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Loss of motoneuron-specific microRNA-218 causes systemic neuromuscular failure

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Loss of motoneuron microRNA-218 results in systemic neuromuscular failure

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by

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DEDICATION

To my family, to my friends, and to all those others who have shared their knowledge, insights, passion, and experiences with me over these years spent in graduate school - you have brought life to my educational journey and kept strong my drive for scientific exploration.

EPIGRAPH

Shall I tell you the secret of the true scholar?

It is this: Every man I meet is my master in some point,

and in that I learn of him.

-Ralph Waldo Emerson

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LIST OF ABBREVIATIONS

218DKO	Mice harboring the genotype: miR-218-1$^{-/-}$2$^{-/-}$
3'UTR	3' untranslated region
AChR	acetylcholine receptor
ALS	amyotrophic lateral sclerosis
bp	base pair
C9ORF72	chromosome 9, open reading frame 72
ChAT	choline acetyltransferase
ChIP	chromatin immunoprecipitation
CNS	central nervous system
CPN	callosal projection neuron
Cre	Cre recombinase
CRISPR	clustered regularly interspersed short palindromic repeats
CthPN	corticothalamic/subplate projection neuron
DeCoN	The Developing Cortical Neuron Transcriptome Resource
DNA	deoxyribonucleic acid
E12.5	embryonic day 12.5
eGFP	enhanced green fluorescent protein
FACS	fluorescent activated cell sorting
FUS	fused in sarcoma, gene
GFP	green fluorescent protein

HxRE	hexamer response element
kb	kilobase
kDa	kilodalton
LMC	lateral motor column
MEF	mouse embryonic fibroblasts
mES, mESC(s)	mouse embryonic stem cell(s)
miR-218	microRNA-218
miRNA	microRNA
MMC	medial motor column
MMCl	lateral medial motor column
MMCm	medial medial motor column
MN	motoneuron, motor neuron
NF	neurofilament
p	progenitor domain
PGC	preganglionic motor column
polyA	poly adenylation
RNA	ribonucleic acid
ScPN	subcerebral projection neuron
sgRNA	single guide RNA
SMA	spinal muscular atrophy
SMN	survival of motoneuron gene

SYN	synaptophysin
TDP43	transactive response DNA binding protein 43kDa
tdTomato	tandem tomato fluorescent protein
V	ventral interneuron subtype

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ABSTRACT OF THE DISSERTATION

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Evidence is mounting that defective RNA metabolism is central to the pathogenesis of diseases affecting motoneurons (e.g. amyotrophic lateral

sclerosis and spinal muscular atrophy). Yet, our understanding of motoneuron-specific gene regulatory pathways is largely limited to those mediated by transcription factors. Investigations into motoneuron-specific, RNA-mediated regulatory pathways (such as those involving microRNAs), may provide novel insights into potential pathogenic mechanisms. In this thesis, I identify a single microRNA (miR-218) that is both highly enriched and abundantly expressed in murine motoneurons. Using a combination of RNA sequencing and mouse genetics, I identify novel alternative promoters embedded within the Slit2/3 genes that contribute to miR-218's specific expression in brainstem and spinal motoneurons.

My most informative and exciting experiments derive from investigation of miR-218 knockout mice, generated by CRISPR-mediated multiplexed deletions of all four miR-218 alleles. Motoneurons in these mice exhibit dramatic neuromuscular synaptic failure, hyperexcitability, and cellular degeneration – the hallmarks of motoneuron diseases. Without miR-218, mice exhibit flaccid paralysis and neonatal death, firmly demonstrating that this microRNA is indispensable to motoneuron function and survival. How can a single, small non-coding RNA have such a fundamental importance to motoneuron gene regulation? Gene profiling wild type and knockout motoneurons uncovers an impressive network of hundreds of mRNAs that are under miR-218 mediated repression. Using differential expression and unbiased 3'UTR motif-enrichment analysis, I find that miR-218 target genes are expressed lower in motoneurons versus other subpopulations of spinal and cortical neurons. Moreover, I find that miR-218 doesn't merely reinforce/potentiate target genes' reduced expression (as has been suggested for microRNAs in general), but instead constitutively and independently drives the repression of its target network in motoneurons.

In summary, this thesis (1) details the identification of one of the most dramatic examples of a neuronal subtype-specific microRNA in mammals, (2) establishes that loss of miR-218 results in neuromuscular failure and motoneuron degeneration, and (3) reveals that motoneurons use miR-218 to tune-down a genetic network expressed across other neuronal cell populations.

Chapter 1

Introduction

Overview

Motoneurons are a neuronal subpopulation located within the spinal cord and brainstem. Their axons navigate through peripheral tissues during embryonic development to form specialized synaptic connections with muscle, a process essential for the ability of the nervous system to control body movements. The grave importance of motor neurons to our daily lives can be appreciated through the lens of human diseases such as amyotrophic lateral sclerosis (ALS) in which motor neurons degenerate, leaving patients progressively unable to control over their muscles until critical processes such as breathing are compromised. In other disorders, such as spinal muscular atrophy (SMA), motor neurons fail to form appropriate connections with muscle during embryonic development resulting in paralysis starting from birth or childhood followed by degeneration in early life. The genetic regulation of motor neurons' embryonic generation from progenitors, the development of their specialized neuronal synapses, and the selective vulnerability of motor neurons to disease are questions of fundamental importance to developmental biology, neuroscience, translational medicine, and the countless families and patients affected by ALS, SMA, and related disorders affecting motor neurons.

