has to be administered directly into the cerebrospinal fluid through lumbar puncture. Also, injection has to be renewed every 4 months. There is currently an ongoing study called NURTURE that follows 25 infants with SMA who received their first dose of Spinraza before six weeks old, thus before pre-symptomatic stage. So far, this study shows that all infants were alive and none of them required any permanent ventilation system. Every patient was able to sit unassisted and 88% can walk alone. The CHOP INTEND (Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders) was used to assess motor function (ranging from 0 to 64). The score from treated patients revealed they were close to the highest score (64) compared to SMA type I patients that usually have a 20 score. Furthermore, treatment was well tolerated by the patients so far (De Vivo *et al.*, 2019). In clinical studies, lower respiratory infections are the most common side effects. Also, due to the type of administration into the system, patients can develop post-lumbar puncture syndrome, which can lead to severe headaches after the procedure (32%). If left untreated, fatal outcomes can arise through hematoma and seizures. (Ahmed *et al.*, 2006).

Zolgensma, or Abeparvovec, is a gene therapy that was approved in 2019. This treatment was designed to treat all types of SMA in patients up to 2 years of age with a single dose intravenous administration. It is a genetically engineered virus, an Adeno-Associated virus 9 delivering system (AAV9) that carries a functional *SMN1* copy. This transgene will be able to cross blood-brain barrier and allow the expression of

SMN protein throughout the central nervous system (CNS) to be produced in a sustainable way in the cells of interest. In a clinical trial, 15 SMA type 1 patient received one single dose of AAV9-SMN. Three patients received a low dose (6.7×10^{13} vg per kilogram of body weight) and 12 received a high dose (2.0×10^{14} vg per kilogram). All patients had two copies of *SMN2* and were between 0.9 and 7.9 months of age. The study began in May 2015 and in August 2017, no patients have died or needed ventilation assistance. Moreover, 11 out of the 12 high dose patients were sitting unassisted and two of them were walking. The CHOP INTEND scale showed an increase of 9.8 points at 1 month and 15.4 points at 3 months (Mendell *et al.*, 2017). Another study showed reduced pulmonary and nutritional support as well as motor function improvements in treated patients with AAV9-SMN (Al-Zaidy *et al.*, 2019). More clinical studies are ongoing to evaluate pre-symptomatic treatment (NCT03505099) and intrathecal administration (NCT03381729).

Risdiplam is an orally administered therapy still under investigation, with FDA approval expected in 2020. This treatment will be able to treat all patients with SMA type I, II and III. Risdiplam also work as a splicing modulators, just like Spinraza. It will modulate pre-mRNA splicing of SMN2 to promote full length transcript and thus elevate functional SMN protein levels. This treatment shows great promise with a less invasive administration than Spinraza and with distribution in CNS as well as in peripheral tissues (Poirier *et al.*, 2018). Studies using the severe mouse model and the *SMNΔ7* showed increased lifespan, elevated SMN levels and improvements in motor function (Naryshkin *et al.*, 2014, Palacino *et al.*, 2015). Four clinical studies (FIREFISH: NCT02913482, SUNFISH: NCT02908685, JEWELFISH: NCT03032172 and RAINBOWFISH : NCT03779334) are currently ongoing to investigate Risdiplam's efficacy and safety on different types of SMA patients.

1.1.4.2. SMN independent therapies

All the therapies described above enhance SMN levels. Indeed, it helps a lot of patients, but there are still a certain number of them that do not respond to those treatments. Moreover, they only show limited beneficial effects, which lead to think that other combinatorial therapies may be needed. SMN- independent therapies will be helpful to address the pathological irreversible damage in SMA. A variety of pathways have been approached, for instance actin dynamics. They are regulators of multiple altered processes in SMA, like neurite outgrowth, axonal branching or synaptic stability. Actin dynamics have been studied in SMA models (Nolle *et al.*, 2011, Oprea *et al.*, 2008, Sharma *et al.*, 2005). More precisely, the RhoA/ROCK pathway was one of the first independent therapies to have shown beneficial effects. ROCK activity was shown to be upregulated in the spinal cord of the intermediate $SMN^{2B}/-$ mouse model as well as in SMN depleted rodent neuronal cells. Treating mice with a ROCK inhibitor showed improvement of their survival as well in NMJ

maturation and muscle fiber size (Bowerman *et al.*, 2010, Bowerman *et al.*, 2007). Moreover, inactivation of ROCK was found to promote neuronal outgrowth and guidance (Jia *et al.*, 2016, Mueller, 1999). Thus, a group, (Coque *et al.*, 2014), suggested that inhibition of ROCK could improve the maintenance of functional NMJs via increasing acting plasticity. Other actin dynamics modulators were studied such as profilin and plastin3 (Bowerman *et al.*, 2007, Oprea *et al.*, 2008). As an actin-bundling protein, plastin3 (PLS3) was shown to play a role in axonogenesis by increasing the levels of F-actin. The knockdown of this gene in a zebrafish model using MO resulted in 90% lethality. Furthermore, overexpression of PLS3 in a Smn knockdown zebrafish model rescued axonal growth defects (Oprea *et al.*, 2008). In an SMA mouse model overexpressing PLS3, results showed rescued synaptic vesicles aberrations, delayed axon pruning and improvement of neuromuscular transmission (Ackermann *et al.*, 2013). Although these are promising results for PLS3 as an SMA modifier, its expression may be different depending on age or sex of patients (Stratigopoulos *et al.*, 2010). Thus, this therapeutic strategy may only be for a specific type of SMA patient.

Other SMA-modifiers are being studied like the ubiquitin-like modifier activating enzyme 1 (UBA1). A mutation in this gene causes X-linked infantile SMA. UBA1 was shown to directly interact with SMN. Moreover, in spinal cords of SMA mouse model, UBA1 splicing was altered. A group showed that UBA1 knockdown using MO in zebrafish could recapitulate an SMA-like neuromuscular pathology. They did also demonstrate that levels of β-catenin, which plays a role in the subsequent UBA1 pathway, were increased. Inhibition of that protein ameliorated the neuromuscular pathology in zebrafish, drosophila and mouse models of SMA (Wishart *et al.*, 2014). Another group used an AAV9-UBA1 gene therapy in SMA mice. With a systemic delivery, they demonstrated increased survival and motor performance as well as an overall improvement in neuromuscular and organ pathology (Powis *et al.*, 2016a).

Other pathways are being studied for SMN-independent therapies. As a clinically heterogenous disease, multiple aspects have to be taken in charge. Within the era of high throughput technologies, the number of novel molecular candidates will only grow. Combinatorial therapy (SMN-dependent and independent) could be an efficient way to treat the wide spectrum of SMA patients.

1.2. SURVIVAL MOTOR NEURON

1.2.1. GENETIC FUNDAMENTALS OF SMA

SMA is an autosomic recessive neurodegenerative disorder caused by either a deletion (95%) or a mutation (5%) in the *SMN*1 gene on the chromosome 5q13. Furthermore, humans also have an *SMN2* copy that is almost identical to *SMN1*. Studies have reported a very high degree of similarity between the two copies even in their introns (Monani *et al.*, 1999). The only major difference lies within the exon 7 (Figure 2). There is a transition from a C to T that results in exon 7 skipping. This slight change causes a disruption of an exon splice enhancer (ESE) that results in *SMN2* to produce more of the truncated form (*SMN*Δ7). (Lorson *et al.*, 2000). Thus, *SMN2* contribute poorly to overall SMN levels, because its major form is not functional. Still, a small amount of the *SMN2* transcript (10%) can produce a full-length and functional protein which can help manage disease severity. In SMA patients, where the *SMN1* gene is non-functional, SMN levels only relies on *SMN2* copy number (1-8), which was shown to ameliorate disease severity (Mailman *et al.*, 2002). *SMN2* copy numbers varies for each individuals and explains why SMA has such a wide spectrum of severities.

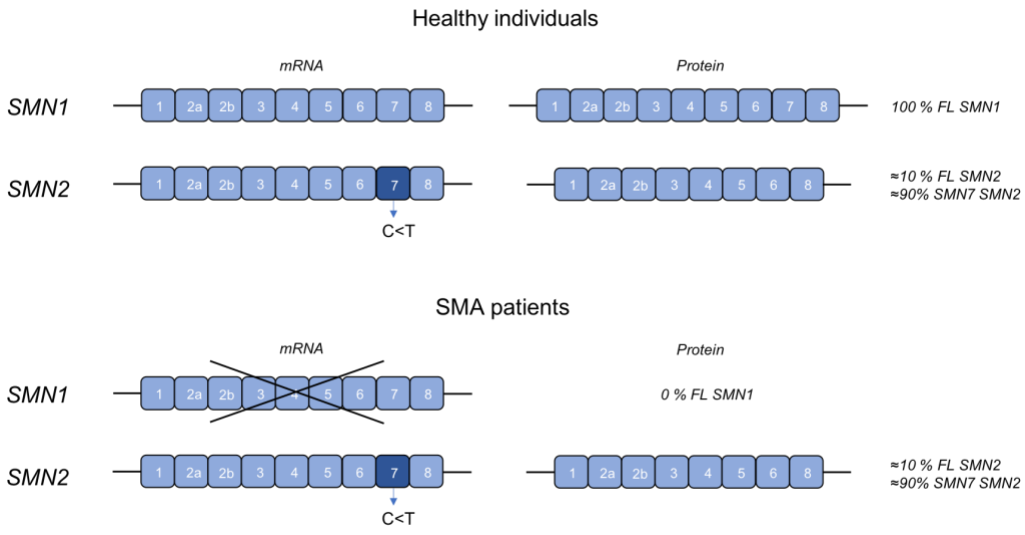

Figure 2: Genetic origins of SMA patient.
Healthy individuals have two copies of *SMN*, *SMN1* and SMN2. *SMN*1 produces 100% of the functional full-length SMN protein while SMN2 only produces approximately 10% of the full-length protein. This event is caused by a mutation in SMN2 that leads to exon7 skipping resulting in a truncating SMN. In SMA patients, the *SMN1* gene is mutated which causes low levels of the full-length SMN protein. SMN2 full-length protein levels are not sufficient to alleviate the burden of the deficient *SMN1* gene, which leads to SMA.

1.2.2. FUNCTIONS OF SMN PROTEIN

1.2.2.1. Axonal transport

The SMN protein is 38 kD with 294 amino acids and is expressed ubiquitously. It is present in the cytoplasm and in the nucleus of all cells. SMN is an RNA binding protein (RBP) that plays multiple roles in RNA metabolism like intracellular trafficking or translation. This specific role was first revealed with electron microscopy showing SMN localization in dendrites and axons of motor neurons in developing rat spinal cord (Pagliardini *et al.*, 2000). This group hypothesized that low levels of SMN could lead to a lack of transport of specifics mRNAs through the axons up to NMJs. Moreover, it was suggested that SMN expression shifts during precise developmental stages. First, SMN is more abundant during embryonic stages than in adult CNS, but its localization also seems to be more nuclear during development and then slowly shifts to be more cytoplasmic as neurons are maturing (Giavazzi *et al.*, 2006). Since SMN is a RBP, it can directly bind to mRNAs or other RBPs to transport polyA-tailed mRNAs for local translation along axons and dendrites (Fallini *et al.*, 2014). The same group also showed that translation within the axon of primary neurons derived from *SMN* -/- mice was dysregulated *in vitro*. Axonal defects were rescued by overexpression of other RBPs like HuD and IMP1, molecular partners of SMN (Fallini *et al.*, 2016). Moreover, using live cell imaging, it was shown that SMN granules were able to achieve rapid bidirectional movements (anterograde and retrograde) and that they were dependent on microtubules in cultured neurons (H. L. Zhang *et al.*, 2003). All of these results demonstrate an extensive role for SMN in mRNA axonal trafficking and translation.

1.2.2.2. RNA metabolism

SMN roles in axonal transport are well documented but its actual most characterized function is in small nuclear ribonucleoproteins (snRNP) assembly, which is important in RNA processing like pre-mRNA splicing. SMN is found to interact with a number of proteins called Gemin (2-8) and together form the SMN complex. This complex is found in cytoplasm and in nuclear gems (Gemini of Cajal Bodies). More precisely, gems are a nuclear structure where there is an accumulation of SMN (Clelland *et al.*, 2009). They are found to be associated with Cajal bodies. Although they are two distinct entities, they are similar in size and number and exert a dynamic relationship together (Q. Liu *et al.*, 1996). Furthermore, the Tudor domain of the SMN protein was shown to interact with coilin, a marker for Cajal bodies (Hebert *et al.*, 2001). The SMN complex interacts with numerous proteins. One of them are the Sm and Sm-like (Lsm) proteins. The SMN complex facilitate the assembly of Sm proteins, in an ATP-dependent manner, on their site of the snRNA (small nuclear RNA) to produce functional snRNPs, more precisely UsnRNPs (Battle *et al.*, 2006).

The Sm site is a uridine rich sequence present in the U snRNAs. There are 7 Sm proteins to form a functional Sm core. Once the core is assembled onto its snRNA, hypermethylation of the 5'cap is also needed for the snRNP translocation into the nucleus. The snRNPs (U1,U2,U4,U5,U6) then assemble with other specific snRNP proteins to form the complex functional machinery called the spliceosome. Then, it can exert its function in the removal of non-coding introns to form mRNAs (Fischer *et al.*, 1993, Mattaj, 1986, Will *et al.*, 2001). Multiple studies have shown splicing defect occurring in SMA models. A group demonstrated a negative feedback loop in SMA depleted mammalian cells that decreases exon 7 inclusion, thus further disrupting its own mRNA splicing. They also confirmed the results in severe-SMA mice with a decreased exon 7 inclusion specifically in their motoneurons (Ruggiu *et al.*, 2012). Furthermore, in SMN-deficient mouse tissues, a group demonstrated unexpected cell type-specific effects on the repertoire of snRNAs and mRNAs. Their findings showed widespread pre-mRNA splicing defects in numerous transcripts (Z. Zhang *et al.*, 2008).

1.2.3. PROTEINS AND RNA PARTNERS OF SMN

As SMN is an RNA-binding protein, it will obviously have numerous mRNA and other protein partners. Its role was established in RNA metabolism, previously described, and in axonal transport. Unraveling SMN's interactions could lead to a better understanding of its function as well as how SMN contributes to specific motor neuron death in SMA. Evidently, SMN was shown to interact with a number of Gemin proteins, because of the known aggregation in gems in the nucleus. As a partner of SMN, gemins can modulate the expression of the SMN complex. A team demonstrated that reducing SMN levels, with RNA interference (RNAi) in HeLA cells, lead to a severe reduction of multiple Gemins. Moreover, a reduction of Gemin 2 or Gemin 6 displayed a decrease in the activity of SMN complex as well (Feng *et al.*, 2005). SMN was also shown to bind to Sm proteins via its Tudor domain, as they are a part of the SMN complex. A missense mutation within the tudor domain (E134K) showed a disruption in the interaction of SMN and Sm proteins, further affecting the assembly of snRNPs (Bühler *et al.*, 1999). This represents only a glimpse of the important researches made on the components of the SMN complex. Multiple studies on SMN and its interacting partners in this complex, specially gemins, were of great help to better understand its role in RNA metabolism and in SMA pathogenesis (Campbell *et al.*, 2000, Charroux *et al.*, 1999, Charroux *et al.*, 2000, Fischer *et al.*, 1997, Q. Liu *et al.*, 1997).

Another very interesting interactions between SMN and FUS were demonstrated. Just like SMN, FUS is an RNA-binding protein, which causes amyotrophic lateral sclerosis (ALS), another motor neuron disease. A team reported that FUS associated with the SMN complex via u1 snRNP and with a direct interaction between FUS and SMN. They showed that FUS was required for Gem formation in HeLa cells. Furthermore,

in ALS patient fibroblasts with either a *FUS* or *TDP-43* mutation, there was a reduction in the number of gems. This interaction is particularly interesting for which it suggests a potentially common and disrupted pathway in both SMA and ALS (Yamazaki *et al.*, 2012).

SMN's role in actin dynamics and axonal transport has been identified and described previously. This function is particularly known, because of the many partners of SMN in this pathway. SMN's exons contain proline-rich motifs at the COOH terminal. These motifs can bind to a known group of actin-binding protein ; profilins. A team has shown an interaction between SMN and two isoform of profilin, but mostly profilin II, through a yeast two-hybrid system and a coimmunoprecipitation experiments (Giesemann *et al.*, 1999). Another group later showed that the two proteins (and others like gemin2, gemin6, gemin7 and unrip) colocalize in the cytoplasm of differentiating rat PC12 cells and at the growth cones of neurite-like extensions. (Sharma *et al.*, 2005). SMN was also shown to interact with the β-actin mRNA-binding protein IMP1 (Fallini *et al.*, 2014) as well as hnRNP-R, which binds the 3'-UTR of β-actin mRNA and colocalize in the growth cones of motoneurons (Rossoll *et al.*, 2002). Moreover, a novel interaction was found between SMN and HuD. The latter is a neuron-specific RBP that is involved in post-transcriptional regulation of mRNAs important for neural development (Pascale *et al.*, 2004). HuD was shown to coimmunoprecipitate with the SMN complex in primary embryonic cortical neurons. In a zebrafish model, both proteins were shown to interact in motoneurons during formation of axonal branches and dendrites. *HuD* mutant zebrafish models displayed decreased motor axon branches as well as fewer dendrite and locomotion defects, similar to SMA phenotypes (Hao le *et al.*, 2017). Partners of the SMN/HuD complex were also investigated. The mRNA CPG15, which promotes neuritogenesis (Naeve *et al.*, 1997), was shown to interact with both HuD and SMN in spinal motor axons. Smn deficiency reduces the mRNA levels of CPG15 and its overexpression ameliorated the phenotype of a *smn*-MO zebrafish model (Akten *et al.*, 2011). Another mRNA involved in axonal outgrowth, known as Gap43, was shown to be decrease in both *smn* and *HuD* zebrafish mutants. Expression of *HuD* in an *smn* zebrafish mutant was able to rescue motoneurons defects as well as Gap43 mRNA levels(Hao le *et al.*, 2017). These results demonstrated that both proteins seem important in motoneuron development and mRNA stability in neurons. Unravelling SMN's partners is key for understanding its multiple functions. Discovering new partners could bring further knowledge into SMA pathogenesis as well.

1.2.4. MODELS OF SPINAL MUSCULAR ATROPHY

Having a good model is key for the understanding of a disease. Many SMA models in various types of animals have been generated, but they all share a common obstacle: they have one copy of *SMN*. Indeed, there is only an *SMN2* copy in humans. It is then more challenging to create a model that recapitulate all types of SMA and their corresponding phenotypes, but many genetic techniques have been used to handle this difficulty and allow a great model.

1.2.4.1. Mouse models

One of the most popular SMA model is the mouse. Although they are very closely conserved to humans, it still has some limitations. They possess one copy of *SMN* that is 82% identical to humans (Viollet *et al.*, 1997). The homozygous knockout (*Smn*-/- or *Smn* null mice) results in embryonic lethal animals at the 8-cell stage (Schrank *et al.*, 1997), which demonstrate that SMN is essential and very important for development. Additionally, a heterozygous knockout of SMN did not show all the SMA hallmarks and have a normal lifespan (Jablonka *et al.*, 2000). It is now clear that other genetic manipulations had to be coupled with the *Smn* null mice since only SMA patients can rely on the SMN levels of the *SMN2* copy. A severe SMA mouse model (*Smn* -/- ; *SMN2* $^{+}/^{+}$) was created by adding human *SMN2* transgene onto the *Smn* null mice. The copy number of *SMN2*, just like in humans, can modulate the disease severity. *SMN2* results mostly in the production of *SMNΔ7*, but still produced a small amount of full-length SMN as well. By adding only one or two copies, researchers have showed that it could rescue embryonic lethality. The mice showed a normal number of motor neurons at birth, but they were reduced at postnatal day 5 and the mice died at postnatal day 7 (Monani *et al.*, 2000b). This model represents a more severe form of SMA, like SMA type I in humans, and is then called the "severe SMA model". A full rescue can be done by adding eight copies of the *SMN2* transgene. Moreover, another team generated a "mild SMA model" using the same logic. They showed that by adding different *SMN2* copy number into the *Smn* null mice, the whole SMA spectrum could be modelled. Their SMA-like mice showed fewer muscles fibers, atrophic muscle bundles as well as a loss of large motor neurons in the anterior horn of the spinal cords, similar to SMA patients (Hsieh-Li *et al.*, 2000).

Another very similar model was generated by adding human *SMNΔ7* cDNA into the severe SMA model, known as the SMNΔ7 model (*Smn*$^{-/-}$; *SMN2*$^{+/+}$; *SMNΔ7*$^{+/+}$). They showed that it could increase lifespan from 5 days to 13 days postnatal, which allows scientists a larger window to study SMA in mice. This team also demonstrated in cultured cells that SMN and SMNΔ7 associate with each other by forming heterotypic complexes. Theses complexes could potentially stabilize SMNΔ7, ameliorating SMA severity (Le *et al.*,

2005). Another team used a different interesting approach to generate an intermediate SMA model (Bowerman *et al.*, 2009). They created a knock-in transgenic mouse line ($Smn^{2B/-}$) with mutations within the exon splicing enhancer (ESE) of exon 7 on the endogenous *Smn*. This causes alternative splicing of *Smn* resulting in smaller ratio of full-length *Smn*/*SMNΔ7*, with approximately 15 % of the full-length protein. Their lifespan is around one month of age and show a reduction in motor neuron in the brain stem and spinal cord at P21. Various other mice models are available at this moment (Bebee *et al.*, 2012, Monani *et al.*, 2000a, Sleigh *et al.*, 2011). Multiple other transgenics can be utilized to model this disease. For instance, a common *SMN1* mutation found in patients, A2G, was added to a severe SMA model (Monani *et al.*, 2003). Also, tissue-specific model were generated to assess SMN role into a specific cell population with the Cre-Lox system (Cifuentes-Diaz *et al.*, 2001, Park *et al.*, 2010, Vitte *et al.*, 2004). Finally, the great variety of these SMA mouse models allows us to better understand the complex pathogenesis of SMA and brings us closer to new therapeutic strategies.