Spinal muscular atrophy

SMA is the result of abnormal motor neuron development in addition to a component of neuronal degeneration with onset as soon as the neonatal period or as late as adulthood. In the most severe variant of SMA called SMA I or Werdnig-Hoffman disease, newborns and infants demonstrate rapidly-progressing loss of motor function resulting in death typically within the first two years of life [1]. These patients are typically diagnosed after proximal muscle weakness and hypotonicity becomes severe enough that babies appear floppy to caretakers and are unable to support their heads. Intermediate forms of SMA (SMA II) present as weakness around the first year of life and afflicted patients typically never gain the ability to stand or walk, relying on wheelchairs for the extent of their life. These patients are at risk of succumbing to respiratory failure secondary to slowly progressing disease, though they often live into adulthood with support of their caretakers. Less severe forms of disease can affect patients in later childhood, adolescence or even adulthood. While they typically do not significantly hasten death, these mild SMA variants contribute to mobility impairment [1].

SMA is an autosomal recessive genetic disease most frequently caused by either a deletion of the survival of motor neuron (SMN1) gene or a deletion of its exon 7 that compromises its protein-coding function. A duplicated copy of the gene is present in humans called SMN2 which generates significantly lower protein quantity than the SMN1 gene can produce [1,2]. In patients with nonsense mutations in SMN1, the SMN2 gene generates sufficient protein to sustain life. However, it is the person-to-person variability in copy number variation and subsequent expression of this otherwise redundant SMN2 gene that contributes to the variable severity of phenotypes associated with disruption of the primary SMN1 gene [1]. SMA patients with 4 or more copies of SMN2 are more likely to survive into adulthood compared with those with 1 or 2 copies [1].

Typically, a splice site of SMN2's exon 7 is not recognized for incorporation into the final mRNA product due to a weak 3' splicing recognition site [3]. This produces SMN2 mRNA transcripts that lack exon 7 and are thereby unable to produce functional protein. However, a small fraction of pre-mRNA transcripts splice correctly and are able to incorporate exon 7 which allows for some small level of SMN2 mRNA that is able to produce useful protein [3]. Novel small molecule oligonucleotide therapeutic agents are now under development to tip the balance of SMN2's exon 7 splicing with the intention of increasing functional SMN protein production. One such oligonucleotide was designed to target a previously identified splicing inhibitor element located near exon 7, and delivery of this oligonucleotide in mice was able to increase exon 7 incorporation and improve protein output [4]. Though many therapeutic strategies are under development for SMA [5], this oligonucleotide-based therapy is the first to reach phase III clinical trials, raising hopes for a breakthrough in the treatment of this disease [6]. The results of phase 1 trials of the therapy (called nusinersin being developed by

Ionis Pharmaceuticals) were encouraging for its safety profile, tolerability, and significantly increased functional motor scores in SMA patients within 3 months versus placebo [7]. This strategy in approaching neurological disease has already validated the importance of investigating mechanisms of RNA processing, as research in this area is already influencing translational research in novel and potentially transformative ways [8].

SMN is known to be a component of a large protein complex found in the cytoplasm and in nuclear foci called gems. These complexes include proteins of the Gemin family and have important roles in the assembly of small nuclear ribonucleoproteins (snRNPs) involved in splicing machinery [9]. However, SMN is likely also involved in the assembly of snRNPs for a diverse range of cellular processes and SMN potentially has other effects on RNA metabolic processes such as splicing [9], microRNA biogenesis [10,11], mRNA localization, and local protein translation [12]. The SMN protein is not specifically enriched in motor neurons and is rather a ubiquitously expressed gene. This fact poses a simple yet central question: why are motor neurons selectively affected when a ubiquitously expressed gene involved in snRNP assembly and RNA processing is defective in every cell of the body in SMA patients? Satisfying answers have proven to be elusive, though it is likely that motor neurons rely upon SMN for one or more cell type-specific processes that are more sensitive than other cell types to decreased levels of SMN [11,13]. The assembly of snRNPs and RNA metabolic events is known to vary in a cell type-specific manner [13], though there is a relatively poor understanding of the impact of decreased SMN expression specifically on motor neurons. A better understanding of snRNP assembly and RNA metabolism specifically in the context of motor neurons could yield important insights into the selective vulnerability of this cell type in SMA patients.

Degenerative motor neuron diseases

While SMA is primarily considered a disease of defective motor neuron development with a neurodegenerative component, other motor neuron diseases such as ALS are primarily neurodegenerative in nature. In patients affected by ALS, motor neuron development occurs normally and patients live and function without motor dysfunction until the onset of symptoms, peaking between 58 to 63 years of age and a decade earlier in familial cases of ALS [14]. Symptoms characteristically begin with motor dysfunction in a limb which is perceived by patients as a focal muscular weakness of a particular arm or leg [15]. Symptoms progress with weakness spreading to adjacent limbs over the course of months to years, leaving patients eventually wheel chair-bound and unable to swallow oral secretions, vocalize their needs, and breathe. The disease progresses to fatally compromise vital functions and within 30 months of diagnosis, 50% of patients succumb [14]. Only one medication, riluzole, has been approved by the FDA to treat ALS, though it has only been shown to increase longevity by months [16]. Its mechanism of action is to inhibit glutamate transmission which is theorized to reduce glutamate-related neurotoxicity[16]. Medical management primarily consists of assistive devices, such as advanced wheelchairs, and nursing support since patients are will become unable to perform activities of daily living such as showering, feeding, or using the bathroom [14].

Unlike SMA which primarily affects lower motor neurons located in the spinal cord, ALS variably affects both lower motor neurons and corticospinal neurons which project axons from the motor cortex to the spinal cord to regulate motor networks [14]. ALS manifests clinically as spasticity, hyperreflexia, and hypertonia if upper motor neurons are predominantly involved or hypotonia, hyporeflexia, and atrophy if lower motor neurons are primarily affected [15]. Patients may have upper or lower motor neuron predominant disease in all areas of their body, though most patients have variable levels of upper and lower motor neuron-associated signs in different areas of their body simultaneously [15].