1.2.4.2. iPSC

Induced pluripotent stem cells (iPSCs) can also be used as a model for SMA. What makes it an interesting model is that easily available cells, like fibroblasts, can be harvested from SMA patients and be reprogrammed to a pluripotent state using specific factors. Then, with the appropriate conditions, they can be derived into a variety of cell types, including motor neurons. A team has demonstrated the feasibility of this technique and also that newly derived motoneurons were able to maintain the disease genotypes and defects (Ebert *et al.*, 2009). A major disadvantage of this model lies in the method of reprogramming. Main pitfalls consist of viral DNA integration into the host genome or a very low yield of efficiency (Medvedev *et al.*, 2010). Patients derived iPSCs allow an ethical human model system that is very useful the development of cell therapy, disease modelling and drug screening. Researchers were able to recapitulate the disease phenotype in iPSCs. A team showed that the capacity to form motoneurons was reduced in SMA iPSCs as well as defects in neurite outgrowth. They were able to rescue these phenotypes by adding wild-type SMN, showing that the abnormalities were indeed caused by SMN deficiency and not by clonal variability (Chang *et al.*, 2011). Moreover, another team used iPSCs in which *SMN* was knockdown to demonstrate impaired axonal outgrowth of spinal motor neurons and motor neuron loss. By restoring the expression of the *FL-SMN*, they were able to ameliorate disease phenotypes (Wang *et al.*, 2013). A proteomic study of iPSCs-derived motor neurons SMA patient revealed reduced expression of proteins involved in developmental and differentiation pathways. They compared these cells with the genetically matched patient's fibroblasts to identify distinct pattern of expression that could explain the specific motoneuron's vulnerability. They have identified depleted levels of UBA1, a known SMA modifier, further

confirming its role in the SMA pathogenesis (Fuller *et al.*, 2015). Finally, the general idea of having iPSCs as a model is to use it as a therapy. Briefly, they would generate iPSCs from SMA patient's fibroblast and correct them in vitro before reimplantation into the patient. A team tested this idea by converting the SMN2 gene to an *SMN*1-like gene into the iPSCs from patient's fibroblasts. They were then derived into motor neurons and transplanted into an SMA mouse model spinal cord to address motoneuron degeneration. The results show an increase in the animal's lifespan as well as improved disease phenotypes (Corti *et al.*, 2012).

1.2.4.3. Drosophilia

The fruit fly, *Drosophilia Melanogaster*, has also been used to model SMA. This model organism has a short life cycle of 12 days and is easy and inexpensive to grow or maintain. The ease of forward and reverse genetic approaches allows for rapid and efficient modelling of diseases. Indeed, null *smn* mutation is also larval lethal in this organism, because of its unique copy. As mentioned before, a team was able to demonstrate neuromuscular defects in *smn* zygotic mutants, further confirming the role of Smn at the NMJ (Chan *et al.*, 2003). Another team used hypomorphic *smn* mutants (Rajendra *et al.*, 2007). They showed an inability to fly or jump as well as neuromuscular defects. Interestingly, they also put forward Smn's role as a sarcomeric protein, which implies a muscle-specific function. A colocalization of dSmn with sarcomeric actin forming a complex with α-actinin was shown. Furthermore, in *smn* mutant, myofibers failed to form thin filaments and, interestingly, their phenotypes were very similar to *Act88F* mutants, which is a flight muscle-specific actin isoform.

1.2.4.4. C. elegans

The nematode *Caenorhabditis elegans* (*C.elegans*) has also been used to model SMA. Although it has a very simplified nervous system, it is still very useful and easy to work with. Its fully sequenced genome contains one *SMN* ortholog, *smn-1*, producing a protein that is 36% identical to the human SMN (Anonyme, 1998, Bertrandy *et al.*, 1999, Miguel-Aliaga *et al.*, 1999). Moreover, this model allows straightforward genetic manipulation. For instance, the knockdown of a gene can be achieved via feeding the worms with RNAi (RNA interference). It has been shown that decreasing endogenous levels of *smn-1* with RNAi in *C. elegans* caused embryonic lethality showing an essential role for SMN in development once again (Miguel-Aliaga *et al.*, 1999). The few surviving worms showed a number of severe deficiencies like uncoordination, paralysis, lack of muscle tone and sterility. Furthermore, they generated a green fluorescent protein fusion construct containing the full-length *smn-1* and its upstream sequence. They showed fluorescence in the nervous system, body wall, vulval muscle cells, hypodermal and gut cells as well as the excretory cells. Fluorescence in the nuclei of germ cells was also noted from the zygotic stage. With these results, they have

showed that the SMN-1 protein and its mRNA were transmitted as a maternal contribution. Another team characterized the *smn-1*(*ok355*) deletion allele that removes the majority of *smn-1* with its translation start codon. It resulted in late larval arrest, short lifespan as well as impaired locomotion. Because of the maternal contribution of SMN-1, early larval development was normal (Briese *et al.*, 2009). With the ease of genetic and highthroughput manipulation, *C. elegans* is a key model for the finding of new potential modifiers and for high throughput drug screening. It may allow for a fast and efficient way to find new therapeutic targets for SMA.

1.2.4.5. Zebrafish

The ease and use of zebrafish in modeling neurodegenerative disease will be discussed in later sections. Many different genetic tools can be used to generate SMA zebrafish models. Like the other animal models of SMA, it only has one gene similar to SMN1 in humans, thus complete deletion of this gene will lead to embryonic lethality. Although maternal contribution of mRNAs and proteins in zebrafish have also been suggested (Giraldez *et al.*, 2006). Knockdown approaches with MO or RNAi have been used widely in zebrafish, but MO's specificity is still controversial and under investigation (Kok *et al.*, 2015). Moreover, transcription activator-like effector nucleases (TALEN), Zinc-finger nucleases (ZFN) or CRISPR/Cas9 are also utilized to study a gene's function in zebrafish (Hwang *et al.*, 2013). A team has used MO to transiently knockdown *smn* in zebrafish in order to assess Smn's function in motoneuron development. Results showed spinal motor axon-specific truncations and branches. They further reduced Smn levels in single motoneurons to demonstrate that these defects were a cell-autonomous function of Smn in motoneurons (McWhorter *et al.*, 2003). Furthermore, RNAi approach was also used to recapitulate different SMA severities into the zebrafish. By using three synthetic miRNA-based backbones, they were able to reduce Smn protein up to 90%. These mutants displayed very early and severe phenotypes including abnormal motor neurons and swimming as well as shorter size and lifespan. They have also developed a less severe SMA model with a smaller reduction of *smn1*. These mutants displayed later onset phenotypes including abnormal swimming behavior, scoliosis and weight loss (Giacomotto *et al.*, 2015). Another very interesting and relevant zebrafish model was generated by crossing a fish containing a human *SMN2* transgene with the smnY262stop$^{-/+}$ line that was previously characterized (Boon *et al.*, 2009). With this new model, they have showed a modest increase of Smn levels as well as survival compared to mutants without *hSMN2* and delayed presynaptic defects(Hao le *et al.*, 2011).

1.3. CRISPR/CAS9

1.3.1. CRISPR/CAS9 MECHANISMS

At first, the CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats-Cas9) system was known to be a part of the adaptive immunity of bacterias and archaeas. They used short RNA sequences coupled with an endonuclease to target and inactivate the intruders (viral) genetic materials. They also kept that information as a genetic memory in case of further infections from phages (Marraffini *et al.*, 2010). The novel CRISPR/Cas9 system is now known all over the world to be a powerful genome editing technique. Indeed, it is still widely under investigation for its therapeutic role, but its use in the laboratory is very efficient so far. Briefly, CRISPR/Cas9 will allow a double-strand break onto a specific region in the genome through Watson-Crick base pairing (Figure 3). CRISPR/Cas9 consists of multiple important components. Firstly, the single-guide RNA (sgRNA) is a specific RNA sequence that represents a fusion of two elements ; crispr RNA (crRNA) and trans-activating crRNA (tracrRNA). The crRNA part of the sgRNA is the complementary sequence of your target gene and the tracrRNA will allow binding to the Cas9. Secondly, the Cas9 can only bind to its specific sequence in the presence of the PAM sequence (Protospacer Adjacent Motif). It is usually a 3 base pair (5'-NGG-3'), but is specific to each Cas nuclease. The break will occur upstream of the PAM sequence. Finally, the CRISPR-associated protein (Cas9) is an endonuclease that will be directed onto the specific DNA, with the help of the sgRNA, to make a double-stranded break. Following the cut, and without any repair template, the cell will undergo the Non-Homologous-End-Joining pathway (NHEJ). This mechanism is prone to error, thus can result in random indel mutations. These mutations can cause a frameshift that will result in a premature stop codon and affect the functionality of the protein (Ran *et al.*, 2013).

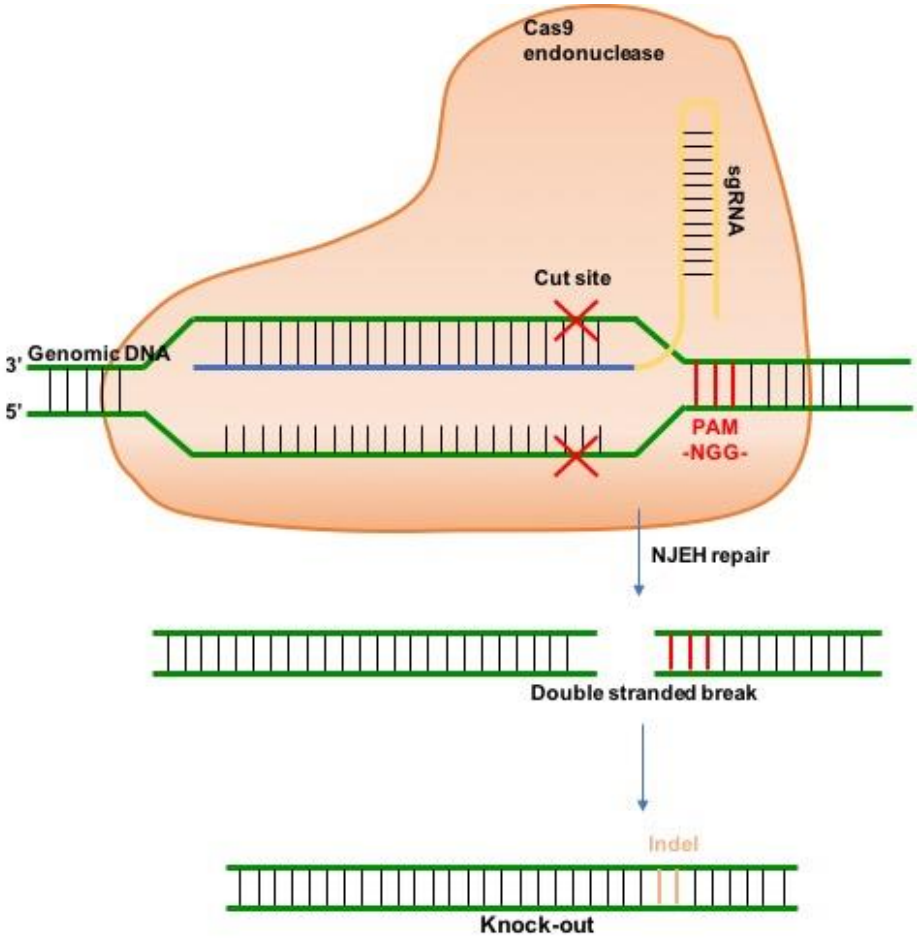

Figure 3: CRISPR/Cas9 mechanisms.
The sgRNA will hybridize to its target genomic DNA sequence preceding the PAM sequence (5'-NGG-3'). This will lead to a double-strand DNA break 3 base pair upstream of the PAM sequence. Repair mehanism will then occur through non homologous end joining if no template is added. Since this mechanism is prone to error, indels may be generated further causing frameshift mutation leading to a loss-of-function or knock-out model.

1.3.2. DISEASE MODELING USING CRISPR/CAS9 IN ZEBRAFISH

Before the CRISPR/Cas9 era, other genome engineering tools were used for targeted mutations, mostly ZFNs and TALENS . But their several weak points, like high cost, design difficulty or long cloning steps, led them to be slowly set aside. Moreover, MOs were frequently used in zebrafish for gene knockdown purpose. As an RNA molecule, they will sterically block translation in a sequence specific manner and then reduce the outcome protein levels. However, because of its RNA nature, it will be easily degraded or diluted which leads to only a transient knockdown. Many reviews have focused on the specificity of MOs and suggest not relying completely on this genetic tool (Kok *et al.*, 2015, Robu *et al.*, 2007).

With the advent and popularity of the CRISPR/Cas9 system, it has now become a technique of choice for targeted mutations. Hwang et al. were the first to demonstrate the feasibility of generating in vivo zebrafish models using the CRISPR/Cas9 system. They showed that using customizable sgRNAs with the Cas9 could disrupt endogenous genes in zebrafish embryos. They were able to successfully introduce site-specific indels (insertion/deletion) mutations with a frequency between 24 % and 59%. Their sgRNA:Cas9 system was also able to target 80% of the sites for which they tested (Hwang *et al.*, 2013). It was also demonstrated that multiple sgRNAs could be used to generate multiple genome modifications (Ota *et al.*, 2014).

Briefly, the sgRNA and Cas9 can be directly injected into the one-cell stage of zebrafish embryos. To generate a stable knockout line, mutations in the germline are necessary and can be achieved through genetic mosaicism. F0 loss-of-function mutations carriers will be allowed to grow and crossed to generate heterozygous F1 carriers. The F2 homozygous larvae will have the desired mutation in every cells and can be further used for multiple applications as an isogenic stable knockout line (Cornet *et al.*, 2018). This fast and effective method allows researchers to study a wide range of genetic diseases as well as phenotypic drug screening. Furthermore, CRISPR/Cas9 cannot only be used for stable knockout line, but also several other genetic modifications. A group, (Auer *et al.*, 2014), has reported to have successfully generated a knock-in zebrafish model. By adding a donor plasmid with the sgRNA and Cas9, they showed an efficient targeted integration through the homology-independent double-strand break repair pathway. Moreover, the CRISPR/Cas9 system can also be modified for other genetic purposes. For instance, CRISPR interference (CRISPRi) can be used to repress genes. By using one or multiple sgRNAs, it is possible to moderate the level of gene expression. It will sterically repress transcription by blocking either its initiation or elongation due to a catalytically inactive Cas9 (dCas9)(Larson *et al.*, 2013, Long *et al.*, 2015).

1.4. USING ZEBRAFISH TO MODEL SMA

1.4.1. EASE OF GENETIC MANIPULATION IN ZEBRAFISH

The zebrafish (*Danio Rerio*) is a powerful model organism for many reasons. First, on a more logistic basis, using zebrafish is cheaper than other vertebrates. They are also small and reproduce rapidly, thus a great number of fish can be maintained in a proper facility. They can produce hundreds of eggs each week, which allows scientists to perform many experiments rapidly with powerful statistic values. Moreover, zebrafish embryos have a fast extrauterine development. They are transparent, which allow to see their development very easily and study it with a simple microscope. Zebrafish is an ideal model to study early development because of those aspects. Secondly, on a genetic basis, approximately 70% of human genes have homologs in zebrafish and 84 % of human genes are known to be related to disease have a zebrafish counterpart (Howe *et al.*, 2013). Zebrafish fully sequenced genome is available as well as multiple open source databases making it really easy to work with when using RNA-sequencing or other bioinformatic tools. Genome editing is also straightforward using zebrafish to investigate the function of a gene making it a leading model for vertebrate genetics. Several techniques can be used for genetic loss-of-function like MO for transitory knockdown, miRNA based knockdown, TALEN or CRISPR/Cas9 system for global knockdown. Genetic gain of function can also be assessed by CRISPR knockin approaches or injecting blastocysts with mRNA for instance or with inducible genomic regulatory elements (e.g. GAL4/UAS system), which can target a smaller set of cells. Based on these aspects, the zebrafish model allows researchers to generate rapid and efficient genetic model organisms.

1.4.2. STUDYING NEURODEGENERATIVE DISEASE USING ZEBRAFISH

As mentioned before, many human genes related to diseases have a zebrafish counterpart. It applies for neurological diseases as well. In fact, zebrafish possesses highly conserved genes and physiological process involved in the nervous system. For this reason, many researchers are now using the zebrafish for modelling of neurodegenerative disorders (Bandmann *et al.*, 2010). Its nervous system is also very well characterized, which makes it a great model for these types of diseases (Ince *et al.*, 1995). The zebrafish possess two different types of motoneurons. First, the primary motoneurons possess large cell bodies and appear around 24 hpf. There are three distinct primary motoneurons per spinal cord hemisegment (CaP, MiP and RoP) that innervate either dorsal, rostral or ventral region of each myotome. Secondary motoneurons begin extending their axons a little later during development. They are usually smaller but more numerous, approximately 30, and their axons extend ventrally and laterally of the Mauthner axon to the ventral root (Beattie, 2000, Myers *et al.*, 1986). This model also provides a clear method for the visualization of motoneurons during

development and is really useful for studying the neuromuscular system in development and diseases. Furthermore, the zebrafish spinal cord at embryonic larval stage is functionally and also anatomically similar to humans (Eisen *et al.*, 1986). The transparency of embryos and early larval stages makes it even easier to work with and develop useful methods like whole-mount imaging to study changes in motor neurons. Numerous other applicable *in vivo* tools can be used with the zebrafish like easy behavioral tests to analyse motor activity, gene expression modulation and simple chemical drug testing.

1.4.3. DRUG SCREENING

Another interesting aspects of the zebrafish model is that, drugs can be added to their water or injected into the cells which makes toxicological screening very easy. Drug screening with the zebrafish is rapid, efficient and allows for the advance of preclinical trials. Once tested in the fish, lead drugs can next be translated into an *in vivo* rodent model. With the high quantity of embryos, multiple compounds can be tested simultaneously. Moreover, they have a wide range of disease-associated phenotypes and signs of toxicity are easily noted under a microscope. For instance, their physiology, motor activity, metabolism or behavior allows crucial knowledge about the effects of a drug. As a whole living organism, the pharmalogical information about absorption, distribution, metabolism and excretion become readily available. Furthermore, they have a fully functional organ system that function just as their human counterparts for the most part, which makes it a much better model than cultured cells (MacRae *et al.*, 2015). With the advent of genome editing and high-throughput technologies, drug discovery projects with the zebrafish will be key for finding new therapeutic strategies or repurposing existing drugs to a variety of diseases.

To conclude, these are the major advantages why zebrafish is an important model system. Although rodent models are very popular and used in research, they are not well suited for high-throughput screenings. Drug screenings in zebrafish allow for identifying potential drugs in a less expensive and faster way. Furthermore, just like rodents, behavioral assays, like investigating learning, memory, sleep, stress or social experiment can be done in zebrafish as well (Basnet *et al.*, 2019, Norton *et al.*, 2010). These aspects further confirm the essential relevance and usefulness of using zebrafish in research.

2. ARTICLE

Modeling SMA in zebrafish: A robust *in vivo* model for screening candidate therapeutic compounds

Marilou Préville[1], Alexandra Lissouba, Shunmoogum A. Patten[1,2*]

Affiliations:

1. INRS- Centre Armand-Frappier Santé et Biotechnologie, Laval, Canada.

2. Centre d'Excellence en Recherche sur les Maladies Orphelines - Fondation Courtois (CERMO-FC), Université du Québec à Montréal (UQAM), Montréal, QC , Canada.

Author contributions

MP and SP designed the experiments, interpreted the results and wrote the manuscript. MP collected, analyzed and interpreted the results. AL helped in the generation of the *Hb9:smn* and *Alx:smn* lines. All authors read and edited the manuscript.

Due to the COVID-19 pandemic, we encountered some unexpected delays and the manuscript is still in preparation for submission to *Biomolecules*.

Abstract

Spinal Muscular Atrophy (SMA) is a rare autosomal recessive neurodegenerative disease primarily caused by mutations in *SMN1*. It is the leading genetic cause of infant mortality for which there is currently no effective cure. Despite recent advances in the SMA therapeutics, there remains a need to identify potential therapeutic compounds that can exert their effects via non-SMN dependent mechanisms. Morpholino-based knockdown is commonly used in zebrafish to study SMA pathogenesis. However, this smn knockdown model is transient and not appropriate for efficient drug discovery purposes. Thus, development of a stable model for screening candidate drugs would be valuable for identifying of lead compounds as potential therapeutics for SMA. Here we generated and characterized an *smn* KO zebrafish mutant using the CRISPR/Cas9 genome targeting. The *smn* mutants displayed impaired locomotor activity, axon branching defects and abnormal NMJ structures. We demonstrated that loss-of-function of *smn* specifically in motoneuron is principally the cause of motor deficits, motoneuron loss and muscle atrophy in zebrafish. As a proof-of-principal, we showed that treatment of the *smn* mutant larvae with salubrinal significantly improved locomotor activity. These findings show the potential value of using this zebrafish SMA model for efficient in vivo screening of potential therapeutic compounds.

INTRODUCTION

Spinal muscular atrophy (SMA) is an autosomal recessive inherited disorder characterized by the degeneration of the anterior horn cell from the spinal cord. SMA is caused by the homozygous deletion (>95%) or mutation of the telomeric Survival Motor Neuron-1 (*SMN1*) gene on chromosome 5q13 (Lefebvre *et al.*, 1995, Melki *et al.*, 1990). There are several types of SMA categorized from I to IV ranging from severe to mild and mainly based on clinical criteria and the age of onset. In humans there are two copies of the *Survival Motor Neuron* gene, *SMN1* and *SMN2*. *SMN2* is a copy gene of *SMN1*, but differs by lacking the exon 7, which produces weaker levels of full-length functional protein. Although mutations in *SMN2* are not responsible for clinical phenotypes, several studies have demonstrated that SMN2 copy number has an effect on the severity of the disease(Campbell *et al.*, 1997, Mailman *et al.*, 2002).