Most cases of ALS are not thought to be hereditary and are instead called sporadic (sALS). Though increasing age and male gender are risk factors for developing sALS, inciting events are not known in the vast majority of cases. Increased incidence of ALS has been noted in Italian soccer players and American football players though epidemiological supporting data is considered weak [17-19]. Relatedly, anecdotal evidence and small scale studies have suggested that ALS occurs more frequently in patients that vigorously exercise or have a history of physical trauma affecting the brain or spine [18]. Worldwide, the prevalence of ALS exhibits only modest variation with one notable exception: the United States territory of Guam. There, the native people were found to have a 100 times higher prevalence of an ALS variant called Lytico-Bodig disease [20]. Investigations into these high rates and the cultural practices of the native people have led to one hypothesis that the consumption of local fruit bat was increasing human exposure to a known neurotoxin called beta-methylamino-L-alanine that could cause disease [20]. In aggregate, these epidemiological data potentially implicate environmental exposure, physical activity, and trauma as risk factors in the development of sporadic forms of ALS.

In less than 10 percent of ALS cases, a family history of disease is present, and in the majority of these patients, an inherited genetic mutation has been identified [21]. Nearly one quarter of these familial cases are caused by mutations in the SOD1 gene, 4-5% in the TARDBP gene, and 4-5% in the FUS gene [22]. Just within the last 5 years, a mutation in the C9ORF72 gene has been identified as the most common mutation attributed to familial ALS [23,24], strikingly accounting for nearly 50% of these cases [22]. Unlike other genes implicated in ALS, the C9ORF72 mutant allele involves a trinucleotide expansion repeat rather than a point mutation. Mutations in over 20 other genes have been implicated in small numbers of familial cases of ALS [22], reflecting a significant diversity in the genetic basis of familial forms of ALS, unlike the monogenic basis of SMA.

Of note, many of the genes mutated in ALS patients are known to have function in RNA metabolism, leading to the hypothesis that the molecular pathogenesis of ALS involves defects in RNA-related processes [25]. For example, TARDBP and FUS both have RNA binding domains and have been implicated in alternative splicing and RNA processing, and the expansion repeat in C9ORF72 may be inciting toxicity by sequestering RNA binding proteins that have affinity for the expanded sequence [26]. Like SMN, these genes are expressed in cells throughout the body, and yet their dysfunction in patients has a greater impact on motor neurons than other cell types, raising the possibility that motor neurons rely upon these genes more so than other cell types in the body.

microRNA dysregulation in motor neuron disease

In both ALS and SMA, ubiquitously expressed RNA binding proteins are mutated causing selective motor neuron loss. Though a common function of these mutated genes is not known, one possibility that has been proposed is a shared involvement in the biogenesis of microRNAs [27]. microRNAs are short ~20 nucleotide RNA molecules that function as post-transcriptional repressors of gene expression. microRNAs have been shown to be dysregulated in the spinal cords and laser-captured motor neurons of patients with familial and sporadic forms of ALS [28-30]. In one mouse model of SMA in which levels of the SMN protein are reduced, spinal cords exhibited dysregulation of many microRNAs as well [31]. Many of these studies investigating links between microRNAs and motor neuron disease have suggested that microRNAs in general are expressed at overall lower levels in disease states and that very few microRNAs demonstrate upregulation, suggesting that there may be a deficit in the common microRNA processing pathways in motor neurons or alternatively. Regardless, it is not clearly understood whether specific microRNAs might be particularly affected in disease states.

Both TDP43 and FUS have been shown to interact with the core proteins involved in microRNA processing, and knock down of these ALS-associated proteins caused many microRNAs to be dysregulated [10,27,32,33]. However, these studies were performed in cell culture systems using cancer cell lines, and it has not been shown whether this is also true in motor neurons. The interpretation of these studies is limited by the use of non-motor neuron cell types in studying motor neuron disease-associated proteins and a lack of clear mechanisms for how these proteins influence microRNA processing. It has also been hypothesized that the formation of protein aggregates observed in ALS may also be sequestering a wider spectrum of RNA binding proteins (including those that are not mutated in patients) that can in turn influence microRNA biogenesis [27]. A better understanding of the factors that influence the biogenesis of microRNAs and the role that specific microRNAs play in motor neuron gene regulation are questions of importance to critically evaluate the role that microRNA dysregulation plays in motor neuron diseases.

What are microRNAs and how are they generated?

microRNAs are a class of non-protein coding RNAs that are typically 21-24 nucleotides in length, are found in both plants and animals, and mediate repression of protein synthesis predominantly via the degradation of mRNA transcripts. The production of microRNAs occurs via several well characterized steps, beginning with transcription of primary microRNA transcripts (pri-microRNAs) by RNA polymerase. Most frequently, microRNAs are transcribed by RNA polymerase II, the same polymerase used to generate capped and polyadenylated protein-coding transcripts. The microRNA sequence can be located intronically, exonically, or in untranslated regions of coding or non-coding RNAs [34], and depending on its location, can influence the stability of the RNA from which it is derived [35]. In one case, the human microRNA-198 is encoded within in the 3'UTR of the FSTL1 gene [36]. When the microRNA is processed, the production of protein is diminished resulting in a "see-saw" switch between microRNA production and protein production from the same precursor transcript. This switch is activated by the expression of an RNA binding protein KSRP and is involved in epithelialization in wound healing [36]. Most frequently, microRNAs are located within introns of coding genes and it is an open question whether microRNA processing of intronic microRNAs influence the production of the mRNA, outside of the context of microRNAs generated from whole introns termed mirtrons [37]. Additionally, some evidence suggests that microRNAs are processed co-transcriptionally [32,38,39], and thus many of the same proteins involved in other RNA processing events such as splicing may also interact with and influence microRNA biogenesis. Though microRNA and mRNA processing machinery are influenced by an overlapping cohort of RNA binding proteins and splicing factors, it is unclear to what extent and in which situations these interactions may be relevant in physiological contexts.

After transcription of the primary microRNA sequence (pri-microRNA), the Drosha Microprocessor complex will associate with and cleave highly characteristic RNA hairpin secondary structures in which microRNAs are located. The minimal components of the Drosha Microprocessor include the DiGeorge Complex Regulator 8 (DCGR8), which recognizes the base of RNA hairpins, and Drosha, the RNA nuclease component [35]. This cleavage step releases an approximately 50nt RNA stem-loop (termed a pre-microRNA, or pre-miR) which associates with exportin-5 to be exported from the nucleus to the cytoplasm where the nuclease Dicer cleaves the RNA between the stem and loop. The loop is degraded and the resulting double-stranded RNA (miR:miR*) consists of the mature microRNA (miR) imperfectly Watson-Crick base paired with a passenger strand (miR*) [35]. miR:miR* will associate with Argonaut proteins which determine which strand will become the functional sequence, and which strand will be degraded. Most frequently, only one strand is used for post-transcriptional repression, though rarely, both strands can be used for targeted repression.

Achieving specificity: how do microRNAs repress a select set of mRNAs?

Argonaut:miR complexes mediate mRNA silencing by binding the 3'UTR of target mRNAs to mediate their degradation [40]. microRNAs do not directly instruct Argonaut to cleave target mRNAs, though these proteins have nuclease capacity. Instead microRNAs instruct the de-adenylation of the poly-adenosine tail of mRNAs or the association of mRNAs with the exosome that mediates RNA turnover. The end result is the destruction of specific mRNA targets to the effect of preventing translation and protein generation [41]. Though microRNAs mediate additional repressive effects by blocking translation by ribosomes [42], the relative contribution of mRNA degradation versus translational blockade to observations of decreased protein cellular content is still debated.

Despite their average length of ~21 nucleotides, microRNAs predominantly achieve target specificity via Watson-Crick base pairing of nucleotides 2 through 7 (considered the 'seed' region or 'seed' sequence) to the 3' UTRs of mRNAs [43]. Owing to the high likelihood of finding a 6 base pair match among the massive collection of mRNA sequences within a given cell, a single microRNA may target hundreds of mRNAs for repression. Additional base pairing of one or two nucleotides at locations 1 and 8 of the microRNA can expand the seed region to seven or eight base pairs and will confer greater magnitude of repression [44]. While a match between a microRNA's seed region and target mRNAs is typically a minimal requirement for interaction, greater base pairing outside of the seed region can additionally increase the likelihood and strength of microRNA mediated repression. The 'rules' of microRNA target finding have been extensively investigated by iteratively combining bioinformatics prediction models with experimental findings from microRNA overexpression or knockdown studies in vitro and in vivo. Currently, computational models of microRNA-mRNA targeting can identify a list of predicted mRNA targets for a given microRNA along with a prediction of efficacy of repression [44]. However, the breadth and extent of the microRNA mediated repression on these targets is influenced by the cellular abundance of both the microRNA and mRNA targets in question, and prediction models cannot indicate whether a specific microRNA and mRNA are actually simultaneously expressed together in a given cell or whether other cell-type specific contexts can alter microRNA-mRNA interactions.

Network properties of microRNA repression

Many outstanding questions remain regarding the role that microRNAs play within the larger scheme of genetic regulation because they have many idiosyncratic features that distinguish them from other gene regulatory modalities. While many well studied developmental TFs can activate or repress gene expression by magnitudes of a hundred-fold or greater, highly expressed microRNAs routinely post-transcriptionally repress mRNA expression on the order of 10% to 2-fold [45,46]. The levels of repression for even the most potent microRNAs are decidedly modest (~4-fold) compared to the activity of many transcription factors. Therefore, microRNAs are not thought to have the ability to mediate large ‘on/off’ switches for single genes, at least at physiologic levels found in vivo. Modest repression of individual genes can nonetheless have large implications on the physiology of the cell, and the impact of modest repression across hundreds of microRNA targets could be amplified if these genes are part of the same or complementary pathways.

Based upon gene expression studies, several theories have been proposed as to the regulatory function of microRNAs during cell fate specification in embryonic development. In an early study, mRNA gene profiling was performed in a variety of tissues known to express a unique, tissue-specific microRNA [47]. They observed that the expression of mRNAs with binding sites for a particular mRNA was expressed at very low level in tissues in which this microRNA was expressed. They concluded that microRNA targets were repressing targets that were already expressed at low levels, and thus, microRNAs are used as a fail-safe or backup system to ensure these transcripts are not re-expressed. Other studies have suggested that microRNAs counteract transcriptional activation of target genes to fine-tune or buffer the level of mRNA transcripts [48,49]. This push-pull idea is modelled as an incoherent feed forward loop and may serve to assure the precise level of expression of key genes. Other studies have suggested microRNAs can set threshold levels for mRNA expression [50]. These models of microRNA regulation are based off a select few developmentally expressed microRNAs, and thus it is not certain whether these models are exclusive or apply to microRNAs expressed in other contexts.

microRNAs in nervous system development

It has become clear in the past 15 years of research that microRNA mediated repression can also have a significant impact upon gene regulation in ways that are unique from other gene expression regulators such as transcription factors. Indeed, several microRNAs have been studied in the context of nervous system development and many more microRNAs have been identified that may yet reveal important functions. microRNAs are thought to influence all of the major cellular and biological processes appreciated in neuroscience such as neuronal differentiation, neuronal subtype identity, electrophysiological properties, neurotransmitter responsiveness, and even neurological disease and behavior.