SMA affects approximately one in 6000-10,000 live births and has a carrier frequency of one in 50, which makes it the leading genetic cause of infant death (Ogino *et al.*, 2004, Roberts *et al.*, 1970). The early symptomatic stages of SMA are characterized by abnormalities of neuromusular junction (NMJ)(Cifuentes-Diaz *et al.*, 2002, Dachs *et al.*, 2011, Kariya *et al.*, 2008, Kong *et al.*, 2009, Ling *et al.*, 2010, Murray *et al.*, 2008, Torres-Benito *et al.*, 2011). The SMN1 protein is ubiquitously expressed, despite many investigations; it is still unclear how low levels of SMN1 can directly affects motoneurons and ultimately neurotransmission at NMJs. Importantly, current therapeutic strategies under development are almost exclusively based on increasing functional SMN protein(Benkhelifa-Ziyyat *et al.*, 2013, Brichta *et al.*, 2003, Hua *et al.*, 2010, Mattis *et al.*, 2009, Riessland *et al.*, 2010, Rigo *et al.*, 2012). Other therapeutic strategies directed to SMN-independent targets to induce neuroprotection and myotrophic effects(Bowerman *et al.*, 2012a, Merlini *et al.*, 2003, Nizzardo *et al.*, 2011) have also been explored but considerably less advancements have been made in search of non-SMN dependent treatments. Given the divergence in prognoses due to SMA type designation, the application of a combination of non-SMN dependent and SMN restoring therapies, would likely be essential to treat SMA. Therefore, there is an urgent need to develop and assess new non-SMN therapeutics for this incurable disease, which could be particularly effective at early stages of SMA, potentially acting at NMJ.

Zebrafish has been used as an alternative vertebrate model for the cellular and molecular characterization of SMA mechanisms. Motor axonal and behavioural defects are observed in zebrafish *smn* knockdown models (Ali *et al.*, 2000, Brent *et al.*, 2002). Zebrafish as a model has robustly complemented studies in mice and has provided important insights in the etiology of SMA(Hao le *et al.*, 2015, Hao le *et al.*, 2012, See *et al.*, 2014, Wishart *et al.*, 2014). However, the majority of these findings were based on morpholino

knockdown of zebrafish *smn*. This approach is transient, limited to the first few days of development and the specificity of morpholinos has also been the subject of debates recently. The development of CRISPR/Cas9-mediated gene targeting technology has greatly facilitated the use of zebrafish for modelling human genetic diseases. In addition, in the last few years, it has become apparent that zebrafish is a powerful model organism amenable to high-throughput drug screening *in vivo* at embryonic and larval stages (Yeh *et al.*, 2009). It is also becoming valuable in the preclinical pipeline to bridge the gap between *in vitro* assays, which allows for a very rapid first drug screenings, and more costly screens in mammals (Giacomotto *et al.*, 2010). Furthermore, a large amount of molecules can firstly be tested in worms, *C. elegans*, and a selection of them can be translated into a more complex neurobiological system like the zebrafish. Several groups, including us, have explored the potential of zebrafish as a model system for drug screening(Asnani *et al.*, 2014, Tat *et al.*, 2013, Vaccaro *et al.*, 2013, Vaccaro *et al.*, 2012).

To facilitate drug discovery for SMA, we developed a genetic zebrafish *smn* model utilizing CRISPR/Cas9 approach. The *smn* mutant zebrafish exhibit behavioural and neuromuscular defects. We also showed that loss of *smn* specifically in the motoneurons replicates aspects of SMA in zebrafish. Furthermore, we demonstrate the utility of the *smn* mutant zebrafish model system to be used as a rapid *in vivo* screen of potential therapeutic drugs.

MATERIALS AND METHODS

Zebrafish Maintenance

Adult zebrafish (Danio rerio) were maintained at 28°C at a light/dark cycle of 12/12 h in accordance and they were bred according to standard procedures (Westerfield, 1993). Embryos were raised at 28.5 °C, and collected and staged as previously described (Kimmel et al., 1995). All experiments were performed in compliance with the guidelines of the Canadian Council for Animal Care and our local animal care committee.

CRISPR/Cas9 generated mutagenesis

A specific single guide RNA (sgRNA) with a T7 promoter and a NGG PAM site was designed using the CRISPRSCAN algorithm (https://www.crisprscan.org). The following gRNA sequence targeting exon 5 of smn was used (PAM site is indicated in brackets): GGCGGTCCAGGTGGGAAACT(GGG). To synthesize the sgRNA, a PCR amplification was performed and followed by in vitro transcription. The following PCR protocol was used : 1 X 95°C for 3 min followed by 30 cycles of 95°C for 30 s, 58°C for 30s, 72°C for 20s and finally 1 cycle of 72°C for 5 min. In vitro Transcription was performed using the AmpliScribe™ T7-

Flash™ Transcription Kit (Lucigen; ASF3257). Cas9 mRNA was synthesized using the mMESSAGE mMACHINE T3 kit (Invitrogen; AM1348) from pT3TS-nCas9n plasmid (Addgene #46757) linearized with Xba1. A volume of 1 nl containing a mix of 100 ng/µl Cas9 mRNA and 60 ng/µl sgRNA was injected into one-cell stage embryos using the Femtojet4i. Genotyping of smn+/+ (wild-type), +/− (heterozygous) and −/− (homozygous) fish was performed by high resolution melting (HRM) analysis using genomic DNA extracted by boiling larva/clipped caudal fin in 50 mM NaOH for 10 minutes and then neutralized in 0.1 M Tris HCl (pH8).

miRNA cell-specific smn knockdown

The miRNA (miRsmn1414) construct targeting zebrafish smn (Giacomotto et al., 2015) was kindly obtained from Dr. Jean Giacomotto. The miRNA construct was cloned downstream of the upstream activating sequence (UAS) in a mini-tol2 R4R2 plasmid and injected into one-cell stage zebrafish embryos to generate transgenic line. The downstream EGFP-cmlc2, a germ-line transmission of a myocardium specific GFP, assures us the correct insertion of miRNA. Cell-specific smn knockdown is achieved by crossing UAS:miRsmn1414 zebrafish with other fish expressing GAL4 specifically in motoneurons (Hb9:Gal4) or interneurons (Alx:Gal4).

Gross Morphology and survival assessment

Gross morphological observations of embryos and larvae were performed at 2 dpf and 6 dpf using a stereomicroscope (Leica S6E). Survival rate was also assessed during the larval developmental stages.

Behavioural Analysis

Zebrafish larvae (5 dpf) were placed individually into a 96-well plate containing 200µl of E3 media and habituated in the Daniovision® recording chamber (Noldus) for 30 min before start of experiment. Larval locomotor activity was monitored over two hours using the Daniovision® apparatus. Analysis was performed using the Ethovision XT12 software (Noldus) to quantify the total swimming distance.

Motoneuron and muscle immunostainings

Immunohistochemical analyses were performed on 2 dpf zebrafish to visualize muscle morphology and the presynaptic and postsynaptic clusters using the markers SV2 and α-bungarotoxin, respectively.. In adult zebrafish tissues, choline acetyltransferase staining (ChAT) and Hematoxylin/Eosin (H&E) staining were performed.

First, embryos were dechorionated and fixed in 4% paraformaldehyde (PFA) overnight at 4 °C. They were then washed in PBS-Tween 0.1 % four times for 15 minutes. Next, they were incubated in collagenase (1mg/ml in PBS) for 30 min. Embryos were rinsed in PBS-Triton 0.5% three times for 10 min. Next, they were incubated for 1h in blocking solution (2% NGS, 1% BSA, 1% DMSO, 1% Triton) and then incubated for 30 min with PBS-Tween containing 10 ug/ml sulforhodamine-conjugated α-bungarotoxin (Molecular Probes) to bind acetylcholine receptors. Embryos were rinsed with PBS-Tween three times for 10 min, then incubated with the blocking solution for 1 h. The first antibody (anti-SV2 1:200) was then added overnight at 4 °C in the blocking solution. The next day, the embryos were washed in PBS-Tween four times for 15min. Secondary antibody (Alexa fluor 488, 1:1000, Invitrogen) was added in fresh blocking solution for 4h at room temperature. Embryos were washed in PBS-Tween overnight at 4 °C.

For the phallodin staining of actin filaments on muscle fibers, 2dpf embryos were fixed as described previously. They were then washed in PBS-Tween (0.1%) three times for 10 minutes. They were incubated for 1.5 h in PBS-Triton (2%). Then, PBS-Triton containing phalloidin (1:100 Alexa Fluor 488 phalloidin ; A12379) was added overnight at 4 °C. The next day, embryos were washed in PBS-Tween and imaged.

ChAT staining was performed on adult zebrafish spinal cord's section (10 µm). Briefly, to remove parrafin slides were put in xylene two times for 5 minutes. Then, they were rehydrated in solution of ethanol and water for 5 minutes each (100%, 95%, 70%, 50%) and incubated in water for 5 minutes. Next, the slides were washed in PBS for 5 minutes, following by a 68°C citrate bath for 20 minutes. Once the slide were cooled down, they were washed once again for 5 minutes followed by an incubation of 0.3% Triton in PBS for 30 minutes. They were then washed in PBS and blocked for 1h at room temperature (1% Donkey serum, 0.4% Triton in PBS). First antibody was added overnight at 4 °C (1:500 anti-mouse). The next day, slides were washed in PBS and second antibody was added for 2h at room temperature (1:750 Donkey anti-goat AlexaFluor 488). Finally, slides were washed and Prolong gold antifade reagent with DAPI was added before mounting.

For the hematoxylin and eosin staining of the muscles and spinal cord, whole fishes were fixed in 4% PFA for 72h. Fishes were then embedded in paraffin and 15 µm sections were obtained using microtome. Paraffin was removed by incubating slides in xylene twice followed by rehydration with 4 successive baths of respectively 100%, 95%, 70% and 50% ethanol in distilled water. Hematoxylin and eosin staining was then performed.

RT-qPCR

Zebrafish embryos were dechorionated at 48 hpf. Total RNA was extracted with Trizol/Chloroform. Concentrations were determined by Nanodrop. cDNA synthesis was performed using SuperScript™ VILO™ Synthesis Kit (Thermofisher; 11754050) with 1µg of RNA. qPCR was performed using the iTaq™ Universal SYBR® Green Supermix (Bio-Rad; 1725121) in a 20 µL reaction mix. The following qPCR protocol was used: 1 X 95°C for 3 min followed by 30 cycles of 95°C for 30 s, 58°C for 30s, 72°C for 20s and finally 1 cycle of 72°C for 5 min. Experiments were done in duplicate and normalized with the EF1 gene using the ΔΔ CT method.

Western Blotting

Thirty 2dpf embryos were dechorionated and grind on ice in 300 µL RIPA solution with 10% Protease Inhibitor Cocktail (Sigma-Aldrich ; P8340) for 1 min. They were then incubated on ice for 10 min before centrifuge 10 min at 10 000 rpm at 4 °C. Supernatants were transferred in new tubes and stored at -20 °C. Protein dosage was done with the Pierce™ BSA Protein Assay Kit (Thermo Scientific ; 23225). Gels were prepared with the TGX Stain-Free FastCast Acrylamide Kit, 7.5% (BioRad Cat #1610181). 20 µg of proteins was used and mixed with same volume of Laemmli solution with β-mercaptoethanol (5%). Samples were incubated at 95 °C for 5 min and loaded into the gel. Next, we used a standard 10 min. transfer protocol (2.5 A, 25V, 10mm). Membranes were then blocked in 5% milk for 1h. First antibody (MANSMA12 1:500 and anti α-tubulin mouse 1:5000 sigma Aldrich T6199) was added in 2.5% milk and incubated overnight at 4°C. The next day, membranes were washed several times in PBS-Tween (0.1%) and then incubated with the secondary antibody (1:8000 anti-mouse IgG ; A4416) at room temperature for 1 h. Membranes were rinsed several times with PBS-Tween and then revealed with the Clarity™ Western ECL Substrate kit (Bio-Rad Cat#170-5060). Membranes were imaged with the ChemiDoc XRS from BioRad.

Drug treatment

Zebrafish embryos at 4 hpf were treated with salubrinal (50 µM) in embryo medium (E3). The medium was changed daily and behavioural assay was performed at 5 dpf using the Daniovision (Noldus) apparatus.

Statistical Analysis

Data are presented as means ± S.E.M. Significance was determined by using unpaired parametric t-test analysis and One-way ANOVA followed by a Tukey post-hoc multiple comparisons test for normally distributed and equal variance data. Statistical analysis was carried out using Prism-GraphPad® (GraphPad, San Diego, CA, USA).

RESULTS

Generation of *smn* zebrafish mutant

An ortholog telomeric *SMN1* gene is found in the zebrafish genome, known as *smn.* By taking advantage of the CRISPR/Cas9 genome editing technique, we generated a *smn* mutant co-injected a specific sgRNA, targeting the 5th exon of the *smn* gene for disruption, and Cas9 mRNA into one-cell stage zebrafish embryos. DNA Sanger sequencing of target-specific PCR products revealed that the *smn* targeted allele bear a deletion of 7 bases (GTTTCCC) (Figure 1A), resulting in a frameshift mutation and premature stop codon (Figure 1B) in F0 CRISPR mutants or crispants. The mutation affected the functional domains of the protein. We confirmed that the expression of *smn* mRNA and protein were significantly reduced in *smn* zebrafish mutant fish by RT-qPCR and Western blotting, respectively (Figure 1C,D). These results demonstrate a successful generation of a transgenic line of *smn*-depletion zebrafish. F0 CRISPR mutants with the mentioned frameshift have then been used to generate a stable *smn* mutant line.

Morphological analysis of smn zebrafish mutant fish

To examine the consequences of *smn* depletion during zebrafish development, we assessed survival rates and gross morphological changes in *smn* zebrafish mutant. Compared to controls, a significant proportion of *smn* mutant embryos (40%) died prematurely (Figure. 1E). In addition, *smn* mutant embryos exhibited morphological defects such as slight curvature of the body axis at 2 days postfertilization (2dpf) (Figure. 2A) and reduced fish length (Figure. 2B). However, at larval stages, we did not observe any differences in the fish length in 6 dpf controls and mutants (Figure. 2C), suggesting developmental delay in *smn* mutant fish.

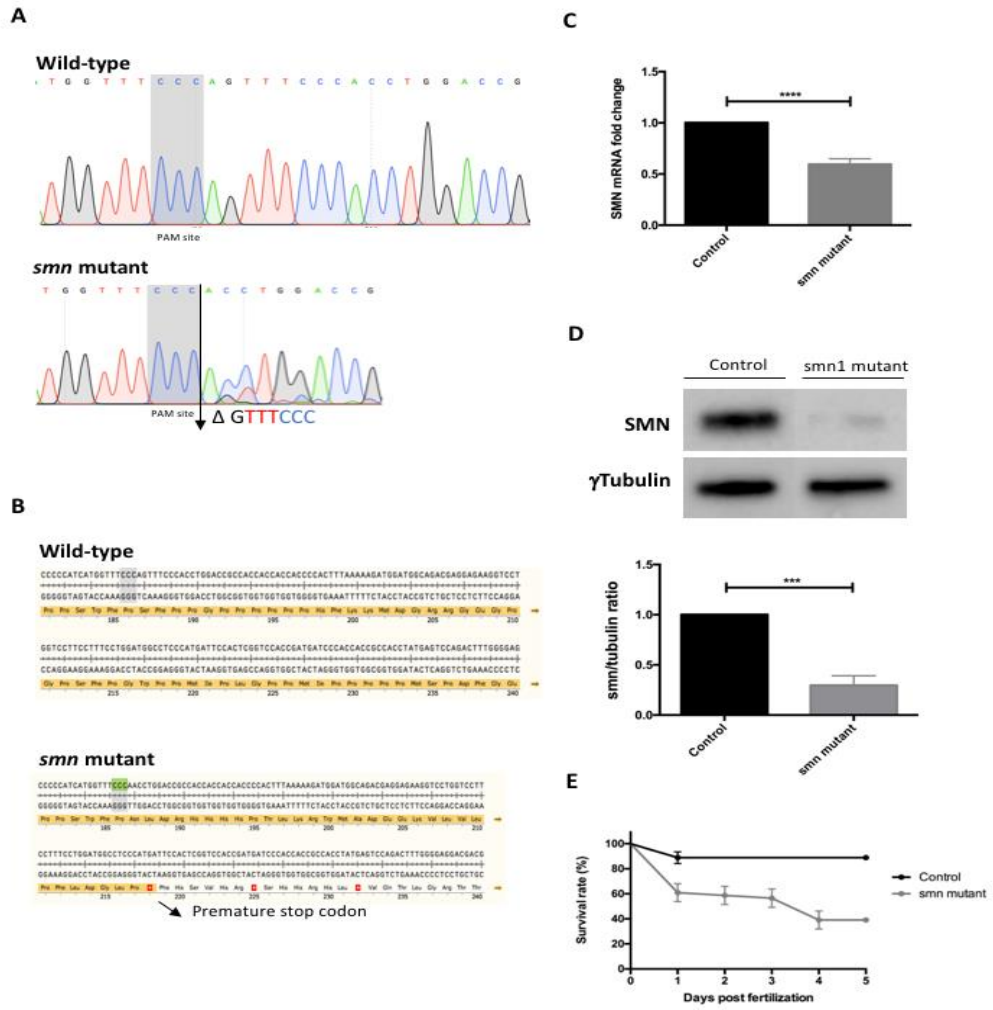

Figure 1. Generation of a zebrafish smn mutant line. (A) Representation of the sequencing results showing a 7 base pair deletion in the *smn* gene (ΔGTTTCCC) causing a premature stop codon (B). Western Blot (C) and mRNA expression (D) analyses of the significantly reduced SMN protein and mRNA respectively in the *smn* mutant zebrafish. For the qPCR, all samples were normalized using the EF1 gene with the ΔΔ CT method. (E) Survival analysis upon injection of sgRNA until 5dpf (n=45). Every experiments was compared with control fish. ** represents a significance of $p < 0.01$, **** represents a significance of $p < 0.0001$. Significance was assessed using an unpaired Student's t-test and all data are represented as mean ± SEM.

Préville & al, Figure. 2

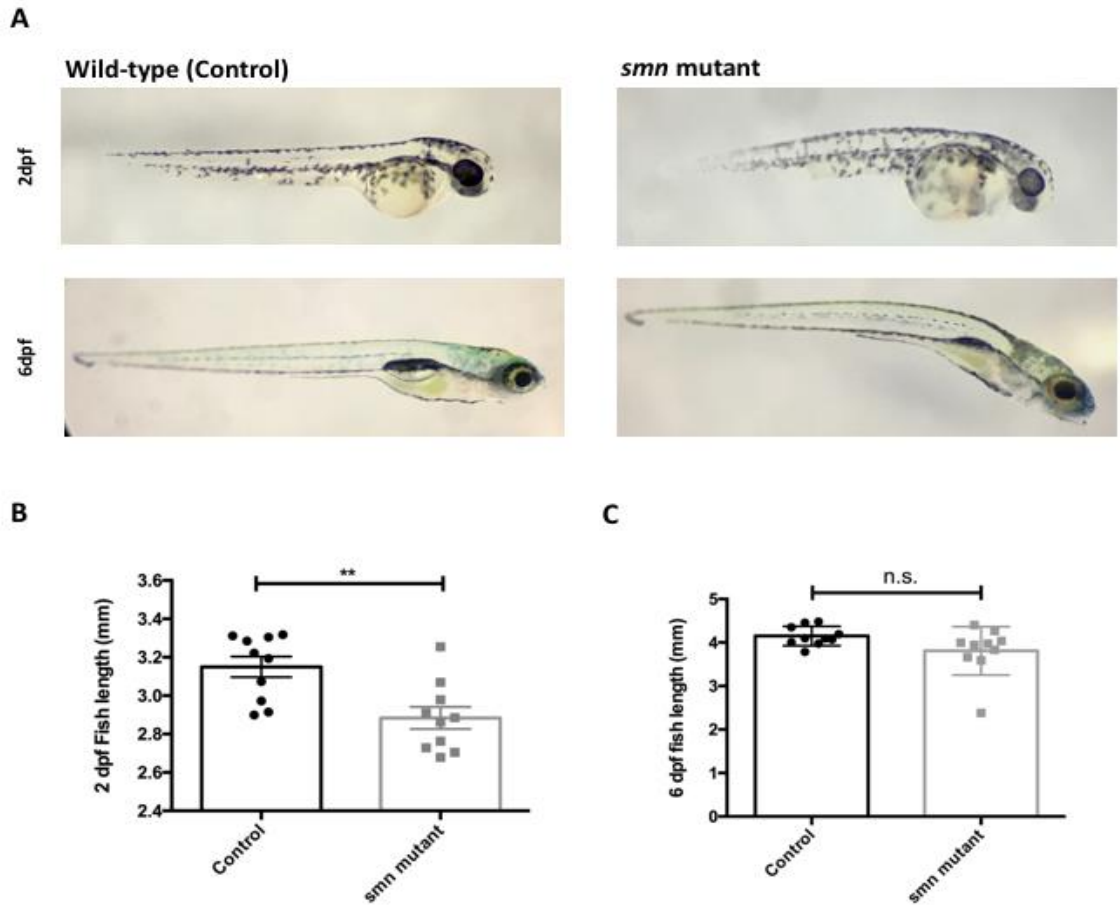

Figure 2. Characterization of the zebrafish smn mutant line (A) Gross morphology assessed with a stereomicroscope at 2 dpf and 6 dpf shows morphological defects occurring as a slight curved spine. Fish length was significantly reduced in *smn* mutant at 2 dpf (n=10). (B) Larvae body length in controls and *smn* mutants at 2 dpf and (C) 6dpf. ** represents a significance of $p < 0.01$. n.s=not significant. Significance was assessed using an unpaired Student's t-test and all data are represented as mean ± SEM.

smn mutant larvae displayed impaired locomotor activity

One of the key features of SMA is the restrained motor ability in patients(De Sanctis *et al.*, 2016, Kariya *et al.*, 2008). To gain insight into changes in motor function in mutant *smn* zebrafish, we monitored and quantified swimming activity in control and mutant fish over a period of 2 h. We observed that *smn* mutant larvae exhibited a significantly impaired locomotion compared to controls (Figure. 3A). To further our understanding of the underlying cause of motor impairment, we next sought to investigate the motor axon projections and NMJ integrity in *smn* mutant fish. We performed a co-staining of SV2 (a component of synaptic vesicles) and α-bungarotoxin (which binds irreversibly to acetylcholine receptors). Compared to controls, *smn* mutants displayed reduced motor axon length and branching (Figure. 3B,C). In addition, we observed the normal tight juxtaposition of presynaptic and postsynaptic components at NMJs in wild-type zebrafish, while in *smn* mutant fish, a significant decreased in the presynaptic and postsynaptic puncta as well as reduced colocalized of these puncta at NMJs (Figure. 3D,E,F). Altogether, these results demonstrate defective NMJ integrity and motor function in the *smn* mutant zebrafish. Our observations are consistent with phenotypes reported in morpholino-based *smn* knockdown zebrafish model (McWhorter *et al.*, 2003) and other zebrafish model of motor neuron diseases(Patten *et al.*, 2014). The muscle morphology of *smn* mutant zebrafish was also examined using the phalloidin staining of the actin filament on muscle fibers. We found no differences in muscle fiber size between control and mutant fish (Figure. 3G). However, the density of the fibers in the *smn* mutant were noticeably less, suggesting the possible early signs of muscle atrophy, which is a key component of SMA. Altogether, our data show that *smn* mutant zebrafish accurately recapitulates key hallmarks of SMA .