Hundreds of microRNAs are expressed in the vertebrate nervous system with spatially and temporally restricted expression patterns. Two nervous system enriched microRNAs, mir-9 and mir-124, were identified as being important for neurogenesis in early studies [51] and have since been heavily investigated. mir-124, is strikingly expressed at high levels in the developing neural tube while being largely absent from other tissues [52]. It is induced in neurons as they differentiate from neural progenitors during embryonic development [53]. miR-218 is a critical genetic regulator of the neural differentiation program partly through its direct actions on repressing PTBP1, thus activating neuronal splicing switch which affects a large portion of the transcriptome [53]. miR-124 also represses BAF complexes that mediate a switch in chromatin state [54,55]. A knockout mouse model in which miR-124 levels were reduced by 60% demonstrated a smaller brain size and defects in photoreceptors, though the authors cast doubt on an essential role for miR-124 in neurogenesis [56]. mir-9 is also enriched in the developing vertebrate nervous system [57] and also targets BAF complexes [54]. Mir-9 is encoded in three genetic loci, and when either miR-9-2 or miR-9-3 were knocked out, no phenotype was observed. In combined mir-9-2/9-3 knockout mice in which levels of mature mir-9 are reduced by 75%, mice die within a week of birth and demonstrated defects in a variety of brain regions [58].

While miR-124 and miR-9 have influential roles in neurogenesis, miR-128 is another brain-enriched microRNA that was shown to govern neuronal excitability and ion channel properties through regulating components of the ERK2 pathway [59], indicating that microRNAs have key roles outside the context of embryonic development. Additionally, dysregulation of miR-124 expression was observed in a mouse model of frontotemporal dementia and was found to regulate AMPA receptors in adult mice [60]. Thus, a function of a CNS-enriched microRNA can be highly varied and could potentially impact both developmental and mature neuronal processes and characteristics, and may be subject to pathogenesis of neurological disorders.

microRNA modify spinal cord development

Gene expression programs that determine the physiological properties, synaptic connectivity, and other fundamental biological characteristics of spinal neuron subtypes are known to be regulated by basic helix loop helix (bHLH) and homeodomain (HD) transcription factors [61]. The combinatorial expression of these TFs drive the expression of specific axon guidance molecules, neurotransmitters, and cell surface receptors that define specific spinal neuronal populations. Decades of work [62-65] have identified and distilled the transcription factors needed for motor neuron specification from neural progenitors to just three: Neurogenin2, Lhx3 and Isl1 to generate spinal motor neurons, and Neurogenin2, Phox2 and Isl1 to generate cranial motoneurons [66].

microRNAs can modify the expression of transcription factors to contribute robustness to certain aspects of spinal neurogenesis. For example, miR-17-3p was shown to regulate Olig2 and Irx3 through a cross repressive loop [67]. The spatial restriction of miR-17-3p to the dorsal half of the spinal cord ensures the repression of Olig2 in this dorsal domain, while leaving its expression in the ventral spinal cord uninhibited. Another group has shown that miR-9 can tune the regulation of another transcription factor, FoxP1, that is expressed in a subpopulation of motor neurons [57]. However, due to the vast number of mRNA targets for a single microRNA, it is likely that the repression of transcription factors by microRNAs is just one of many potential regulatory modalities.

Concluding thoughts

Considerable amount of research has investigated the role that microRNAs may be playing in the context of motor neuron development and disease, and yet, many outstanding questions remain. Do specific microRNAs demonstrate unique or exclusive expression pattern in motor neurons? Do individual microRNAs have an outsized role in motor neuron function? What might the mode of regulation of such microRNAs be, and how do these regulatory modalities differ from the action of transcription factors? The next chapter investigates the answers to these questions, and these answers raise significantly more questions about the unique role that microRNAs play in motor neuron biology.

Chapter 2

Loss of motoneuron microRNA-218 results in systemic neuromuscular failure

Abstract

Dysfunction of microRNA (miRNA) metabolism is thought to underlie diseases affecting motoneurons, however, the identity and regulatory capacity of microRNAs involved in motoneuron survival remain unknown. Here, we identify the selective and abundant expression of a single microRNA, miR-218, in motoneurons. Mutant mice lacking miR-218 die neonatally and exhibit neuromuscular junction defects, motoneuron hyperexcitability, and progressive motoneuron cell loss – hallmarks of motoneuron diseases. Gene profiling reveals that miR-218 represses an extensive array of mRNA transcripts in motoneurons. Only with miR-218 expression, these genes are expressed at low levels in motoneurons relative to other profiled neuronal subpopulations. Thus, the targets of miR-218 represent a neuronal gene network whose coordinated post-transcriptional repression in motoneurons is essential for their synaptogenesis, function, and survival.

Introduction

Motoneurons are a specialized neuronal subpopulation within the central nervous system (CNS) that establish synaptic connections with muscles to regulate movement. The pathophysiology of diseases affecting motoneurons such as amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA) is not well understood, however, defective RNA metabolism is thought to underlie a common pathogenic mechanism [27,31,68,69]. Genes associated with motoneuron disease in humans (e.g. TDP43, FUS, SMN) are known to regulate the biogenesis of microRNAs (microRNAs) [10,27,31-33,70], post-transcriptional repressors with *in vivo* roles in nervous system development and function [56,59,60,71]. While the transcriptional regulation of motoneurons has been extensively studied [66,72,73], it is not well appreciated whether motoneurons specifically depend upon individual microRNAs for post-transcriptional genetic regulation, function, and survival.

Results

To identify motoneuron-enriched microRNAs, we performed small RNA sequencing of fluorescence-activated cell sorting (FACS)-purified *Hb9::gfp*$^+$ motoneurons from spinal cords of E10.5 mouse embryos. The largest fraction of total microRNA reads, 19%, aligned to the mature nucleotide sequence of miR-218. This microRNA was ~27-fold enriched in motoneurons versus *Hb9::gfp-* non-motoneurons (Fig. 1A). Interestingly, we did not detect other microRNAs with comparable levels of enrichment and abundance in E12.5 FACS-purified V2a and V3 spinal interneurons (fig. S1A), suggesting motoneurons may be a unique neuronal subpopulation with respect to microRNA expression.

Motoneurons are highly heterogeneous and have been classified based on their soma position, muscle target, cell body size, and physiological firing pattern [66,73-76]. We detected miR-218 expression by *in situ* hybridization in lateral limb- and medial axial-innervating somatic spinal motoneurons (LMC and MMC), preganglionic visceral spinal motoneurons (PGC), and brainstem visceral and somatic motoneurons at E18.5 (Fig. 1B, fig. S1B). At P10, miR-218 expression was detected in choline acetyl transferase$^+$ (ChAT$^+$) α- and γ-motoneurons (Fig. 1C, fig. S1C), though not in ChAT$^+$ interneuron populations (fig. S1D). Likewise, we could not detect miR-218 expression in other embryonic CNS and non-CNS tissues by *in situ* hybridization (Fig 1D, fig. S1E). miR-218 expression has been documented in zebrafish [77] and chick [78], and we found that motoneuron-specific expression is conserved to mouse and human (fig. S1F). Compared with the extensive catalog of protein markers that delineate motoneuron subtypes [61], miR-218 is remarkable for its expression spanning motoneuron classes from embryonic stages into adulthood (fig. S1G) and its low or undetectable expression in other tissues.

miR-218 is encoded within introns of the *Slit2* and *Slit3* genes [79]. In contrast to miR-218, Slit2 and Slit3 mRNA expression has been detected in a vast multitude of embryonic tissues [80-83]. We explored the transcription of these genes by performing polyA$^+$ RNA-sequencing of FACS-isolated motoneurons and floor plate tissue (Fig. 1E), a distinct ventral neural tube cell population with well-established Slit2 and Slit3 signaling roles. Instead of transcription starting from exon 1 – as observed in the floor plate – we discovered that Slit2 and Slit3 were expressed from exon 6 in motoneurons only (Fig. 1F, fig S2A and B). Robust chromatin immunoprecipitation (ChIP) peaks (data from [66]) and highly conserved hexamer DNA response elements (HxREs, [84]) for the motoneuron-specifying transcription factors Isl1/2, Lhx3 and Phox2a overlapped proximal to exon 6 (Fig. 1F), suggesting the presence of motoneuron-specific promoters (Fig. 1G). To test this hypothesis, we generated a transgenic mouse line, tg(*218-2::eGFP*) that contained a 7.4 kilobase (kb) sequence with highly conserved promoter and enhancer elements likely to be active in motoneuron (fig. S2C). *In vivo*, eGFP was expressed robustly and specifically in tg(*218-2::eGFP*) spinal and cranial motoneurons, exquisitely reproducing miR-218's expression pattern (Fig. 1H and I, fig. S2C). These findings demonstrate that primary miR-218 transcripts are under independent activation in motoneurons by alternative, non-canonical promoters embedded within *Slit2* and *Slit3*.

To identify miR-218's biological role *in vivo*, we used CRISPR/Cas9 gene editing [85,86] to create microdeletions of miR-218-1 and miR-218-2 precursor sequences (Fig. 2A, fig. S3A-D). miR-218 expression was detected in miR-218-1$^{-/-}$ and at lower levels in miR-218-2$^{-/-}$ motoneurons, but was undetectable in miR-218-1$^{-/-}$2$^{-/-}$ double knockout (218^{DKO}) motoneurons (Fig. 2B, fig. S3E-H). 218^{DKO} embryos were observed in Mendelian ratios at E18.5 (fig. S3I), but strikingly, 218^{DKO} mice were never found to be viable postnatally. Furthermore, E18.5 218^{DKO} embryos exhibited akinesia, kyphosis, and weak or absent responses to pain stimulation after caesarean delivery and died within minutes due to an apparent lack of respiration (Fig. 2C) – a phenotype similar to mice carrying null alleles of critical neuromuscular components [87].

To exclude the possibility that Slit2 and Slit3 function are affected in 218^{DKO} mice, we investigated phenotypes associated with their canonical functions as secreted chemorepellents mediating neuronal branching and axon guidance [83,88,89]. The embryonic growth of neurofilament^{+} ophthalmic sensory axons and Tag1^{+} commissural axons are sensitive to *Slit2* and *Slit3* disruption [90,91], however these nerves did not exhibit differences in projection or branching patterns in 218^{DKO} mutants (fig. S3J). Additionally, *Slit2* mutants are not viable two weeks after birth [82], whereas miR-218-1$^{-/-}$ mice were viable without behavioral abnormalities. Taken together, these data indicate that the lethal phenotype of 218^{DKO} mice arises from the specific loss of miR-218.

Neuronal microRNAs, including miR-218 [78], have been reported to affect neuronal patterning and differentiation from progenitors during embryonic development [53,67,92]. However, miR-218 expression is initiated in post-migratory motoneurons (fig. S4A) making it unlikely that miR-218 could affect motoneuron specification *in vivo*. Consistently, the patterns of transcription factor expression that defines motoneuron and interneuron subtype identity were not affected in 218^{DKO} mutants (fig S4B-F). Subsequent stages of motor axon spinal exiting, outgrowth, and pathfinding were also indistinguishable between Hb9::*gfp*$^{+}$ control and 218^{DKO} embryos (fig. S5A), suggesting miR-218 impacts later stages of motoneuron maturation such as the establishment of peripheral synaptic connectivity.

Neuromuscular synaptogenesis is an intricate process in which motor nerves first innervate muscle and subsequently form pre-synaptic specializations with post-synaptic acetylcholine receptors (AChRs) expressed by muscle [93]. We examined tg(*218-2::eGFP*) motor nerves in glycerol-cleared E14.5 limb tissue and found that the deep peroneal nerve of 218^{DKO} motoneurons reached limb targets at E14.5 but fewer penetrating, fine intra-muscular branches were observable (Fig. 2D, fig. S5B). Across intercostal, diaphragm and limb muscle groups, pre-synaptic motor axons (immunolabelled with synaptophysin (SYN) antibodies) weakly arborize within muscle and are incompletely apposed with post-synaptic $AChR^+$ clusters in 218^{DKO} embryos (identified by alpha-bungarotoxin staining) (Fig 2E, fig. S6A-C). At E18.5, the majority of $AChR^+$ clusters are aneural in 218^{DKO} limb muscles (Fig. 2F) reflecting a gross failure of motoneurons to establish neuromuscular junctions needed for the control of body movements.