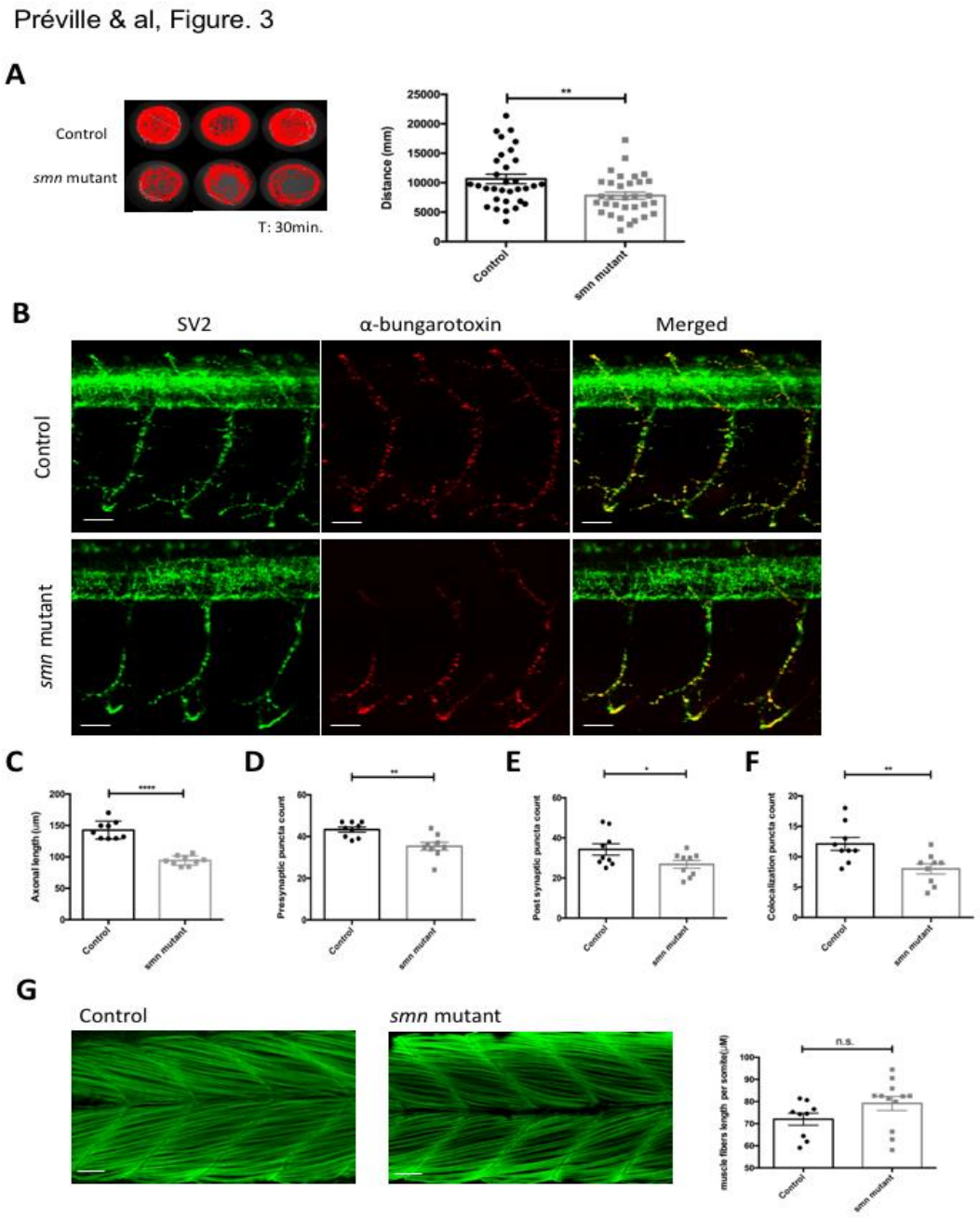

Figure 3. Behavioural and neuromuscular defects in smn mutants (A) Traces (red) of representative swimming activity in 5dpf control and *smn* mutant over 30 min recording are shown in *left panel*. Motor activity analysis revealed a significant reduction of the *smn* mutant swim distance (n=32) (*Right panel*). (B) Co-staining of SV2 (pre-synaptic) and α-bungarotoxin (post-synaptic) of the neuromuscular junctions shows significant differences in motor axonal length (C) and the count of pre-synaptic and post-synaptic and colocalized puncta (n=9) compared to control fish (E-F) * represents a significance of $p < 0.05$, ** represents a significance of $p < 0.01$ and ***denotes $p < 0.001$. Significance was assessed using an unpaired parametric t.test and all data are represented as mean ± SEM. (G) Phalloidin staining of the actin filaments on muscle fibers shows no significant differences in the *smn* mutant at 2 dpf (control ; n=9, *smn* mutant ; n=12). Scale bar= 25 µm.

Spinal and muscular defects upon loss of function of smn specifically in motoneurons

In order to assess if the SMA hallmarks in zebrafish was either because the *smn* expression was specifically affected in motor neurons, or not, we employed the *Hb9:Gal4* driver line and an UAS:miR*smn* line to induce specific silencing of *smn* in motor neurons and *Alx:Gal4* driver line to induce specific silencing of *smn* in upstream in the motor system in glutamatergic interneurons.

To generate the UAS:*smn* miRNA line, we used synthetic micro-RNAs (miRNA) targeting the 3'-UTR of the endogenous zebrafish *smn* gene, sown previously to be effective in knockdown *smn(Giacomotto et al., 2015)* and cloned them downstream of the upstream activating sequence (UAS) in a mini-tol2-R4R2 destination plasmid (Figure 4A). To generate a transgenic line, the DNA construct (UAS:miR*smn*) was microinjected into one-cell stage zebrafish embryos. We then generated tissue-specific knockdown of *smn*, by crossing UAS:miR*smn* zebrafish with these fish either expressing GAL4 specifically in motoneurons (*Hb9:Gal4*) or glutamatergic interneurons (*Alx:Gal4*) (Figure 4B). Furthermore, GFP under a cardiac promoter (*cmlc2*) was included to confirm an efficient insertion of the construct (Figure 4C). UAS:miR*smn*:: *Hb9:Gal4* (hereafter referred as *Hb9:smn*) and UAS:miR*smn*:: *Alx:Gal4* (hereafter referred as *Alx:smn*) founders with a green heart were selected to generate a stable *smn* depletion-cell-specific line.

We did not observe any overt morphological abnormalities during early development (0-5 dpf) in the *smn* depletion-cell-specific line. However, a significant decrease in motor activity was observed in *Hb9:smn* 5 dpf larvae compared to wild-type controls and *Alx:smn* larvae (Figure 4D). At later stages, in addition to impaired locomotion in *Hb9:smn* fish (Supplemental Video), we also observed a significant reduction in the muscle fiber length (Figure 5A,D). Although, *Alx:smn* mutant fish had normal swimming behaviour (Supplemental Video), the muscle fiber length was also reduced in size but at significantly lesser extent than in *Hb9:smn* fish. Analysis of the spinal cord morphology revealed anomalies in *Hb9:smn* fish compared compared to controls and *Alx:smn* mutant fish (Figure 5B,E). Choline acetyltransferase (ChAT) staining is a hallmark feature of cholinergic motor neurons. ChAT immunostaining was performed on the spinal cord sections of controls, *Hb9:smn* and *Alx:smn* fish. We observed a significant decreased in the number of motor neurons in spinal cords of the *Hb9:smn* mutant compared to *Alx:smn* and *controls* (Figure 5C,F). Altogether, these results demonstrate that *smn* depletion only in motoneurons recapitulate SMA hallmarks.

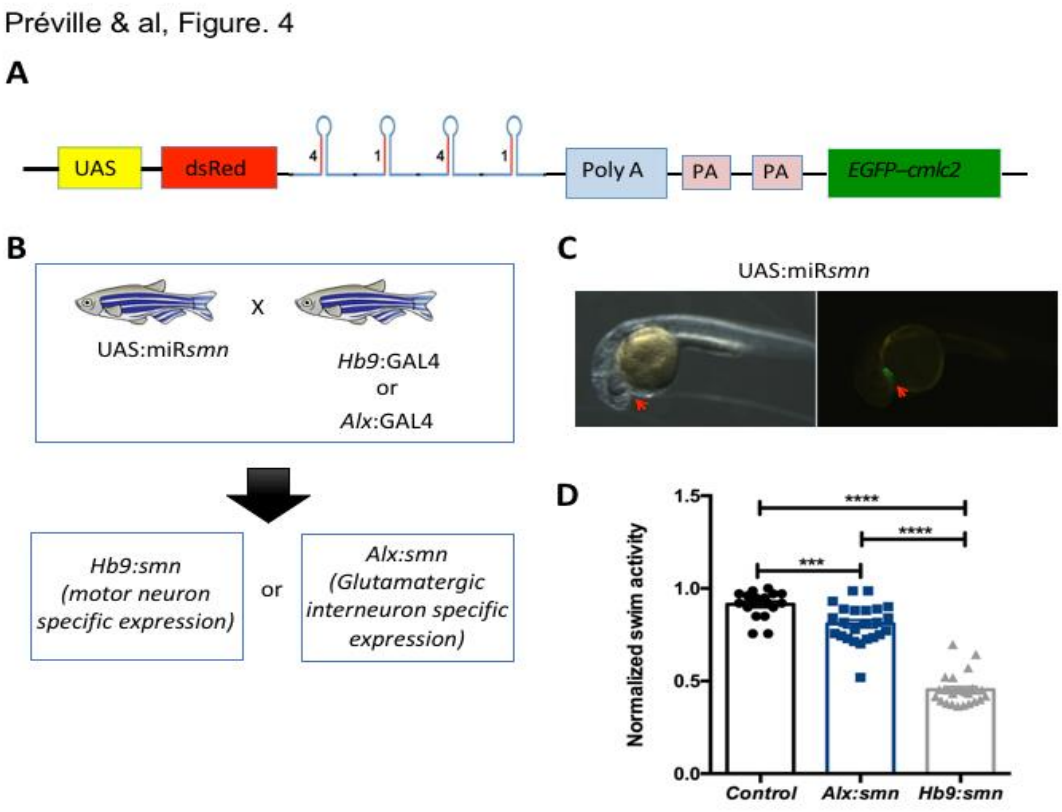

Figure 4. Generation of cell-specific smn knockdown (A) miRNAs targeting zebrafish *smn* (miRsmn1414) was cloned downstream of the upstream activating sequence (UAS) in a mini-tol2 R4R2 plasmid. (B) Cell-specific *smn* knockdown was achieved by crossing UAS:miR*smn* zebrafish with other fish expressing GAL4 specifically in motoneurons (*Hb9*:Gal4) or glutamatergic interneuron interneurons (*Alx*:Gal4). (C) A cardiac GFP reporter was used to select founders with successful integration of the UAS:mIR*smn* construct. (D) Impaired locomotor activity in 5dpf larvae upon loss of function *smn* specifically in motoneurons. Significance was assessed using a One-way ANOVA followed by a Tukey post-hoc multiple comparisons test for normally distributed and equal variance date, ***denotes $p < 0.001$.**** denotes $p < 0.0001$.

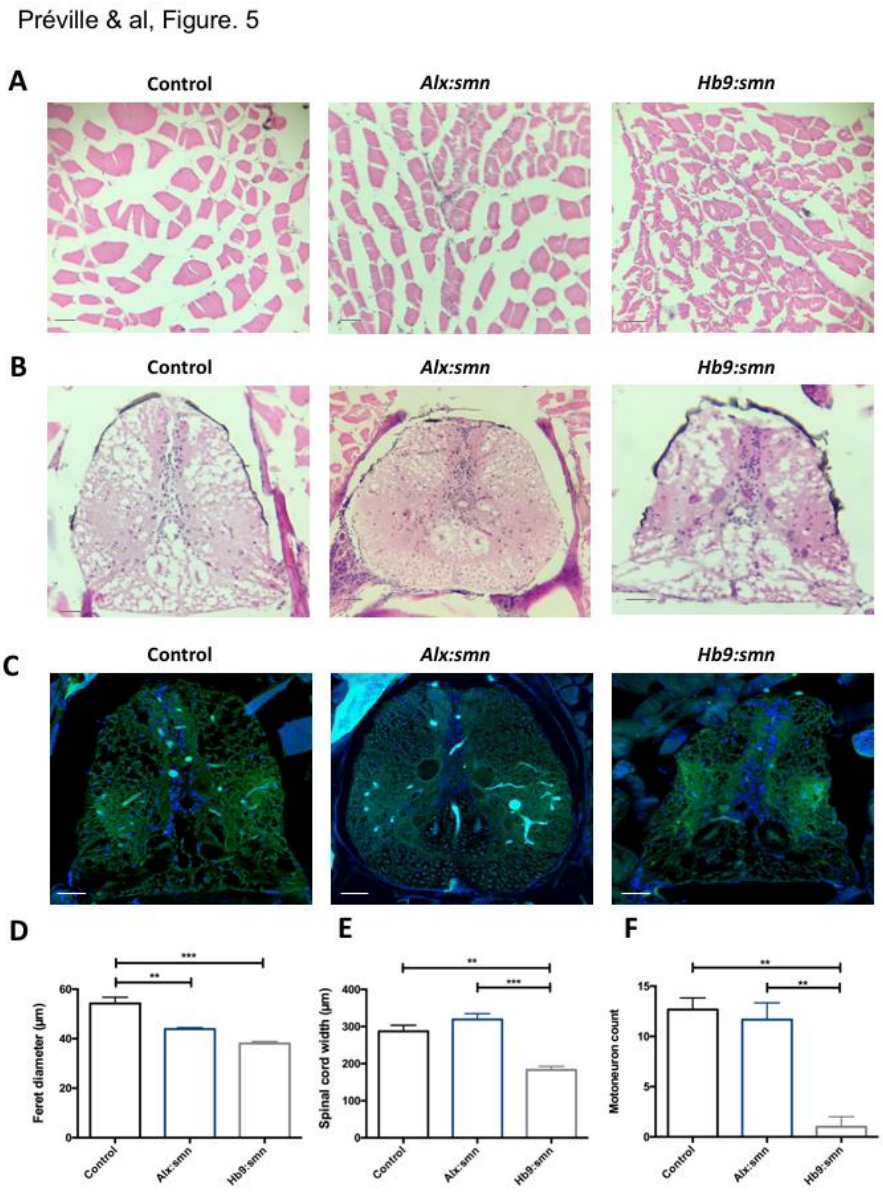

Figure 5. Spinal and muscular defects upon loss of *smn* in motoneurons but not in *smn*-interneuron specific knockdown. (A) Histology analysis with hematoxylin and eosin staining reveals severe atrophy of the muscles and (B) spinal cord in *smn* mutant fish (n=3). (C) ChAT staining of the motoneurons revealed a reduced numbers of motoneurons in *smn* mutant fish compared to controls and *Alx:smn* fish (n=3). ** represents a significance of $p < 0.01$. ***denotes $p < 0.001$. Significance was assessed using a One-way ANOVA followed by a Tukey post-hoc multiple comparisons test for normally distributed and equal variance date. All data are represented as mean ± SEM. Scale bar= 50 µm.

Salubrinal ameliorates locomotor activity in s*mn* mutant zebrafish

Automated monitoring of locomotor activity in zebrafish can be a powerful readout for drug efficacy in an i*n vivo* drug screen. Indeed, we previously demonstrated the power this readout in zebrafish models of amyotrohic lateral sclerosis (ALS) for the validation of neuroprotective compounds that can rapidly be translated into preclinical testing and clinical trial (Patten *et al.*, 2017). We also identified ER stress as a potential therapeutic target in ALS and showed that compounds such as salubrinal can correct motor deficits in *C. elegans* and zebrafish models of ALS(Vaccaro *et al.*, 2013).

A key feature in SMA is the presence of ER stress, which arises from the abnormal splicing of ER chaperones(Ng *et al.*, 2015). Given that ALS and SMA share many clinical and molecular features(Comley *et al.*, 2016), as a proof of principle, we tested the feasibility of using *smn* mutants to screen for potential drugs using salubrinal. Zebrafish *smn* mutants and controls were treated with either salubrinal or vehicle (E3 medium) for five days. After the five days of treatment, we assessed for locomotor activity using the Danio. Interestingly, salubrinal was found to significantly improve motor behavioural defects in salubrinal-treated *smn* mutant larvae compared to vehicle-treated larvae (Figure. 6). The assessment of the effects of salubrinal on the motor axonal phenotypes and synaptic clusters at NMJs was interrupted by the closure due to the COVID-19 pandemic. These experiments have now been resumed and upon completion these findings will be included in the manuscript prior to submission.

Préville & al, Figure. 6

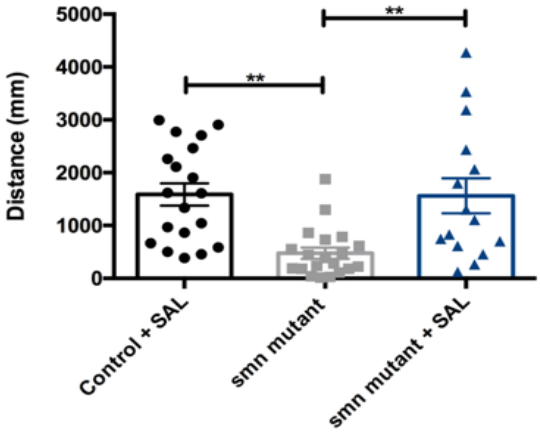

Figure 6. Salubrinal ameliorates motor phenotypes in zebrafish *smn* mutants. Motor activity analysis from 5dpf zebrafish larvae assessed with the Ethiovision software shows a significant difference between *smn* mutants (n=20) swim distance and controls (n=19). Salubrinal treatment (50 μM) on *smn* mutant larvae (n=15) ameliorated motor phenotype. ** represents a significance of $p < 0.005$. Significance was assessed using a One-way ANOVA followed by a Tukey post-hoc multiple comparisons test for normally distributed and equal variance date. All data are represented as mean ± SEM.

DISCUSSION

In this study, we generated the first *smn* loss-of-function mutation in zebrafish using the CRISPR/Cas9 gene editing method and reported the morphological, behavioral and neurological characterizations of *smn* zebrafish mutants. The s*mn* deficiency led to reduced motoneurons/motor axons, defective neuromuscular junctions and impaired the swimming ability of the zebrafish larvae. This observation is reminiscent of the major hallmarks of SMA patients. We also identify salubrinal as a neuroprotective compound that can ameliorate swimming ability of *smn* mutant zebrafish.

Previous groups have used morpholino to knockdown *smn* expression in zebrafish. These morphants exhibit motor axon branching and motor axon pathfinding defects at early developmental stages (Carrel et al., 2006, McWhorter et al., 2003). Additionally, knockdown of the endogenous *smn* gene with transient overexpression mutant *smn* RNA (smnY262stop, smnG264D and smnL265stop) in zebrafish resulted in shorter lifespan and NMJ defects (Boon et al., 2009). Similarly, a miRNA-based smn1 silencing in zebrafish

led to locomotor deficits and motor axonal abnormalities as well as premature death (Giacomotto et al., 2015). Our stable mutant line results in the same motor axonal phenotype and behavioural defects, suggesting the specificity and reproducibility of the phenotype upon loss of function of *smn* in zebrafish.

We also showed evidence that only a specific knockdown of *smn* in motoneuron displayed SMA hallmarks in zebrafish. Our results are consistent with the smn1 knockdown in zebrafish motoneuron using miRNAs showed to induce late-onset phenotypes like muscle atrophy, scoliosis as well as a reduced number of motoneurons(Laird et al., 2016). Additionally, similar phenotypes were also reported in a mice model where SMN was depleted in motor neuronal progenitors and the model displayed SMA-like phenotype like neuromuscular defects, muscle atrophy and motoneuron degeneration(Park et al., 2010). However, in both studies, the defects were modest compared to those observed in ubiquitously low SMN levels zebrafish (Laird et al., 2016) or mice model (Park et al., 2010). This suggests that additional cells, other than motor neurons, may play a role in SMA pathogenesis. A recent study showed that skeletal muscle alone persistently deprived of SMN in a SMA mouse model displayed muscle fiber defects, abnormal NMJs and locomotion as well as shorter lifespan (Kim et al., 2020). However this observation, contradicts results, where muscles were shown to function properly with low levels of SMN (Iyer et al., 2015, Laird et al., 2016). In particular, silencing of *smn* specifically in muscles is not sufficient to reproduce features of SMA in zebrafish (Laird et al., 2016). Here, we report for the first time that loss of function of *smn* at central synapses, particularly in excitatory interneurons, is also is not sufficient to induce motor behavioural deficits and motoneuron defects. Additional studies investigating the complete motor and sensory circuitries are needed to unravel a clear implication of cell types, other than muscles and interneurons, in SMA pathogenesis.

Zebrafish are becoming increasingly valuable as in vivo models for drug screening due to their ability to rapidly assess morphological and/or behavioural readouts in live animals. We found that zebrafish *smn* mutants displayed reduced locomotor activity at 5 dpf. This phenotype can provide an efficient, robust and rapid screen for potential therapeutics. Capitalizing on this advantage of zebrafish, showed that salubrinal can ameliorate the impaired locomotor activity of the smn1 mutant fish. This compound selectively inhibits dephosphorylation of the eukaryotic translation initiation factor 2 (eIF2a) and has a protective effect against endoplasmic reticulum (ER) stress-induced apoptosis (Boyce et al., 2005). ER stress responses have been widely reported in several neurodegenerative diseases (Colla et al., 2012, Huang et al., 2012, Saxena et al., 2009, Walker et al., 2013) including SMA(Ng et al., 2015). Interestingly, salubrinal was found to have protective effects in many of these diseases such as Alzheimer's disease (Huang et al., 2012), Parkinson's disease (Colla et al., 2012) and ALS (Saxena et al., 2009, Walker et al., 2013). For instance, in ALS models,

salubrinal improved their survival as well as reduced the overall reduction of motoneuron phenotypes and paralysis. Similarly, motoneurons generated from SMA patient iPSCs revealed high levels of apoptotic and ER stress genes and loss of SMN specifically activates the UPR. Importantly, treatment of these motoneurons with various inhibitors of ER stress, including salubrinal improved their survival. Furthermore, treatment with salubrinal reduced the activation of ER stress associated apoptosis in spinal and bulbar muscular atrophy (SBMA)(Montague et al., 2014). These findings raise the possibility that ER stress could play a role in motoneuron degeneration. Targeting this pathway could be a promising strategy to consider for SMA.

In this study, we developed a stable genetic zebrafish SMA model that displays robust SMA behavioral characteristics and is useful for efficient and rapid in vivo screening of candidate therapeutic compounds. It provides a new model system to complements other SMA models in gaining insights into pathological processes contributing to SMA pathogenesis. Importantly, it provides a tool to facilitate the identification of potential non-SMN dependent therapies that can be translated to ultimately to SMA patients.

Acknowledgments

SP is supported by the Natural Science and Engineering Research Council, Canadian Institutes of Health Research (CIHR), an ALS Canada-Brain Canada Career Transition Award and a FRQS Junior 1 research scholar. SP holds the Anna Sforza Djoukhadjian research chair in ALS. The authors would like to summer student Gabriel Desrosiers for his assistance in some of the zebrafish experiments and Dr. Jean Giacomotto for the miRNA *smn* construct.

Conflicts of interest

The authors declare no competing interests.

3. DISCUSSION

3.1. GENERAL DISCUSSION

In this study, we generated and characterized a CRISPR zebrafish Smn knockout. We identified a redundant 7bp deletion causing a frameshift mutation that resulted in low levels of the Smn protein as well as reduced mRNA expression in our CRISPR model. This *smn* mutant displayed several SMA hallmarks such as gross morphological defects like curvature of the spine, reduced motor activity, abnormal axon branching as well as fewer NMJs. Altogether, there results put forward a novel *smn* KO zebrafish model that we will further use to study SMA pathogenesis and for drug screenings. These findings are consistent with those reported in SMA zebrafish models (Boon *et al.*, 2009, Giacomotto *et al.*, 2015, Hao le *et al.*, 2011, McWhorter *et al.*, 2003). For instance, an established MO based knockdown SMA zebrafish model exhibited a strong motor axon phenotype at early development stages. They have noticed morphological abnormalities specifically in motoneuron development, while lowering Smn levels in the entire fish, that causes axon branching and motor axon pathfinding defects (Carrel *et al.*, 2006, McWhorter *et al.*, 2003). Moreover, another team (Boon *et al.*, 2009) used MO to knockdown the endogenous *smn* gene while injecting mutant *smn* RNA as well (i.e. *smnY262stop, smnG264D* and *smnL265stop*). Their model had a shorter lifespan and displayed NMJ defects, more precisely a decrease in SV2 protein at the presynaptic terminals. These specific phenotypes seen in SMA morphants can also be seen in our *smn* mutant with premature death, abnormal branching patterns as well as reduced SV2 markers. Similarly, a miRNA-based *smn* silencing resulted in zebrafish exhibiting strong motor phenotypes such as locomotor and motor neuron abnormalities as well as premature death (Giacomotto *et al.*, 2015). These phenotypes were displayed in their most severe model with a reduction of the Smn protein up to 90%. Again, these phenotypes are consistent to those observed in our *smn* mutants, which demonstrate the relevance of our model.

Our *smn* mutant zebrafish model will elegantly complement the existing SMA models. Having a stable line allows to have a large number of embryos to assess multiple experiments rapidly as well as drug screenings. The continuous expression of a disrupted Smn protein will assure stable and reliable results. Our model will be the first SMA zebrafish model generated with CRISPR/Cas9. Usually, miRNA or MO models are widely used to model various genetic diseases in zebrafish, including SMA. Although, these two methods are transient and only able to knockdown a gene by repressing or blocking transcription. Furthermore, MO's specificity is still controversial and under investigation (Kok *et al.*, 2015). Using miRNA is still very attractive for its simple designing steps and was considered easier than sgRNA for CRISPR/Cas9. But recent advances have also make it very easy and cleaner to use CRISPR/Cas9 to model SMA. As mentioned before,

algorithms have been created to help counter the negative aspects of CRISPR/Cas9, which are off-target effects and specificity due to PAM sequence. Experimental set-ups are mostly similar for every technique, which requires micro-injection of zebrafish embryos. Overall, CRISPR/Cas9 is one of the most versatile genetic manipulation techniques. Although homozygous Smn mutation is lethal, our stable heterozygous (*smn* +/-) line can viably provide homozygous larvae to be analysed within the first week of development. By taking advantages of the existing SMA zebrafish models (Hao le *et al.*, 2011), we could also improve our CRISPR model and cross it with a *hSMN2* transgenic line to model a more intermediate phenotype.

Currently, the transient zebrafish MO model is heavily used to understand SMA pathogenesis (Boyd *et al.*, 2017, McGovern *et al.*, 2015, Powis *et al.*, 2016b, See *et al.*, 2014, Sleigh *et al.*, 2014). Our goal was to create a more stable model while knocking out the *smn* gene. To generate a stable knock-out *smn* mutant zebrafish line, we first designed the sgRNA with the crisprscan algorithm (https://www.crisprscan.org). One known limitations of the CRISPR/Cas9 system is the off-target effects that consists of undesired mutations (Ran *et al.*, 2013). The *crisprscan* website allows for a rapid and efficient designing of a specific sgRNA while considering the off-target activity. This algorithm also considers a 20-nt sequence onto the gene of interest that will immediately be followed by a -NGG- PAM sequence. These characteristics are very important in order to generate a specific sgRNA. We also analysed the *smn* gene and protein sequence to search for an optimal region to direct the mutation. The Smn protein contains a unique domain, which is known as Tudor. This domain facilitates the SMN-Sm protein interactions for its role in the assembly of snRNP (Selenko *et al.*, 2001). Our first sgRNA was directed on that specific region of the *smn* gene to affect the functionality of the protein, but no conclusive results was observed. Another sgRNA was designed outside the Tudor domain showing great results, hence it was used for our experimentations. To make sure that micro-injections were properly done, we used a positive control. A team, (Irion *et al.*, 2014), showed efficient genome editing in the gene responsible for pigmentation in zebrafish using the CRISPR/Cas9 system. The efficiency of directed mutation can be easily verified since crispants will show lesser or no pigmentation and have an albino phenotype. We then injected the albino sgRNA in another subset of embryos, parallel to our *smn* sgRNA, in order to confirm a successful micro-injection technique.

Of note most of our experiments were done with the F0 line, because of time limitations. Although it allows for rapid results, it does not represent a stable line and some aspects must be considered. Firstly, mosaicism is an effect that makes the generation of a stable line challenging. For a mutation to be transmitted to offspring, it needs to occur into the germline cells of the founder fish. Even though injections were done at the same stage, the Cas9 can cut at different timelines and cell population, thus have different effects. Since SMN is important during embryonic development (Burlet *et al.*, 1998), different timepoint mutations can

have an important impact in subsequent development as well as the mutant fish phenotype severity (Yen *et al.*, 2014). Mutations had to be verified and scrutinized by sequencing every possible founder. A common mutation was identified and chosen to continue further steps of the generation of a stable line. Then, by outcrossing these fish with wild-types and sequencing the resulting embryos, it was possible to identify founders that possess the desired mutation in their germline. Secondly, Only a certain percentage of the injected fish have the desired mutation and nothing can differentiate one and other rapidly at early stages. Thus, all the injected fish were always pooled for experiments. More precisely, it means that in every experiment representing the *smn* mutants, there were probably a certain percentage of fish without the mutation that could decrease the severity of phenotypes. Moreover, it could also suggest the presence of genetic compensation as a result of a loss-of-function of the Smn protein. Studies in multiple model organisms, including zebrafish, addressed the possibility of a less severe phenotype in knockout compared to knockdown models as a result of endogenous genetic perturbations (De Souza *et al.*, 2006, El-Brolosy *et al.*, 2017, Evers *et al.*, 2016, Teng *et al.*, 2013). For instance, a group observed compensatory network in a zebrafish egfl7 knockout model, but not in their respective knockdown model and showed considerable differences in their phenotypes and proteome/transcriptome analysis (Rossi *et al.*, 2015). Although this phenomenon exists, we still showed great similarities between our knockout and knockdown zebrafish models.

Our results allowed for the characterization of a novel zebrafish model, but it would be interesting and necessary to assess experiments in our stable F2 *smn* mutant zebrafish larvae to address exactly the impact of the disrupted Smn protein. Furthermore, we used sequencing to genotype and identify our founder fishes, but another very efficient and faster technique is also available. HRM, High Resolution Melting, is a post-PCR analysis that allows identification of mutation through a shift in the DNA melt-curve profiles. Briefly, this method is based on the dissociation of double-stranded DNA when increasing temperature. Knowing that a single mutation can have an impact on the melting temperature, it allows the distinction between heterozygote, homozygote and wild-type genotypes. Thus, this technique is very useful for reliable detection of CRISPR/Cas9 induced indels in fish (Samarut *et al.*, 2016). Unfortunately, we were not able to optimize an efficient HRM detection of our mutation in the *smn* zebrafish. DNA preparation as well as primer design were optimized according to known guidelines (Słomka *et al.*, 2017), but no conclusive results could be considered and presented.

In this dissertation, we also developed a motoneuron-specific *smn* knockdown model and showed SMA hallmarks like fewer motoneuron counts, abnormal spinal cord morphology and muscle atrophy compared to controls and our interneuron-specific *smn* knockdown. We also showed evidence that only a specific

knockdown of *smn* in motoneuron displayed SMA hallmarks in zebrafish. Other teams have also focused on investigating defects upon cell-specific Smn depletion. For instance, researchers showed that *smn* knockdown in zebrafish motoneuron using miRNAs induced late-onset phenotypes like muscle atrophy, scoliosis as well as a reduction of motoneurons counts (Laird *et al.*, 2016). Our results coincide well with these phenotypes, which confirms the relevance of our model. Moreover, these phenotypes were also seen in a mice model were SMN was depleted in motor neuronal progenitors. They displayed SMA-like phenotype like neuromuscular defects, muscle atrophy and motoneuron degeneration. Interestingly, they noted that these defects were modest compared to those observed in ubiquitously low SMN levels mice model (Park *et al.*, 2010). This brings out the possibility that other cell types could play a role in SMA pathogenesis. Our investigation showed that depleting *smn* in interneurons was not enough to cause SMA-like phenotypes. But other cell types showed interesting results when depleting *smn* levels. For instance, a recent study showed that skeletal muscle alone persistently deprived of SMN in a SMA mouse model displayed muscle fiber defects, abnormal NMJs and locomotion as well as shorter lifespan (Kim *et al.*, 2020). Very interestingly, other researches showed contradictory results, although muscles are severely affected in SMA, they seems to function properly with low levels of SMN (Iyer *et al.*, 2015, Laird *et al.*, 2016). In another study, they reported that only a small increase in full-length SMN in neurons of the severe SMA mouse model could impact greatly SMA phenotype whereas no major impact could be seen by correcting SMN expression in skeletal muscle alone (Gavrilina *et al.*, 2008). Interestingly, sensory nerve pathology was reported in severely affected SMA patients (Type I) (Rudnik-Schöneborn *et al.*, 2003). In SMA *Drosophila* mutants, restoration in muscles or motoneurons wasn't able to rescue SMA-like phenotypes such as reduced muscle size, defective locomotion, motor rhythm and motoneuron neurotransmission. But they did expressed Smn in the motor circuit, more precisely in proprioceptive neurons and interneurons, and were able to notice rescue in muscle and motoneurons (Imlach *et al.*, 2012). A study using deep RNA-sequencing from the spinal cord of SMA mouse model at early presymptomatic stage, before seeing motoneuron pathology, revealed dysregulation of essential sensory-motor circuitry mRNAs (Z. Zhang *et al.*, 2013). These suggests that these dysfunctions would precede motoneuron defects. Other studies suggest that SMA phenotypes would autonomously originate in motoneurons, thus would not be a cause of motoneuron degeneration (Gogliotti *et al.*, 2012, Thirumalai *et al.*, 2013). Although it is clear that sensory-motor defect are present, the uncertainty relies on whether it would be a cause or consequence of motoneuron loss. This ambiguity can only suggest that more studies in the complete motor circuitry are needed to unravel a clear timeline of events in SMA pathogenesis.

Zebrafish's locomotor activity starts at 2-3 dpf, following hatching, with small burst of swimming activity. Once dechorionated, they can also have vigorous response to touch as early as 27 hpf. They possess a swim

bladder, which is a gas-filled organ, that allows them to swim properly. It is fully developed around 4-5 dpf, thus zebrafish have a mature swimming at this precise stage (Saint-Amant *et al.*, 1998). Many neurobehavioral aspects can be studied in the zebrafish. Among them is locomotor behavior that allows for rapid assessment of motor abnormalities. In our study also showed the usefulness of a stable zebrafish SMA model for drug discovery. In particular, we demonstrate that assessment of locomotor activity is a key phenotypic component that can be used for rapid drug screening. Locomotion, or the lack of locomotion, can be used as a phenotypic screen to test a large variety of compounds. Thousands of molecules can firstly be tested in worms, *C. elegans*, and then a selection of them can be translated into a more complex neurobiological system like the zebrafish. Similar screening methods have been very useful to study the effects of small molecules or drugs on motor abilities in disease context. For instance, it can be assessed by multiples experiments such as a touch-evoked escape response, swim distance and velocity measurements, electrophysiology as well as immunohistochemical analyses of NMJ structures. In a recent work, Patten et al., identified thirteen compounds that protect against motor deficits in *C. elegans* and zebrafish model of ALS (Patten *et al.*, 2017). One of which, pimozide, restored both locomotor performance and neuromuscular junction (NMJ) synaptic connectivity in *C. elegans* and zebrafish TDP-43 mutants as well as restore NMJ function in an ALS SOD1 mouse model, showing that findings from simple ALS models such as zebrafish can be rapidly translated to mammals. Pimozide is currently in a Phase II clinical trial for ALS (clinicaltrials.gov Identifier: NCT02463825). We are hopeful that future use of our model as a drug discovery tool can accelerate the identification of non-SMN dependent candidate compounds for clinical trials.

Interestingly, in our preliminary drug screening work, we found that salubrinal can ameliorate the impaired locomotor activity of the *smn* mutant fish. Salubrinal selectively inhibits dephosphorylation of the eukaryotic translation initiation factor 2 (eIF2a). This compound was shown to have a protective effect against endoplasmic reticulum (ER) stress-induced apoptosis (Boyce *et al.*, 2005). The ER stress response is a necessity in order to maintain protein homeostasis in case of various cellular injuries like inflammation or oxidative damage. Furthermore, misfolded proteins cause ER stress and are taken in charge by the unfolded protein response (UPR) which will eventually trigger cell death if the ER insult is critical (Xu *et al.*, 2005). Salubrinal's protective effects have been studied in a number of neurodegenerative diseases such as Alzheimer's disease (Huang *et al.*, 2012), Parkinson's disease (Colla *et al.*, 2012) and ALS (Saxena *et al.*, 2009, Walker *et al.*, 2013). Markers of ER stress are elevated in motor neurons of $SOD1^{G93A}$ ALS mice and treatment with salubrinal improved their survival (Saxena *et al.*, 2009). It was also able to attenuate loss of muscle force as well as axon pathology, showing a protective effect in FALS mice. Their longitudinal analysis hypothesized that motoneurons could have a specific vulnerability to ER stress and could play a

role in the disease. Moreover, a study done in a mTDP-43 ALS model in *C.elegans* and zebrafish using salubrinal as a neuroprotective agent revealed overall reduction of motoneuron phenotypes and paralysis. Treatment with multiple agents, including salubrinal, showed a reduction in global oxidative stress that would potentially originate within the ER. Insoluble mTDP-43 protein that may contribute to motoneuron degeneration was also reduced (Vaccaro *et al.*, 2013). Interestingly, post-mortem spinal cords of sporadic ALS patients showed the presence of ER stress as well as induction of the UPR (Atkin *et al.*, 2008). This suggest that ER stress could be important in the pathogenesis of ALS but for other motor neuron disease as well, like SMA. Results from a RNA sequencing of purified motoneurons generated from SMA patient iPSCs revealed high levels of apoptotic genes and genes involved in the ER stress pathway. Interestingly, they found that loss of SMN specifically activates the UPR. They have also treated the motoneurons with various inhibitors of ER stress, including salubrinal, and showed a modest increase in their survival (Ng *et al.*, 2015). Another team focused on spinal and bulbar muscular atrophy (SBMA) and showed the presence of ER stress markers in motor neurons from spinal cords of SBMA mice at presymptomatic stage of the disease. This may suggest a very early and important role for ER stress in the pathogenesis of this disease. Again, treatment with salubrinal reduced the activation of ER stress associated apoptosis (Montague *et al.*, 2014). These findings raise the possibility that ER stress could play a role in motoneuron degeneration. Targeting this pathway could be a promising strategy to consider for various neurodegenerative diseases, including SMA.

3.2. FUTURE DIRECTIONS

SMN interacts with a large number of mRNAs and is involved in multiple aspects of RNA processing (Bertrandy *et al.*, 1999, Lagier-Tourenne *et al.*, 2010, Rossoll *et al.*, 2002). With the advent of high throughput sequencing (RNA seq), for the past few years several groups have performed transcriptomic analyses using SMA models(Maeda *et al.*, 2014, Murray *et al.*, 2015, Ng *et al.*, 2015). However, none of these studies have thoroughly investigated synaptic genes that SMN may interact directly with and that are dysregulated in motor neurons in SMA. SMN likely regulates specific synaptic genes and that defining this genetic network will reveal novel molecular pathways in motor function and new drug targets for therapeutic intervention in SMA. Using our model, we can in the future, unraveling SMN's partners at synapses. Capitalizing on the advantages of zebrafish, we can perform transcriptomic analysis on isolated motoneurons from our *smn* mutant model and controls to identify synaptic genes that are potentially regulated by SMN1. This could help us find new targets for dependent or independent SMA therapy.

NMJ alterations have been found in patients (Martinez-Hernandez *et al.*, 2013, Wadman *et al.*, 2012) and in animal models of SMA such that it is now widely recognized that early and extensive nerve sprouting and synaptic remodeling occur at the NMJ long before presentation of clinical symptoms(Dadon-Nachum *et al.*, 2011, Murray *et al.*, 2008). Coincident with (or perhaps causative of) defects in the periphery are abnormalities arising centrally, in particular loss of glutamatergic afferents(Ling *et al.*, 2010, Mentis *et al.*, 2011, Park *et al.*, 2010, Tarabal *et al.*, 2014). The development of agents that target synaptic pathology may represent an attractive approach for therapy for SMA. However, the detailed mechanism(s) involved in NMJ pathology in SMA have not been fully described and considerably less is known about synaptic defects that arise centrally in the spinal cord. This is due in part to the difficulty in recording synaptic activity in the mammalian spinal cord. Our new zebrafish model can provide the ability to monitor defects in synaptic transmission and extend our understanding of SMA pathology as well as uncover new targets for therapeutic interventions. Indeed, electrophysiological experiments can be performed in vivo in zebrafish to unravel interesting changes in biophysical properties of the motoneurons and synaptic transmission at central synapses and NMJs in SMA. For instance, whole cell patch-clamp electrophysiology can be used for analyzing miniature end plate currents (mEPCs) between motoneurons and muscles. These types of currents represent very small and random acetylcholine release that occurs from a resting presynaptic terminal. The quantity, frequency and amplitude of mEPCS can be recorded in wild-type and compared to mutant fish and inform us about the connectivity at NMJ. Drug treatment in restoring aberrant mEPCs can also be assessed to identify neuroprotective small molecules. Addressing mEPCs in the *smn* mutant fish at early stage can help us characterize the neurological changes in SMA. An interesting study showed that SMA motoneurons from iPSCs displayed hyperexcitability and that the reduction of FL-SMN would be the cause. They noted an increase in membrane input resistance, hyperpolarized threshold and a larger action potential amplitude. By knocking down the FL-SMN, they were able to recapitulate those same characteristics. SMA motoneuron also exhibited enhanced sodium channel activities. They were able to correct it by restoring SMN levels in SMA motoneurons (H. Liu *et al.*, 2015). Another study also reported hyperexcitable SMA motoneurons and higher amplitude of action potential (Mentis *et al.*, 2011). Electrophysiology techniques and our model can allow to address circuit and synaptic abnormalities for a better understanding of the disease

Preliminary work from this dissertation show that our zebrafish SMA model represents a powerful drug-discovery tool. Indeed, having access to a large number of the *smn* mutant embryos, we can screen multiple drugs in parallels and test them for their efficacy on rescuing SMA motor phenotypes. This screen can be done using the using the Danio Vision chamber and candidate compounds can be further assessed for their effects on NMJs via simple immunostainings. Lead compounds can be subsequently translated into

preclinical and clinical trials. Taken together, these further directions can provide strategies that can help us to unravel SMA pathogenesis as well as finding a possible treatment in a rapidly efficient way.