In contrast to normal numbers of motoneurons observed in E12.5 spinal cords (Fig 2G), 18 to 36% fewer motoneurons were observed at cervical, thoracic, and lumbar segments of 218^{DKO} spinal cords at E18.5, indicating degenerative cell loss (Fig. 2H; fig. S7A-B). To examine whether the physiology of the remaining motoneurons was altered in mice lacking miR-218, we assessed fictive locomotion [94] and performed intracellular recordings of *Hb9::gfp*$^+$ LMC α-motoneurons from E18.5 lumbar spinal slices (fig. S8A). Left/right and flexor/extensor activation of motor roots was normal in 218^{DKO} spinal cords, and motoneuron resting membrane potentials, capacitances, resistances, and holding currents were similar between control and 218^{DKO} motoneurons (fig. S8B-J). However, action potentials were elicited by a 4.4-fold lower rheobase current in 218^{DKO} motoneurons compared with controls (Fig. 2I and J), indicative of hyperexcitability [95]. Taken together, our findings demonstrate miR-218 is dispensable for early motoneuron development, but it is critical for the regulation of neuromuscular interaction, membrane excitability, and motoneuron survival.

The dramatic phenotypic defects in 218^{DKO} mice suggested that critical aspects of motoneuron-specific genetic regulation depend on miR-218's post-transcriptional repression of target mRNAs. To identify miR-218's gene targets in its *in vivo* cellular context, we performed polyA$^+$ RNA sequencing on FACS-isolated Hb9::*gfp*$^+$ motoneurons from wild type and 218^{DKO} E12.5 spinal cords, before the onset of neuromuscular defects (fig S9A). Using Sylamer [96], we determined that genes expressed higher in 218^{DKO} motoneurons were greatly enriched for 6bp, 7bp and 8bp 3'UTR complementary seed matches to miR-218 (fig. S9 B-E), validating the widespread de-repression of miR-218 target genes.

To identify a high-confidence list of specific miR-218 targets, we examined genes with bioinformatically predicted miR-218 binding sites

(TargetScan6, [97]) and found that 333 of these genes were de-repressed in 218^{DKO} motoneurons with statistical significance (Fig. 3A). This cohort of genes is likely to be under direct miR-218 mediated repression, and we name them *target*^{218} genes. *target*^{218} genes are enriched for neurotransmission and neurotransmitter transport biological processes (fig. S9, F and G), including the most highly upregulated *target*^{218} gene, Slc1a2/GLT-1 (266% increase). Interestingly, the expression of this glutamate reuptake transporter is known to be modulated by riluzole – the only medication approved for the treatment of ALS [98]. On average, *target*^{218} genes were expressed 61.1% higher in 218^{DKO} motoneurons, and 47 genes were increased by at least 2-fold. The wide breadth of target genes affected in 218^{DKO} motoneurons suggests miR-218 plays a fundamental role in shaping the expression of an extensive genetic network, rather than merely modulating a small group of individual genes within a single molecular pathway.

Other microRNA-gene networks have been shown to reinforce the repression of differentiation programs to confer robustness to cell-fate decisions [99-101], however, the lack of cell specification errors in 218^{DKO} embryos indicated miR-218 has a distinct regulatory role from other microRNAs with well-defined network properties. We evaluated whether the *target*^{218} gene network was expressed higher or lower in motoneurons compared with other spinal neuronal subpopulations by gene profiling FACS-purified interneuron subpopulations labelled by genetic reporters: GABAergic-

V1 (En1:Cre), glutamatergic-V2a (Chx10:Cre), and glutamatergic-V3 (Sim1:Cre) spinal interneurons (Fig. 3B, fig. S10A-C). We found that ~80% *target*^{218} genes are expressed lower in wild type motoneurons versus each of V1, V2a, and V3 interneurons (Fig. 3C). Moreover, the majority (69%) of *target*^{218} genes are expressed lower in wild type motoneurons versus all three of the spinal interneurons subpopulations profiled (Fig. 3D), far greater than expected chance (12.5%). These findings suggest miR-218 represses a gene network shared across interneuron subpopulations, but not specific to a single one. Furthermore, hierarchical clustering revealed that 218^{DKO} motoneurons express *target*^{218} genes at levels more similar to V1, V2a and V3 interneurons than to wild type motoneurons (Fig 3E). Thus, rather than reinforcing or potentiating the repression of *target*^{218} genes, miR-218 effectively establishes the characteristically low expression of its target network in motoneurons versus interneurons.