3.3. CONCLUSION

In summary, this study reports the generation and characterization of a novel CRISPR/Cas9 *smn* mutant zebrafish model. Within this stable model, we showed hallmarks of SMA such as NMJ and axon branching defects, reduced locomotor activity, gross morphological defects and major Smn level reduction. Treating *smn* mutant fish with an ER-stress protective drug, known as Salubrinal, was able to rescue the motor phenotype. We wish to screen many more drugs and small molecules while investigating new pathways of Smn with transcriptomic analysis. Our stable model will also allow the investigation of more in depth the synaptic dysfunctions of SMA pathogenesis with electrophysiology techniques. The *smn* mutant represents a more severe form of SMA, but could be used to model an intermediate form by adding a SMN2 transgene. Overall, our versatile and stable SMA zebrafish model will help finding new pathways for alternative therapeutic strategies and for the understanding of SMA pathogenesis as well as neurodegenerative disease in general.

BIBLIOGRAPHY

Ackermann B, Krober S, Torres-Benito L, Borgmann A, Peters M, Hosseini Barkooie SM, Tejero R, Jakubik M, Schreml J, Milbradt J, Wunderlich TF, Riessland M, Tabares L & Wirth B (2013) Plastin 3 ameliorates spinal muscular atrophy via delayed axon pruning and improves neuromuscular junction functionality. *Hum Mol Genet* 22(7):1328-1347.

Ahmed SV, Jayawarna C & Jude E (2006) Post lumbar puncture headache: diagnosis and management. *Postgrad Med J* 82(973):713-716.

Akten B, Kye MJ, Hao le T, Wertz MH, Singh S, Nie D, Huang J, Merianda TT, Twiss JL, Beattie CE, Steen JA & Sahin M (2011) Interaction of survival of motor neuron (SMN) and HuD proteins with mRNA cpg15 rescues motor neuron axonal deficits. *Proc Natl Acad Sci U S A* 108(25):10337-10342.

Al-Zaidy S, Pickard AS, Kotha K, Alfano LN, Lowes L, Paul G, Church K, Lehman K, Sproule DM, Dabbous O, Maru B, Berry K, Arnold WD, Kissel JT, Mendell JR & Shell R (2019) Health outcomes in spinal muscular atrophy type 1 following AVXS-101 gene replacement therapy. *Pediatr Pulmonol* 54(2):179-185.

Ali DW, Buss RR & Drapeau P (2000) Properties of miniature glutamatergic EPSCs in neurons of the locomotor regions of the developing zebrafish. *J Neurophysiol* 83(1):181-191.

Anonyme (1998) Genome sequence of the nematode C. elegans: a platform for investigating biology. *Science* 282(5396):2012-2018.

Asnani A & Peterson RT (2014) The zebrafish as a tool to identify novel therapies for human cardiovascular disease. *Disease models & mechanisms* 7(7):763-767.

Atkin JD, Farg MA, Walker AK, McLean C, Tomas D & Horne MK (2008) Endoplasmic reticulum stress and induction of the unfolded protein response in human sporadic amyotrophic lateral sclerosis. *Neurobiol Dis* 30(3):400-407.

Auer TO, Duroure K, De Cian A, Concordet JP & Del Bene F (2014) Highly efficient CRISPR/Cas9-mediated knock-in in zebrafish by homology-independent DNA repair. *Genome Res* 24(1):142-153.

Bandmann O & Burton EA (2010) Genetic zebrafish models of neurodegenerative diseases. *Neurobiol Dis* 40(1):58-65.

Basnet RM, Zizioli D, Taweedet S, Finazzi D & Memo M (2019) Zebrafish Larvae as a Behavioral Model in Neuropharmacology. *Biomedicines* 7(1).

Battle DJ, Kasim M, Yong J, Lotti F, Lau CK, Mouaikel J, Zhang Z, Han K, Wan L & Dreyfuss G (2006) The SMN complex: an assembly machine for RNPs. *Cold Spring Harb Symp Quant Biol* 71:313-320.

Beattie CE (2000) Control of motor axon guidance in the zebrafish embryo. *Brain Res Bull* 53(5):489-500.

Bebee TW, Dominguez CE & Chandler DS (2012) Mouse models of SMA: tools for disease characterization and therapeutic development. *Hum Genet* 131(8):1277-1293.

Benkhelifa-Ziyyat S, Besse A, Roda M, Duque S, Astord S, Carcenac R, Marais T & Barkats M (2013) Intramuscular scAAV9-SMN injection mediates widespread gene delivery to the spinal cord and decreases disease severity in SMA mice. *Molecular therapy : the journal of the American Society of Gene Therapy* 21(2):282-290.

Bertrandy S, Burlet P, Clermont O, Huber C, Fondrat C, Thierry-Mieg D, Munnich A & Lefebvre S (1999) The RNA-binding properties of SMN: deletion analysis of the zebrafish orthologue defines domains conserved in evolution. *Hum Mol Genet* 8(5):775-782.

Boon KL, Xiao S, McWhorter ML, Donn T, Wolf-Saxon E, Bohnsack MT, Moens CB & Beattie CE (2009) Zebrafish survival motor neuron mutants exhibit presynaptic neuromuscular junction defects. *Hum Mol Genet* 18(19):3615-3625.

Bowerman M, Anderson CL, Beauvais A, Boyl PP, Witke W & Kothary R (2009) SMN, profilin IIa and plastin 3: a link between the deregulation of actin dynamics and SMA pathogenesis. *Mol Cell Neurosci* 42(1):66-74.

Bowerman M, Beauvais A, Anderson CL & Kothary R (2010) Rho-kinase inactivation prolongs survival of an intermediate SMA mouse model. *Hum Mol Genet* 19(8):1468-1478.

Bowerman M, Murray LM, Boyer JG, Anderson CL & Kothary R (2012a) Fasudil improves survival and promotes skeletal muscle development in a mouse model of spinal muscular atrophy. *BMC medicine* 10:24.

Bowerman M, Shafey D & Kothary R (2007) Smn depletion alters profilin II expression and leads to upregulation of the RhoA/ROCK pathway and defects in neuronal integrity. *J Mol Neurosci* 32(2):120-131.

Bowerman M, Swoboda KJ, Michalski JP, Wang GS, Reeks C, Beauvais A, Murphy K, Woulfe J, Screaton RA, Scott FW & Kothary R (2012b) Glucose metabolism and pancreatic defects in spinal muscular atrophy. *Ann Neurol* 72(2):256-268.

Boyce M, Bryant KF, Jousse C, Long K, Harding HP, Scheuner D, Kaufman RJ, Ma D, Coen DM, Ron D & Yuan J (2005) A selective inhibitor of eIF2alpha dephosphorylation protects cells from ER stress. *Science* 307(5711):935-939.

Boyd PJ, Tu WY, Shorrock HK, Groen EJN, Carter RN, Powis RA, Thomson SR, Thomson D, Graham LC, Motyl AAL, Wishart TM, Highley JR, Morton NM, Becker T, Becker CG, Heath PR & Gillingwater TH (2017) Bioenergetic status modulates motor neuron vulnerability and pathogenesis in a zebrafish model of spinal muscular atrophy. *PLoS Genet* 13(4):e1006744.

Brent LJ & Drapeau P (2002) Targeted "knockdown" of channel expression in vivo with an antisense morpholino oligonucleotide. *Neuroscience* 114(2):275-278.

Brichta L, Hofmann Y, Hahnen E, Siebzehnrubl FA, Raschke H, Blumcke I, Eyupoglu IY & Wirth B (2003) Valproic acid increases the SMN2 protein level: a well-known drug as a potential therapy for spinal muscular atrophy. *Human molecular genetics* 12(19):2481-2489.

Briese M, Esmaeili B, Fraboulet S, Burt EC, Christodoulou S, Towers PR, Davies KE & Sattelle DB (2009) Deletion of smn-1, the Caenorhabditis elegans ortholog of the spinal muscular atrophy gene, results in locomotor dysfunction and reduced lifespan. *Hum Mol Genet* 18(1):97-104.

Bühler D, Raker V, Lührmann R & Fischer U (1999) Essential role for the tudor domain of SMN in spliceosomal U snRNP assembly: implications for spinal muscular atrophy. *Hum Mol Genet* 8(13):2351-2357.

Burlet P, Huber C, Bertrandy S, Ludosky MA, Zwaenepoel I, Clermont O, Roume J, Delezoide AL, Cartaud J, Munnich A & Lefebvre S (1998) The distribution of SMN protein complex in human fetal tissues and its alteration in spinal muscular atrophy. *Hum Mol Genet* 7(12):1927-1933.

Butchbach ME (2016a) Copy Number Variations in the Survival Motor Neuron Genes: Implications for Spinal Muscular Atrophy and Other Neurodegenerative Diseases. *Front Mol Biosci* 3:7.

Butchbach MER (2016b) Copy Number Variations in the Survival Motor Neuron Genes: Implications for Spinal Muscular Atrophy and Other Neurodegenerative Diseases. *Frontiers in Molecular Biosciences* 3(7).

Campbell L, Hunter KM, Mohaghegh P, Tinsley JM, Brasch MA & Davies KE (2000) Direct interaction of Smn with dp103, a putative RNA helicase: a role for Smn in transcription regulation? *Hum Mol Genet* 9(7):1093-1100.

Campbell L, Potter A, Ignatius J, Dubowitz V & Davies K (1997) Genomic variation and gene conversion in spinal muscular atrophy: implications for disease process and clinical phenotype. *Am J Hum Genet* 61(1):40-50.

Carrel TL, McWhorter ML, Workman E, Zhang H, Wolstencroft EC, Lorson C, Bassell GJ, Burghes AH & Beattie CE (2006) Survival motor neuron function in motor axons is independent of functions required for small nuclear ribonucleoprotein biogenesis. *J Neurosci* 26(43):11014-11022.

Chan YB, Miguel-Aliaga I, Franks C, Thomas N, Trülzsch B, Sattelle DB, Davies KE & van den Heuvel M (2003) Neuromuscular defects in a Drosophila survival motor neuron gene mutant. *Hum Mol Genet* 12(12):1367-1376.

Chang T, Zheng W, Tsark W, Bates S, Huang H, Lin RJ & Yee JK (2011) Brief report: phenotypic rescue of induced pluripotent stem cell-derived motoneurons of a spinal muscular atrophy patient. *Stem Cells* 29(12):2090-2093.

Charroux B, Pellizzoni L, Perkinson RA, Shevchenko A, Mann M & Dreyfuss G (1999) Gemin3: A novel DEAD box protein that interacts with SMN, the spinal muscular atrophy gene product, and is a component of gems. *J Cell Biol* 147(6):1181-1194.

Charroux B, Pellizzoni L, Perkinson RA, Yong J, Shevchenko A, Mann M & Dreyfuss G (2000) Gemin4. A novel component of the SMN complex that is found in both gems and nucleoli. *J Cell Biol* 148(6):1177-1186.

Cifuentes-Diaz C, Frugier T, Tiziano FD, Lacène E, Roblot N, Joshi V, Moreau MH & Melki J (2001) Deletion of murine SMN exon 7 directed to skeletal muscle leads to severe muscular dystrophy. *J Cell Biol* 152(5):1107-1114.

Cifuentes-Diaz C, Nicole S, Velasco ME, Borra-Cebrian C, Panozzo C, Frugier T, Millet G, Roblot N, Joshi V & Melki J (2002) Neurofilament accumulation at the motor endplate and lack of axonal sprouting in a spinal muscular atrophy mouse model. *Human molecular genetics* 11(12):1439-1447.

Clelland AK, Kinnear NP, Oram L, Burza J & Sleeman JE (2009) The SMN protein is a key regulator of nuclear architecture in differentiating neuroblastoma cells. *Traffic* 10(11):1585-1598.

Colla E, Coune P, Liu Y, Pletnikova O, Troncoso JC, Iwatsubo T, Schneider BL & Lee MK (2012) Endoplasmic reticulum stress is important for the manifestations of α-synucleinopathy in vivo. *J Neurosci* 32(10):3306-3320.

Comley LH, Nijssen J, Frost-Nylen J & Hedlund E (2016) Cross-disease comparison of amyotrophic lateral sclerosis and spinal muscular atrophy reveals conservation of selective vulnerability but differential neuromuscular junction pathology. *J Comp Neurol* 524(7):1424-1442.

Coque E, Raoul C & Bowerman M (2014) ROCK inhibition as a therapy for spinal muscular atrophy: understanding the repercussions on multiple cellular targets. *Front Neurosci* 8:271.

Cornet C, Di Donato V & Terriente J (2018) Combining Zebrafish and CRISPR/Cas9: Toward a More Efficient Drug Discovery Pipeline. *Front Pharmacol* 9:703.

Corti S, Nizzardo M, Simone C, Falcone M, Nardini M, Ronchi D, Donadoni C, Salani S, Riboldi G, Magri F, Menozzi G, Bonaglia C, Rizzo F, Bresolin N & Comi GP (2012) Genetic correction of human induced pluripotent stem cells from patients with spinal muscular atrophy. *Sci Transl Med* 4(165):165ra162.

Cusin V, Clermont O, Gérard B, Chantereau D & Elion J (2003) Prevalence of SMN1 deletion and duplication in carrier and normal populations: implication for genetic counselling. *Journal of Medical Genetics* 40(4):e39-e39.

D'Amico A, Mercuri E, Tiziano FD & Bertini E (2011) Spinal muscular atrophy. *Orphanet J Rare Dis* 6:71.

Dachs E, Hereu M, Piedrafita L, Casanovas A, Calderó J & Esquerda JE (2011) Defective neuromuscular junction organization and postnatal myogenesis in mice with severe spinal muscular atrophy. *J Neuropathol Exp Neurol* 70(6):444-461.

Dadon-Nachum M, Melamed E & Offen D (2011) The "dying-back" phenomenon of motor neurons in ALS. *J Mol Neurosci* 43(3):470-477.

De Sanctis R, Coratti G, Pasternak A, Montes J, Pane M, Mazzone ES, Young SD, Salazar R, Quigley J, Pera MC, Antonaci L, Lapenta L, Glanzman AM, Tiziano D, Muntoni F, Darras BT, De Vivo DC, Finkel R & Mercuri E (2016) Developmental milestones in type I spinal muscular atrophy. *Neuromuscul Disord* 26(11):754-759.

De Souza AT, Dai X, Spencer AG, Reppen T, Menzie A, Roesch PL, He Y, Caguyong MJ, Bloomer S, Herweijer H, Wolff JA, Hagstrom JE, Lewis DL, Linsley PS & Ulrich RG (2006) Transcriptional and phenotypic comparisons of Ppara knockout and siRNA knockdown mice. *Nucleic Acids Res* 34(16):4486-4494.

De Vivo DC, Bertini E, Swoboda KJ, Hwu WL, Crawford TO, Finkel RS, Kirschner J, Kuntz NL, Parsons JA, Ryan MM, Butterfield RJ, Topaloglu H, Ben-Omran T, Sansone VA, Jong YJ, Shu F, Staropoli JF, Kerr D, Sandrock AW, Stebbins C, Petrillo M, Braley G, Johnson K, Foster R, Gheuens S, Bhan I, Reyna SP, Fradette S & Farwell W (2019) Nusinersen initiated in infants during the presymptomatic stage of spinal muscular atrophy: Interim efficacy and safety results from the Phase 2 NURTURE study. *Neuromuscul Disord* 29(11):842-856.

Desarnaud F, Do Thi AN, Brown AM, Lemke G, Suter U, Baulieu EE & Schumacher M (1998) Progesterone stimulates the activity of the promoters of peripheral myelin protein-22 and protein zero genes in Schwann cells. *J Neurochem* 71(4):1765-1768.

Dimitriadi M, Derdowski A, Kalloo G, Maginnis MS, O'Hern P, Bliska B, Sorkaç A, Nguyen KCQ, Cook SJ, Poulogiannis G, Atwood WJ, Hall DH & Hart AC (2016) Decreased function of survival motor neuron protein impairs endocytic pathways. *Proceedings of the National Academy of Sciences* 113(30):E4377-E4386.

Ebert AD, Yu J, Rose FF, Jr., Mattis VB, Lorson CL, Thomson JA & Svendsen CN (2009) Induced pluripotent stem cells from a spinal muscular atrophy patient. *Nature* 457(7227):277-280.

Eisen JS, Myers PZ & Westerfield M (1986) Pathway selection by growth cones of identified motoneurones in live zebra fish embryos. *Nature* 320(6059):269-271.

El-Brolosy MA & Stainier DYR (2017) Genetic compensation: A phenomenon in search of mechanisms. *PLoS Genet* 13(7):e1006780.

Evers B, Jastrzebski K, Heijmans JP, Grernrum W, Beijersbergen RL & Bernards R (2016) CRISPR knockout screening outperforms shRNA and CRISPRi in identifying essential genes. *Nat Biotechnol* 34(6):631-633.

Fallini C, Donlin-Asp PG, Rouanet JP, Bassell GJ & Rossoll W (2016) Deficiency of the Survival of Motor Neuron Protein Impairs mRNA Localization and Local Translation in the Growth Cone of Motor Neurons. *J Neurosci* 36(13):3811-3820.

Fallini C, Rouanet JP, Donlin-Asp PG, Guo P, Zhang H, Singer RH, Rossoll W & Bassell GJ (2014) Dynamics of survival of motor neuron (SMN) protein interaction with the mRNA-binding protein IMP1 facilitates its trafficking into motor neuron axons. *Dev Neurobiol* 74(3):319-332.

Fan L & Simard LR (2002) Survival motor neuron (SMN) protein: role in neurite outgrowth and neuromuscular maturation during neuronal differentiation and development. *Hum Mol Genet* 11(14):1605-1614.

Farrar MA & Kiernan MC (2015) The Genetics of Spinal Muscular Atrophy: Progress and Challenges. *Neurotherapeutics* 12(2):290-302.

Farrar MA, Park SB, Vucic S, Carey KA, Turner BJ, Gillingwater TH, Swoboda KJ & Kiernan MC (2017) Emerging therapies and challenges in spinal muscular atrophy. *Annals of Neurology* 81(3):355-368.

Farrar MA, Vucic S, Johnston HM, du Sart D & Kiernan MC (2013) Pathophysiological insights derived by natural history and motor function of spinal muscular atrophy. *J Pediatr* 162(1):155-159.

Feldkötter M, Schwarzer V, Wirth R, Wienker TF & Wirth B (2002) Quantitative analyses of SMN1 and SMN2 based on real-time lightCycler PCR: fast and highly reliable carrier testing and prediction of severity of spinal muscular atrophy. *Am J Hum Genet* 70(2):358-368.

Feng W, Gubitz AK, Wan L, Battle DJ, Dostie J, Golembe TJ & Dreyfuss G (2005) Gemins modulate the expression and activity of the SMN complex. *Hum Mol Genet* 14(12):1605-1611.

Fischer U, Liu Q & Dreyfuss G (1997) The SMN-SIP1 complex has an essential role in spliceosomal snRNP biogenesis. *Cell* 90(6):1023-1029.

Fischer U, Sumpter V, Sekine M, Satoh T & Luhrmann R (1993) Nucleo-cytoplasmic transport of U snRNPs: definition of a nuclear location signal in the Sm core domain that binds a transport receptor independently of the m3G cap. *EMBO J* 12(2):573-583.

Fuller HR, Mandefro B, Shirran SL, Gross AR, Kaus AS, Botting CH, Morris GE & Sareen D (2015) Spinal Muscular Atrophy Patient iPSC-Derived Motor Neurons Have Reduced Expression of Proteins Important in Neuronal Development. *Front Cell Neurosci* 9:506.

Gavrilina TO, McGovern VL, Workman E, Crawford TO, Gogliotti RG, DiDonato CJ, Monani UR, Morris GE & Burghes AH (2008) Neuronal SMN expression corrects spinal muscular atrophy in severe SMA mice while muscle-specific SMN expression has no phenotypic effect. *Hum Mol Genet* 17(8):1063-1075.

Giacomotto J, Rinkwitz S & Becker TS (2015) Effective heritable gene knockdown in zebrafish using synthetic microRNAs. *Nat Commun* 6:7378.

Giacomotto J & Segalat L (2010) High-throughput screening and small animal models, where are we? *British journal of pharmacology* 160(2):204-216.

Giavazzi A, Setola V, Simonati A & Battaglia G (2006) Neuronal-specific roles of the survival motor neuron protein: evidence from survival motor neuron expression patterns in the developing human central nervous system. *J Neuropathol Exp Neurol* 65(3):267-277.

Giesemann T, Rathke-Hartlieb S, Rothkegel M, Bartsch JW, Buchmeier S, Jockusch BM & Jockusch H (1999) A role for polyproline motifs in the spinal muscular atrophy protein

SMN. Profilins bind to and colocalize with smn in nuclear gems. *J Biol Chem* 274(53):37908-37914.

Giraldez AJ, Mishima Y, Rihel J, Grocock RJ, Van Dongen S, Inoue K, Enright AJ & Schier AF (2006) Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. *Science* 312(5770):75-79.

Glanzman AM, O'Hagen JM, McDermott MP, Martens WB, Flickinger J, Riley S, Quigley J, Montes J, Dunaway S, Deng L, Chung WK, Tawil R, Darras BT, De Vivo DC, Kaufmann P & Finkel RS (2011) Validation of the Expanded Hammersmith Functional Motor Scale in spinal muscular atrophy type II and III. *J Child Neurol* 26(12):1499-1507.

Glass CK, Saijo K, Winner B, Marchetto MC & Gage FH (2010) Mechanisms underlying inflammation in neurodegeneration. *Cell* 140(6):918-934.

Gogliotti RG, Quinlan KA, Barlow CB, Heier CR, Heckman CJ & Didonato CJ (2012) Motor neuron rescue in spinal muscular atrophy mice demonstrates that sensory-motor defects are a consequence, not a cause, of motor neuron dysfunction. *J Neurosci* 32(11):3818-3829.

Hao le T, Burghes AH & Beattie CE (2011) Generation and Characterization of a genetic zebrafish model of SMA carrying the human SMN2 gene. *Mol Neurodegener* 6(1):24.

Hao le T, Duy PQ, An M, Talbot J, Iyer CC, Wolman M & Beattie CE (2017) HuD and the Survival Motor Neuron Protein Interact in Motoneurons and Are Essential for Motoneuron Development, Function, and mRNA Regulation. *J Neurosci* 37(48):11559-11571.

Hao le T, Duy PQ, Jontes JD & Beattie CE (2015) Motoneuron development influences dorsal root ganglia survival and Schwann cell development in a vertebrate model of spinal muscular atrophy. *Human molecular genetics* 24(2):346-360.

Hao le T, Duy PQ, Jontes JD, Wolman M, Granato M & Beattie CE (2013) Temporal requirement for SMN in motoneuron development. *Hum Mol Genet* 22(13):2612-2625.

Hao le T, Wolman M, Granato M & Beattie CE (2012) Survival motor neuron affects plastin 3 protein levels leading to motor defects. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 32(15):5074-5084.

Harada Y, Sutomo R, Sadewa AH, Akutsu T, Takeshima Y, Wada H, Matsuo M & Nishio H (2002) Correlation between SMN2 copy number and clinical phenotype of spinal muscular atrophy: three SMN2 copies fail to rescue some patients from the disease severity. *J Neurol* 249(9):1211-1219.

Hebert MD, Szymczyk PW, Shpargel KB & Matera AG (2001) Coilin forms the bridge between Cajal bodies and SMN, the spinal muscular atrophy protein. *Genes Dev* 15(20):2720-2729.

Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, Collins JE, Humphray S, McLaren K, Matthews L, McLaren S, Sealy I, Caccamo M, Churcher C, Scott C, Barrett JC, Koch R, Rauch GJ, White S, Chow W, Kilian B, Quintais LT, Guerra-Assuncao JA, Zhou Y, Gu Y, Yen J, Vogel JH, Eyre T, Redmond S, Banerjee R, Chi J, Fu B, Langley E, Maguire SF, Laird GK, Lloyd D, Kenyon E, Donaldson S, Sehra H, Almeida-King J, Loveland J, Trevanion S, Jones M, Quail M, Willey D, Hunt A, Burton J, Sims S, McLay K, Plumb B, Davis J, Clee C, Oliver K, Clark R, Riddle C, Elliot D, Threadgold G, Harden G, Ware D, Begum S, Mortimore B, Kerry G, Heath P, Phillimore B, Tracey A, Corby N, Dunn M, Johnson C, Wood J, Clark S, Pelan S, Griffiths G, Smith M, Glithero R, Howden P, Barker N, Lloyd C, Stevens C, Harley J, Holt K, Panagiotidis G, Lovell J, Beasley H, Henderson C, Gordon D, Auger K, Wright D, Collins J, Raisen C, Dyer L,

Leung K, Robertson L, Ambridge K, Leongamornlert D, McGuire S, Gilderthorp R, Griffiths C, Manthravadi D, Nichol S, Barker G, Whitehead S, Kay M, Brown J, Murnane C, Gray E, Humphries M, Sycamore N, Barker D, Saunders D, Wallis J, Babbage A, Hammond S, Mashreghi-Mohammadi M, Barr L, Martin S, Wray P, Ellington A, Matthews N, Ellwood M, Woodmansey R, Clark G, Cooper J, Tromans A, Grafham D, Skuce C, Pandian R, Andrews R, Harrison E, Kimberley A, Garnett J, Fosker N, Hall R, Garner P, Kelly D, Bird C, Palmer S, Gehring I, Berger A, Dooley CM, Ersan-Urun Z, Eser C, Geiger H, Geisler M, Karotki L, Kirn A, Konantz J, Konantz M, Oberlander M, Rudolph-Geiger S, Teucke M, Lanz C, Raddatz G, Osoegawa K, Zhu B, Rapp A, Widaa S, Langford C, Yang F, Schuster SC, Carter NP, Harrow J, Ning Z, Herrero J, Searle SM, Enright A, Geisler R, Plasterk RH, Lee C, Westerfield M, de Jong PJ, Zon LI, Postlethwait JH, Nusslein-Volhard C, Hubbard TJ, Roest Crollius H, Rogers J & Stemple DL (2013) The zebrafish reference genome sequence and its relationship to the human genome. *Nature* 496(7446):498-503.

Hsieh-Li HM, Chang JG, Jong YJ, Wu MH, Wang NM, Tsai CH & Li H (2000) A mouse model for spinal muscular atrophy. *Nat Genet* 24(1):66-70.

Hua Y, Liu YH, Sahashi K, Rigo F, Bennett CF & Krainer AR (2015) Motor neuron cell-nonautonomous rescue of spinal muscular atrophy phenotypes in mild and severe transgenic mouse models. *Genes Dev* 29(3):288-297.

Hua Y, Sahashi K, Hung G, Rigo F, Passini MA, Bennett CF & Krainer AR (2010) Antisense correction of SMN2 splicing in the CNS rescues necrosis in a type III SMA mouse model. *Genes & development* 24(15):1634-1644.

Huang X, Chen Y, Zhang H, Ma Q, Zhang YW & Xu H (2012) Salubrinal attenuates β-amyloid-induced neuronal death and microglial activation by inhibition of the NF-κB pathway. *Neurobiol Aging* 33(5):1007 e1009-1017.

Hunter G, Aghamaleky Sarvestany A, Roche SL, Symes RC & Gillingwater TH (2014) SMN-dependent intrinsic defects in Schwann cells in mouse models of spinal muscular atrophy. *Hum Mol Genet* 23(9):2235-2250.

Hwang WY, Fu Y, Reyon D, Maeder ML, Tsai SQ, Sander JD, Peterson RT, Yeh JR & Joung JK (2013) Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat Biotechnol* 31(3):227-229.

Imlach WL, Beck ES, Choi BJ, Lotti F, Pellizzoni L & McCabe BD (2012) SMN is required for sensory-motor circuit function in Drosophila. *Cell* 151(2):427-439.

Ince PG, Slade J, Chinnery RM, McKenzie J, Royston C, Roberts GW & Shaw PJ (1995) Quantitative study of synaptophysin immunoreactivity of cerebral cortex and spinal cord in motor neuron disease. *J Neuropathol Exp Neurol* 54(5):673-679.

Irion U, Krauss J & Nüsslein-Volhard C (2014) Precise and efficient genome editing in zebrafish using the CRISPR/Cas9 system. *Development* 141(24):4827-4830.

Ito Y, Kumada S, Uchiyama A, Saito K, Osawa M, Yagishita A, Kurata K & Hayashi M (2004) Thalamic lesions in a long-surviving child with spinal muscular atrophy type I: MRI and EEG findings. *Brain Dev* 26(1):53-56.

Iyer CC, McGovern VL, Murray JD, Gombash SE, Zaworski PG, Foust KD, Janssen PM & Burghes AH (2015) Low levels of Survival Motor Neuron protein are sufficient for normal muscle function in the SMNΔ7 mouse model of SMA. *Hum Mol Genet* 24(21):6160-6173.

Jablonka S, Karle K, Sandner B, Andreassi C, von Au K & Sendtner M (2006) Distinct and overlapping alterations in motor and sensory neurons in a mouse model of spinal muscular atrophy. *Hum Mol Genet* 15(3):511-518.

Jablonka S, Schrank B, Kralewski M, Rossoll W & Sendtner M (2000) Reduced survival motor neuron (Smn) gene dose in mice leads to motor neuron degeneration: an animal model for spinal muscular atrophy type III. *Hum Mol Genet* 9(3):341-346.

Jia XF, Ye F, Wang YB & Feng DX (2016) ROCK inhibition enhances neurite outgrowth in neural stem cells by upregulating YAP expression in vitro. *Neural Regen Res* 11(6):983-987.

Kariya S, Park GH, Maeno-Hikichi Y, Leykekhman O, Lutz C, Arkovitz MS, Landmesser LT & Monani UR (2008) Reduced SMN protein impairs maturation of the neuromuscular junctions in mouse models of spinal muscular atrophy. *Hum Mol Genet* 17(16):2552-2569.

Khatri IA, Chaudhry US, Seikaly MG, Browne RH & Iannaccone ST (2008) Low bone mineral density in spinal muscular atrophy. *J Clin Neuromuscul Dis* 10(1):11-17.

Kim JK, Jha NN, Feng Z, Faleiro MR, Chiriboga CA, Wei-Lapierre L, Dirksen RT, Ko CP & Monani UR (2020) Muscle-specific SMN reduction reveals motor neuron-independent disease in spinal muscular atrophy models. *J Clin Invest* 130(3):1271-1287.

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B & Schilling TF (1995) Stages of embryonic development of the zebrafish. *Dev Dyn* 203(3):253-310.

Kinali M, Banks LM, Mercuri E, Manzur AY & Muntoni F (2004) Bone mineral density in a paediatric spinal muscular atrophy population. *Neuropediatrics* 35(6):325-328.

Kok FO, Shin M, Ni CW, Gupta A, Grosse AS, van Impel A, Kirchmaier BC, Peterson-Maduro J, Kourkoulis G, Male I, DeSantis DF, Sheppard-Tindell S, Ebarasi L, Betsholtz C, Schulte-Merker S, Wolfe SA & Lawson ND (2015) Reverse genetic screening reveals poor correlation between morpholino-induced and mutant phenotypes in zebrafish. *Dev Cell* 32(1):97-108.

Kolb SJ & Kissel JT (2015) Spinal Muscular Atrophy. *Neurologic Clinics* 33(4):831-846.

Kong L, Wang X, Choe DW, Polley M, Burnett BG, Bosch-Marce M, Griffin JW, Rich MM & Sumner CJ (2009) Impaired synaptic vesicle release and immaturity of neuromuscular junctions in spinal muscular atrophy mice. *J Neurosci* 29(3):842-851.

Lagier-Tourenne C, Polymenidou M & Cleveland DW (2010) TDP-43 and FUS/TLS: emerging roles in RNA processing and neurodegeneration. *Human molecular genetics* 19(R1):R46-64.

Laird AS, Mackovski N, Rinkwitz S, Becker TS & Giacomotto J (2016) Tissue-specific models of spinal muscular atrophy confirm a critical role of SMN in motor neurons from embryonic to adult stages. *Hum Mol Genet* 25(9):1728-1738.

Larson MH, Gilbert LA, Wang X, Lim WA, Weissman JS & Qi LS (2013) CRISPR interference (CRISPRi) for sequence-specific control of gene expression. *Nat Protoc* 8(11):2180-2196.

Le TT, Pham LT, Butchbach ME, Zhang HL, Monani UR, Coovert DD, Gavrilina TO, Xing L, Bassell GJ & Burghes AH (2005) SMNDelta7, the major product of the centromeric survival motor neuron (SMN2) gene, extends survival in mice with spinal muscular atrophy and associates with full-length SMN. *Hum Mol Genet* 14(6):845-857.

Lee AJ, Awano T, Park GH & Monani UR (2012) Limited phenotypic effects of selectively augmenting the SMN protein in the neurons of a mouse model of severe spinal muscular atrophy. *PLoS One* 7(9):e46353.

Lee YI, Mikesh M, Smith I, Rimer M & Thompson W (2011) Muscles in a mouse model of spinal muscular atrophy show profound defects in neuromuscular development even in the absence of failure in neuromuscular transmission or loss of motor neurons. *Dev Biol* 356(2):432-444.

Lefebvre S, Bürglen L, Reboullet S, Clermont O, Burlet P, Viollet L, Benichou B, Cruaud C, Millasseau P, Zeviani M & et al. (1995) Identification and characterization of a spinal muscular atrophy-determining gene. *Cell* 80(1):155-165.

Ling KK, Lin MY, Zingg B, Feng Z & Ko CP (2010) Synaptic defects in the spinal and neuromuscular circuitry in a mouse model of spinal muscular atrophy. *PLoS One* 5(11):e15457.

Liu H, Lu J, Chen H, Du Z, Li XJ & Zhang SC (2015) Spinal muscular atrophy patient-derived motor neurons exhibit hyperexcitability. *Sci Rep* 5:12189.

Liu Q & Dreyfuss G (1996) A novel nuclear structure containing the survival of motor neurons protein. *EMBO J* 15(14):3555-3565.

Liu Q, Fischer U, Wang F & Dreyfuss G (1997) The spinal muscular atrophy disease gene product, SMN, and its associated protein SIP1 are in a complex with spliceosomal snRNP proteins. *Cell* 90(6):1013-1021.

Long L, Guo H, Yao D, Xiong K, Li Y, Liu P, Zhu Z & Liu D (2015) Regulation of transcriptionally active genes via the catalytically inactive Cas9 in C. elegans and D. rerio. *Cell Res* 25(5):638-641.

Lorson CL & Androphy EJ (2000) An exonic enhancer is required for inclusion of an essential exon in the SMA-determining gene SMN. *Hum Mol Genet* 9(2):259-265.

MacLeod MJ, Taylor JE, Lunt PW, Mathew CG & Robb SA (1999) Prenatal onset spinal muscular atrophy. *Eur J Paediatr Neurol* 3(2):65-72.

MacRae CA & Peterson RT (2015) Zebrafish as tools for drug discovery. *Nat Rev Drug Discov* 14(10):721-731.

Maeda M, Harris AW, Kingham BF, Lumpkin CJ, Opdenaker LM, McCahan SM, Wang W & Butchbach ME (2014) Transcriptome profiling of spinal muscular atrophy motor neurons derived from mouse embryonic stem cells. *PloS one* 9(9):e106818.

Mailman MD, Heinz JW, Papp AC, Snyder PJ, Sedra MS, Wirth B, Burghes AH & Prior TW (2002) Molecular analysis of spinal muscular atrophy and modification of the phenotype by SMN2. *Genet Med* 4(1):20-26.

Marraffini LA & Sontheimer EJ (2010) CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea. *Nat Rev Genet* 11(3):181-190.

Martinez TL, Kong L, Wang X, Osborne MA, Crowder ME, Van Meerbeke JP, Xu X, Davis C, Wooley J, Goldhamer DJ, Lutz CM, Rich MM & Sumner CJ (2012) Survival motor neuron protein in motor neurons determines synaptic integrity in spinal muscular atrophy. *J Neurosci* 32(25):8703-8715.

Martinez-Hernandez R, Bernal S, Also-Rallo E, Alias L, Barcelo MJ, Hereu M, Esquerda JE & Tizzano EF (2013) Synaptic defects in type I spinal muscular atrophy in human development. *J Pathol* 229(1):49-61.

Martinez-Hernandez R, Soler-Botija C, Also E, Alias L, Caselles L, Gich I, Bernal S & Tizzano EF (2009) The developmental pattern of myotubes in spinal muscular atrophy indicates prenatal delay of muscle maturation. *J Neuropathol Exp Neurol* 68(5):474-481.

Mattaj IW (1986) Cap trimethylation of U snRNA is cytoplasmic and dependent on U snRNP protein binding. *Cell* 46(6):905-911.

Mattis VB, Ebert AD, Fosso MY, Chang CW & Lorson CL (2009) Delivery of a read-through inducing compound, TC007, lessens the severity of a spinal muscular atrophy animal model. *Human molecular genetics* 18(20):3906-3913.

Mazzone E, Bianco F, Martinelli D, Glanzman AM, Messina S, De Sanctis R, Main M, Eagle M, Florence J, Krosschell K, Vasco G, Pelliccioni M, Lombardo M, Pane M, Finkel R, Muntoni F, Bertini E & Mercuri E (2011) Assessing upper limb function in nonambulant SMA patients: development of a new module. *Neuromuscul Disord* 21(6):406-412.

McGovern VL, Massoni-Laporte A, Wang X, Le TT, Le HT, Beattie CE, Rich MM & Burghes AH (2015) Plastin 3 Expression Does Not Modify Spinal Muscular Atrophy Severity in the Δ7 SMA Mouse. *PLoS One* 10(7):e0132364.

McWhorter ML, Monani UR, Burghes AH & Beattie CE (2003) Knockdown of the survival motor neuron (Smn) protein in zebrafish causes defects in motor axon outgrowth and pathfinding. *J Cell Biol* 162(5):919-931.

Medvedev SP, Shevchenko AI & Zakian SM (2010) Induced Pluripotent Stem Cells: Problems and Advantages when Applying them in Regenerative Medicine. *Acta Naturae* 2(2):18-28.

Melki J, Abdelhak S, Sheth P, Bachelot MF, Burlet P, Marcadet A, Aicardi J, Barois A, Carriere JP, Fardeau M & et al. (1990) Gene for chronic proximal spinal muscular atrophies maps to chromosome 5q. *Nature* 344(6268):767-768.

Mendell JR, Al-Zaidy S, Shell R, Arnold WD, Rodino-Klapac LR, Prior TW, Lowes L, Alfano L, Berry K, Church K, Kissel JT, Nagendran S, L'Italien J, Sproule DM, Wells C, Cardenas JA, Heitzer MD, Kaspar A, Corcoran S, Braun L, Likhite S, Miranda C, Meyer K, Foust KD, Burghes AHM & Kaspar BK (2017) Single-Dose Gene-Replacement Therapy for Spinal Muscular Atrophy. *N Engl J Med* 377(18):1713-1722.

Mentis GZ, Blivis D, Liu W, Drobac E, Crowder ME, Kong L, Alvarez FJ, Sumner CJ & O'Donovan MJ (2011) Early functional impairment of sensory-motor connectivity in a mouse model of spinal muscular atrophy. *Neuron* 69(3):453-467.

Mercuri E, Bertini E & Iannaccone ST (2012) Childhood spinal muscular atrophy: controversies and challenges. *Lancet Neurol* 11(5):443-452.

Merlini L, Solari A, Vita G, Bertini E, Minetti C, Mongini T, Mazzoni E, Angelini C & Morandi L (2003) Role of gabapentin in spinal muscular atrophy: results of a multicenter, randomized Italian study. *Journal of child neurology* 18(8):537-541.

Miguel-Aliaga I, Culetto E, Walker DS, Baylis HA, Sattelle DB & Davies KE (1999) The Caenorhabditis elegans orthologue of the human gene responsible for spinal muscular atrophy is a maternal product critical for germline maturation and embryonic viability. *Hum Mol Genet* 8(12):2133-2143.

Monani UR, Coovert DD & Burghes AH (2000a) Animal models of spinal muscular atrophy. *Hum Mol Genet* 9(16):2451-2457.

Monani UR, Lorson CL, Parsons DW, Prior TW, Androphy EJ, Burghes AH & McPherson JD (1999) A single nucleotide difference that alters splicing patterns distinguishes the SMA gene SMN1 from the copy gene SMN2. *Hum Mol Genet* 8(7):1177-1183.

Monani UR, Pastore MT, Gavrilina TO, Jablonka S, Le TT, Andreassi C, DiCocco JM, Lorson C, Androphy EJ, Sendtner M, Podell M & Burghes AH (2003) A transgene carrying an A2G missense mutation in the SMN gene modulates phenotypic severity in mice with severe (type I) spinal muscular atrophy. *J Cell Biol* 160(1):41-52.

Monani UR, Sendtner M, Coovert DD, Parsons DW, Andreassi C, Le TT, Jablonka S, Schrank B, Rossoll W, Prior TW, Morris GE & Burghes AH (2000b) The human centromeric

survival motor neuron gene (SMN2) rescues embryonic lethality in Smn(-/-) mice and results in a mouse with spinal muscular atrophy. *Hum Mol Genet* 9(3):333-339.
Montague K, Malik B, Gray AL, La Spada AR, Hanna MG, Szabadkai G & Greensmith L (2014) Endoplasmic reticulum stress in spinal and bulbar muscular atrophy: a potential target for therapy. *Brain* 137(Pt 7):1894-1906.
Mueller BK (1999) Growth cone guidance: first steps towards a deeper understanding. *Annu Rev Neurosci* 22:351-388.
Murray LM, Beauvais A, Gibeault S, Courtney NL & Kothary R (2015) Transcriptional profiling of differentially vulnerable motor neurons at pre-symptomatic stage in the Smn (2b/-) mouse model of spinal muscular atrophy. *Acta neuropathologica communications* 3:55.
Murray LM, Comley LH, Thomson D, Parkinson N, Talbot K & Gillingwater TH (2008) Selective vulnerability of motor neurons and dissociation of pre- and post-synaptic pathology at the neuromuscular junction in mouse models of spinal muscular atrophy. *Human molecular genetics* 17(7):949-962.
Myers PZ, Eisen JS & Westerfield M (1986) Development and axonal outgrowth of identified motoneurons in the zebrafish. *J Neurosci* 6(8):2278-2289.
Naeve GS, Ramakrishnan M, Kramer R, Hevroni D, Citri Y & Theill LE (1997) Neuritin: a gene induced by neural activity and neurotrophins that promotes neuritogenesis. *Proc Natl Acad Sci U S A* 94(6):2648-2653.
Naryshkin NA, Weetall M, Dakka A, Narasimhan J, Zhao X, Feng Z, Ling KK, Karp GM, Qi H, Woll MG, Chen G, Zhang N, Gabbeta V, Vazirani P, Bhattacharyya A, Furia B, Risher N, Sheedy J, Kong R, Ma J, Turpoff A, Lee CS, Zhang X, Moon YC, Trifillis P, Welch EM, Colacino JM, Babiak J, Almstead NG, Peltz SW, Eng LA, Chen KS, Mull JL, Lynes MS, Rubin LL, Fontoura P, Santarelli L, Haehnke D, McCarthy KD, Schmucki R, Ebeling M, Sivaramakrishnan M, Ko CP, Paushkin SV, Ratni H, Gerlach I, Ghosh A & Metzger F (2014) Motor neuron disease. SMN2 splicing modifiers improve motor function and longevity in mice with spinal muscular atrophy. *Science* 345(6197):688-693.
Nelson L, Owens H, Hynan LS & Iannaccone ST (2006) The gross motor function measure is a valid and sensitive outcome measure for spinal muscular atrophy. *Neuromuscul Disord* 16(6):374-380.
Ng SY, Soh BS, Rodriguez-Muela N, Hendrickson DG, Price F, Rinn JL & Rubin LL (2015) Genome-wide RNA-Seq of Human Motor Neurons Implicates Selective ER Stress Activation in Spinal Muscular Atrophy. *Cell Stem Cell* 17(5):569-584.
Nizzardo M, Nardini M, Ronchi D, Salani S, Donadoni C, Fortunato F, Colciago G, Falcone M, Simone C, Riboldi G, Govoni A, Bresolin N, Comi GP & Corti S (2011) Beta-lactam antibiotic offers neuroprotection in a spinal muscular atrophy model by multiple mechanisms: Experimental Neurology 2011; 229: 214-225. *Annals of neurosciences* 18(4):156-157.
Nolle A, Zeug A, van Bergeijk J, Tonges L, Gerhard R, Brinkmann H, Al Rayes S, Hensel N, Schill Y, Apkhazava D, Jablonka S, O'Mer J, Srivastav RK, Baasner A, Lingor P, Wirth B, Ponimaskin E, Niedenthal R, Grothe C & Claus P (2011) The spinal muscular atrophy disease protein SMN is linked to the Rho-kinase pathway via profilin. *Hum Mol Genet* 20(24):4865-4878.
Norton W & Bally-Cuif L (2010) Adult zebrafish as a model organism for behavioural genetics. *BMC Neurosci* 11:90.

Ogino S, Gao S, Leonard DG, Paessler M & Wilson RB (2003) Inverse correlation between SMN1 and SMN2 copy numbers: evidence for gene conversion from SMN2 to SMN1. *Eur J Hum Genet* 11(3):275-277.

Ogino S & Wilson RB (2004) Spinal muscular atrophy: molecular genetics and diagnostics. *Expert Rev Mol Diagn* 4(1):15-29.

Oprea GE, Krober S, McWhorter ML, Rossoll W, Muller S, Krawczak M, Bassell GJ, Beattie CE & Wirth B (2008) Plastin 3 is a protective modifier of autosomal recessive spinal muscular atrophy. *Science* 320(5875):524-527.

Ota S, Hisano Y, Ikawa Y & Kawahara A (2014) Multiple genome modifications by the CRISPR/Cas9 system in zebrafish. *Genes Cells* 19(7):555-564.

Pagliardini S, Giavazzi A, Setola V, Lizier C, Di Luca M, DeBiasi S & Battaglia G (2000) Subcellular localization and axonal transport of the survival motor neuron (SMN) protein in the developing rat spinal cord. *Hum Mol Genet* 9(1):47-56.

Palacino J, Swalley SE, Song C, Cheung AK, Shu L, Zhang X, Van Hoosear M, Shin Y, Chin DN, Keller CG, Beibel M, Renaud NA, Smith TM, Salcius M, Shi X, Hild M, Servais R, Jain M, Deng L, Bullock C, McLellan M, Schuierer S, Murphy L, Blommers MJ, Blaustein C, Berenshteyn F, Lacoste A, Thomas JR, Roma G, Michaud GA, Tseng BS, Porter JA, Myer VE, Tallarico JA, Hamann LG, Curtis D, Fishman MC, Dietrich WF, Dales NA & Sivasankaran R (2015) SMN2 splice modulators enhance U1-pre-mRNA association and rescue SMA mice. *Nat Chem Biol* 11(7):511-517.

Park GH, Maeno-Hikichi Y, Awano T, Landmesser LT & Monani UR (2010) Reduced survival of motor neuron (SMN) protein in motor neuronal progenitors functions cell autonomously to cause spinal muscular atrophy in model mice expressing the human centromeric (SMN2) gene. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 30(36):12005-12019.

Pascale A, Gusev PA, Amadio M, Dottorini T, Govoni S, Alkon DL & Quattrone A (2004) Increase of the RNA-binding protein HuD and posttranscriptional up-regulation of the GAP-43 gene during spatial memory. *Proc Natl Acad Sci U S A* 101(5):1217-1222.

Patten SA, Aggad D, Martinez J, Tremblay E, Petrillo J, Armstrong GA, La Fontaine A, Maios C, Liao M, Ciura S, Wen XY, Rafuse V, Ichida J, Zinman L, Julien JP, Kabashi E, Robitaille R, Korngut L, Parker JA & Drapeau P (2017) Neuroleptics as therapeutic compounds stabilizing neuromuscular transmission in amyotrophic lateral sclerosis. *JCI Insight* 2(22).

Patten SA, Armstrong GA, Lissouba A, Kabashi E, Parker JA & Drapeau P (2014) Fishing for causes and cures of motor neuron disorders. *Dis Model Mech* 7(7):799-809.

Poirier A, Weetall M, Heinig K, Bucheli F, Schoenlein K, Alsenz J, Bassett S, Ullah M, Senn C, Ratni H, Naryshkin N, Paushkin S & Mueller L (2018) Risdiplam distributes and increases SMN protein in both the central nervous system and peripheral organs. *Pharmacol Res Perspect* 6(6):e00447.

Powis RA, Karyka E, Boyd P, Come J, Jones RA, Zheng Y, Szunyogova E, Groen EJ, Hunter G, Thomson D, Wishart TM, Becker CG, Parson SH, Martinat C, Azzouz M & Gillingwater TH (2016a) Systemic restoration of UBA1 ameliorates disease in spinal muscular atrophy. *JCI Insight* 1(11):e87908.

Powis RA, Karyka E, Boyd P, Côme J, Jones RA, Zheng Y, Szunyogova E, Groen EJ, Hunter G, Thomson D, Wishart TM, Becker CG, Parson SH, Martinat C, Azzouz M & Gillingwater TH (2016b) Systemic restoration of UBA1 ameliorates disease in spinal muscular atrophy. *JCI Insight* 1(11):e87908.

Rajendra TK, Gonsalvez GB, Walker MP, Shpargel KB, Salz HK & Matera AG (2007) A Drosophila melanogaster model of spinal muscular atrophy reveals a function for SMN in striated muscle. *J Cell Biol* 176(6):831-841.

Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA & Zhang F (2013) Genome engineering using the CRISPR-Cas9 system. *Nat Protoc* 8(11):2281-2308.

Riessland M, Ackermann B, Forster A, Jakubik M, Hauke J, Garbes L, Fritzsche I, Mende Y, Blumcke I, Hahnen E & Wirth B (2010) SAHA ameliorates the SMA phenotype in two mouse models for spinal muscular atrophy. *Human molecular genetics* 19(8):1492-1506.

Rigo F, Hua Y, Krainer AR & Bennett CF (2012) Antisense-based therapy for the treatment of spinal muscular atrophy. *The Journal of cell biology* 199(1):21-25.

Rindt H, Feng Z, Mazzasette C, Glascock JJ, Valdivia D, Pyles N, Crawford TO, Swoboda KJ, Patitucci TN, Ebert AD, Sumner CJ, Ko CP & Lorson CL (2015) Astrocytes influence the severity of spinal muscular atrophy. *Hum Mol Genet* 24(14):4094-4102.

Roberts DF, Chavez J & Court SD (1970) The genetic component in child mortality. *Arch Dis Child* 45(239):33-38.

Robu ME, Larson JD, Nasevicius A, Beiraghi S, Brenner C, Farber SA & Ekker SC (2007) p53 activation by knockdown technologies. *PLoS Genet* 3(5):e78.

Rossi A, Kontarakis Z, Gerri C, Nolte H, Hölper S, Krüger M & Stainier DY (2015) Genetic compensation induced by deleterious mutations but not gene knockdowns. *Nature* 524(7564):230-233.

Rossoll W, Kröning AK, Ohndorf UM, Steegborn C, Jablonka S & Sendtner M (2002) Specific interaction of Smn, the spinal muscular atrophy determining gene product, with hnRNP-R and gry-rbp/hnRNP-Q: a role for Smn in RNA processing in motor axons? *Hum Mol Genet* 11(1):93-105.

Rudnik-Schöneborn S, Goebel HH, Schlote W, Molaian S, Omran H, Ketelsen U, Korinthenberg R, Wenzel D, Lauffer H, Kreiss-Nachtsheim M, Wirth B & Zerres K (2003) Classical infantile spinal muscular atrophy with SMN deficiency causes sensory neuronopathy. *Neurology* 60(6):983-987.

Rudnik-Schoneborn S, Heller R, Berg C, Betzler C, Grimm T, Eggermann T, Eggermann K, Wirth R, Wirth B & Zerres K (2008) Congenital heart disease is a feature of severe infantile spinal muscular atrophy. *J Med Genet* 45(10):635-638.

Ruggiu M, McGovern VL, Lotti F, Saieva L, Li DK, Kariya S, Monani UR, Burghes AH & Pellizzoni L (2012) A role for SMN exon 7 splicing in the selective vulnerability of motor neurons in spinal muscular atrophy. *Mol Cell Biol* 32(1):126-138.

Saint-Amant L & Drapeau P (1998) Time course of the development of motor behaviors in the zebrafish embryo. *J Neurobiol* 37(4):622-632.

Samarut É, Lissouba A & Drapeau P (2016) A simplified method for identifying early CRISPR-induced indels in zebrafish embryos using High Resolution Melting analysis. *BMC Genomics* 17:547.

Saxena S, Cabuy E & Caroni P (2009) A role for motoneuron subtype-selective ER stress in disease manifestations of FALS mice. *Nat Neurosci* 12(5):627-636.

Schrank B, Gotz R, Gunnersen JM, Ure JM, Toyka KV, Smith AG & Sendtner M (1997) Inactivation of the survival motor neuron gene, a candidate gene for human spinal muscular atrophy, leads to massive cell death in early mouse embryos. *Proc Natl Acad Sci U S A* 94(18):9920-9925.

Schreml J, Riessland M, Paterno M, Garbes L, Rossbach K, Ackermann B, Kramer J, Somers E, Parson SH, Heller R, Berkessel A, Sterner-Kock A & Wirth B (2013) Severe SMA mice

show organ impairment that cannot be rescued by therapy with the HDACi JNJ-26481585. *Eur J Hum Genet* 21(6):643-652.
See K, Yadav P, Giegerich M, Cheong PS, Graf M, Vyas H, Lee SG, Mathavan S, Fischer U, Sendtner M & Winkler C (2014) SMN deficiency alters Nrxn2 expression and splicing in zebrafish and mouse models of spinal muscular atrophy. *Hum Mol Genet* 23(7):1754-1770.
Selenko P, Sprangers R, Stier G, Bühler D, Fischer U & Sattler M (2001) SMN tudor domain structure and its interaction with the Sm proteins. *Nat Struct Biol* 8(1):27-31.
Shababi M, Habibi J, Yang HT, Vale SM, Sewell WA & Lorson CL (2010) Cardiac defects contribute to the pathology of spinal muscular atrophy models. *Hum Mol Genet* 19(20):4059-4071.
Shanmugarajan S, Swoboda KJ, Iannaccone ST, Ries WL, Maria BL & Reddy SV (2007) Congenital bone fractures in spinal muscular atrophy: functional role for SMN protein in bone remodeling. *J Child Neurol* 22(8):967-973.
Shanmugarajan S, Tsuruga E, Swoboda KJ, Maria BL, Ries WL & Reddy SV (2009) Bone loss in survival motor neuron (Smn(-/-) SMN2) genetic mouse model of spinal muscular atrophy. *J Pathol* 219(1):52-60.
Sharma A, Lambrechts A, Hao le T, Le TT, Sewry CA, Ampe C, Burghes AH & Morris GE (2005) A role for complexes of survival of motor neurons (SMN) protein with gemins and profilin in neurite-like cytoplasmic extensions of cultured nerve cells. *Exp Cell Res* 309(1):185-197.
Shishikura K, Hara M, Sasaki Y & Misugi K (1983) A neuropathologic study of Werdnig-Hoffmann disease with special reference to the thalamus and posterior roots. *Acta Neuropathol* 60(1-2):99-106.
Sleigh JN, Barreiro-Iglesias A, Oliver PL, Biba A, Becker T, Davies KE, Becker CG & Talbot K (2014) Chondrolectin affects cell survival and neuronal outgrowth in in vitro and in vivo models of spinal muscular atrophy. *Hum Mol Genet* 23(4):855-869.
Sleigh JN, Gillingwater TH & Talbot K (2011) The contribution of mouse models to understanding the pathogenesis of spinal muscular atrophy. *Dis Model Mech* 4(4):457-467.
Słomka M, Sobalska-Kwapis M, Wachulec M, Bartosz G & Strapagiel D (2017) High Resolution Melting (HRM) for High-Throughput Genotyping-Limitations and Caveats in Practical Case Studies. *Int J Mol Sci* 18(11).
Stratigopoulos G, Lanzano P, Deng L, Guo J, Kaufmann P, Darras B, Finkel R, Tawil R, McDermott MP, Martens W, Devivo DC & Chung WK (2010) Association of plastin 3 expression with disease severity in spinal muscular atrophy only in postpubertal females. *Arch Neurol* 67(10):1252-1256.
Tangsrud SE, Carlsen KCL, Lund-Petersen I & Carlsen K-H (2001) Lung function measurements in young children with spinal muscle atrophy; a cross sectional survey on the effect of position and bracing. *Archives of Disease in Childhood* 84(6):521-524.
Tarabal O, Caraballo-Miralles V, Cardona-Rossinyol A, Correa FJ, Olmos G, Llado J, Esquerda JE & Caldero J (2014) Mechanisms involved in spinal cord central synapse loss in a mouse model of spinal muscular atrophy. *Journal of neuropathology and experimental neurology* 73(6):519-535.
Tat J, Liu M & Wen XY (2013) Zebrafish cancer and metastasis models for in vivo drug discovery. *Drug discovery today. Technologies* 10(1):e83-89.

Teng X, Dayhoff-Brannigan M, Cheng WC, Gilbert CE, Sing CN, Diny NL, Wheelan SJ, Dunham MJ, Boeke JD, Pineda FJ & Hardwick JM (2013) Genome-wide consequences of deleting any single gene. *Mol Cell* 52(4):485-494.

Thirumalai V, Behrend RM, Birineni S, Liu W, Blivis D & O'Donovan MJ (2013) Preservation of VGLUT1 synapses on ventral calbindin-immunoreactive interneurons and normal locomotor function in a mouse model of spinal muscular atrophy. *J Neurophysiol* 109(3):702-710.

Thomas NH & Dubowitz V (1994) The natural history of type I (severe) spinal muscular atrophy. *Neuromuscul Disord* 4(5-6):497-502.

Torres-Benito L, Neher MF, Cano R, Ruiz R & Tabares L (2011) SMN requirement for synaptic vesicle, active zone and microtubule postnatal organization in motor nerve terminals. *PloS one* 6(10):e26164.

Vaccaro A, Patten SA, Aggad D, Julien C, Maios C, Kabashi E, Drapeau P & Parker JA (2013) Pharmacological reduction of ER stress protects against TDP-43 neuronal toxicity in vivo. *Neurobiol Dis* 55:64-75.

Vaccaro A, Patten SA, Ciura S, Maios C, Therrien M, Drapeau P, Kabashi E & Parker JA (2012) Methylene blue protects against TDP-43 and FUS neuronal toxicity in C. elegans and D. rerio. *PloS one* 7(7):e42117.

Verhaart IEC RA, Wilson IJ, Aartsma-Rus A, Cameron S, Jones CC, Cook SF, Lochmüller H. (2017) Prevalence, incidence and carrier frequency of 5q-linked spinal muscular atrophy - a literature review. *Orphanet journal of rare diseases* doi: 10.1186/s13023-017-0671-8.

Viollet L, Bertrandy S, Bueno Brunialti AL, Lefebvre S, Burlet P, Clermont O, Cruaud C, Guenet JL, Munnich A & Melki J (1997) cDNA isolation, expression, and chromosomal localization of the mouse survival motor neuron gene (Smn). *Genomics* 40(1):185-188.

Vitte JM, Davoult B, Roblot N, Mayer M, Joshi V, Courageot S, Tronche F, Vadrot J, Moreau MH, Kemeny F & Melki J (2004) Deletion of murine Smn exon 7 directed to liver leads to severe defect of liver development associated with iron overload. *Am J Pathol* 165(5):1731-1741.

von Gontard A, Zerres K, Backes M, Laufersweiler-Plass C, Wendland C, Melchers P, Lehmkuhl G & Rudnik-Schoneborn S (2002) Intelligence and cognitive function in children and adolescents with spinal muscular atrophy. *Neuromuscul Disord* 12(2):130-136.

Wadman RI, Vrancken AF, van den Berg LH & van der Pol WL (2012) Dysfunction of the neuromuscular junction in spinal muscular atrophy types 2 and 3. *Neurology* 79(20):2050-2055.

Walker AK, Soo KY, Sundaramoorthy V, Parakh S, Ma Y, Farg MA, Wallace RH, Crouch PJ, Turner BJ, Horne MK & Atkin JD (2013) ALS-associated TDP-43 induces endoplasmic reticulum stress, which drives cytoplasmic TDP-43 accumulation and stress granule formation. *PLoS One* 8(11):e81170.

Wang ZB, Zhang X & Li XJ (2013) Recapitulation of spinal motor neuron-specific disease phenotypes in a human cell model of spinal muscular atrophy. *Cell Res* 23(3):378-393.

Westerfield M (1993) *The zebrafish book : a guide for the laboratory use of zebrafish (Brachydanio rerio).* M. Westerfield, Eugene, OR

Will CL & Luhrmann R (2001) Spliceosomal UsnRNP biogenesis, structure and function. *Curr Opin Cell Biol* 13(3):290-301.

Wishart TM, Mutsaers CA, Riessland M, Reimer MM, Hunter G, Hannam ML, Eaton SL, Fuller HR, Roche SL, Somers E, Morse R, Young PJ, Lamont DJ, Hammerschmidt M, Joshi A, Hohenstein P, Morris GE, Parson SH, Skehel PA, Becker T, Robinson IM, Becker CG,

Wirth B & Gillingwater TH (2014) Dysregulation of ubiquitin homeostasis and beta-catenin signaling promote spinal muscular atrophy. *J Clin Invest* 124(4):1821-1834.
Xu C, Bailly-Maitre B & Reed JC (2005) Endoplasmic reticulum stress: cell life and death decisions. *J Clin Invest* 115(10):2656-2664.
Yamazaki T, Chen S, Yu Y, Yan B, Haertlein TC, Carrasco MA, Tapia JC, Zhai B, Das R, Lalancette-Hebert M, Sharma A, Chandran S, Sullivan G, Nishimura AL, Shaw CE, Gygi SP, Shneider NA, Maniatis T & Reed R (2012) FUS-SMN protein interactions link the motor neuron diseases ALS and SMA. *Cell Rep* 2(4):799-806.
Yeh JR, Munson KM, Elagib KE, Goldfarb AN, Sweetser DA & Peterson RT (2009) Discovering chemical modifiers of oncogene-regulated hematopoietic differentiation. *Nature chemical biology* 5(4):236-243.
Yen ST, Zhang M, Deng JM, Usman SJ, Smith CN, Parker-Thornburg J, Swinton PG, Martin JF & Behringer RR (2014) Somatic mosaicism and allele complexity induced by CRISPR/Cas9 RNA injections in mouse zygotes. *Dev Biol* 393(1):3-9.
Zerres K & Rudnik-Schoneborn S (1995) Natural history in proximal spinal muscular atrophy. Clinical analysis of 445 patients and suggestions for a modification of existing classifications. *Arch Neurol* 52(5):518-523.
Zhang HL, Pan F, Hong D, Shenoy SM, Singer RH & Bassell GJ (2003) Active transport of the survival motor neuron protein and the role of exon-7 in cytoplasmic localization. *J Neurosci* 23(16):6627-6637.
Zhang Z, Lotti F, Dittmar K, Younis I, Wan L, Kasim M & Dreyfuss G (2008) SMN deficiency causes tissue-specific perturbations in the repertoire of snRNAs and widespread defects in splicing. *Cell* 133(4):585-600.
Zhang Z, Pinto AM, Wan L, Wang W, Berg MG, Oliva I, Singh LN, Dengler C, Wei Z & Dreyfuss G (2013) Dysregulation of synaptogenesis genes antecedes motor neuron pathology in spinal muscular atrophy. *Proc Natl Acad Sci U S A* 110(48):19348-19353.