To evaluate microRNA-mediated repression in an unbiased manner, we bioinformatically evaluated the statistical enrichment of binding sites for all microRNAs across all differentially expressed genes. Using Sylamer [96], we determined the hypergeometric statistical enrichment of 3'UTR sequences complementary to known 7-mer microRNA seed sequences (microRNA seed matches) in transcripts differentially expressed in motoneurons versus interneurons (Fig. 4A). We find that 3'UTR seed matches to miR-218 are significantly and specifically enriched in transcripts expressed lower in wild type motoneurons versus averaged (Fig. 4A) and individual spinal interneuron subpopulations – and even distantly located cortical subpopulations (isolated by DeCoN [102]) (Fig. 4B). However, 3'UTR seed matches to miR-218 were no longer found to be enriched in genes differentially expressed in 218^{DKO} motoneurons, demonstrating the coordinated repression of these genes in motoneurons is dependent upon miR-218 (Fig. 4C). Interestingly, the 3'UTR seed match to miR-124, a neuronal microRNA abundantly expressed in motoneurons and other CNS neurons [52,53,56], but not 3'UTR seed matches to miR-218, was overrepresented in transcripts expressed lower in motoneurons versus highly-purified motoneuron progenitors differentiated from embryonic stem cells (Fig. 4B and C). Taken together, these bioinformatics analyses reveal that 1) miR-218 represses a genetic network shared across functionally and spatially distinct neuronal cell types, 2) the low relative expression of this gene network in motoneurons is established by miR-218, and 3) while miR-124 and miR-218 are co-expressed in motoneurons, their regulatory roles are strikingly segregated – miR-124 represses a neuronal progenitor-associated gene network, while miR-218 represses a gene network active across other spinal and cortical neuronal subpopulations.

Discussion

It is well appreciated that motoneuron gene expression and cellular identity are shaped by gene regulatory pathways activated by transcription factors [62,66,72,73]. Here, we identify an extensive and previously unappreciated gene network active across neuronal subtypes that is under constitutive repression in motoneurons by a single microRNA. When this network is de-repressed in 218^{DKO} mice, motoneurons exhibit severe neuromuscular junction defects, hyperexcitability and cell loss – the pathological hallmarks of motoneuron diseases such as ALS and SMA [68,95,103,104]. A link between miR-218 and motoneuron disease likely extends beyond phenotypic similarities alone. Patients suffering from motoneuron diseases carry genetic mutations in ubiquitously expressed RNA processing factors (e.g. TDP-43, FUS, SMN) or expansion repeats in C9ORF72 that sequester RNA binding proteins [27], but the biological mechanisms that contribute to motoneuron-specific degeneration are unclear. microRNA processing pathways, and therefore the repression of miR-218's genetic network, could be particularly sensitive to defects in RNA metabolic pathways thought to underlie motoneuron disease. Elucidating the homeostatic mechanisms affecting miR-218's differential biogenesis and the modulation of its genetic network are new and promising lines of investigation that may be critical to understand and tackle these devastating diseases.

Acknowledgements

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Figures

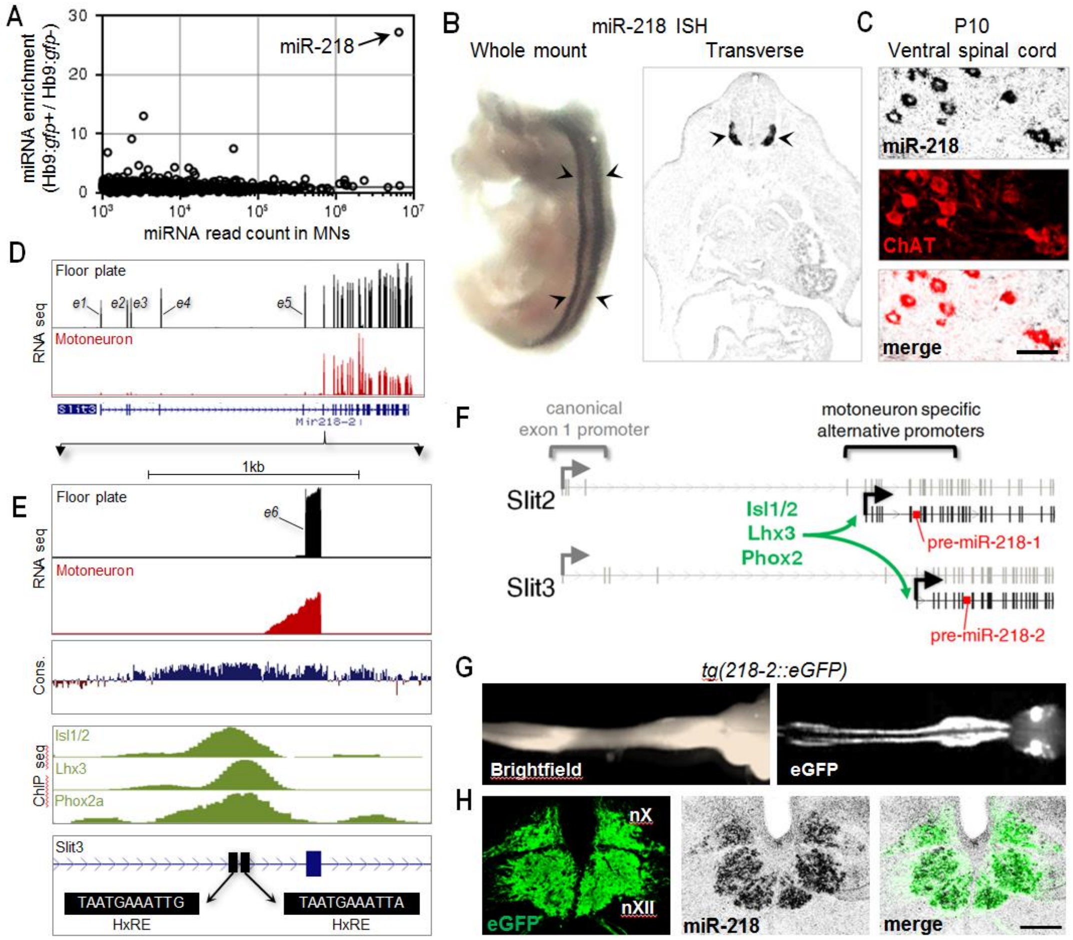


Figure 1 Abundant and specific expression of miR-218 in spinal and cranial motoneuron subtypes. (A) Murine microRNA expression (x-axis) versus enrichment (Hb9::*gfp*$^{+}$ motoneurons versus Hb9::*gfp*$^{-}$ non-motoneurons, y-axis) (n=2). (B) miR-218 *in situ* hybridization in whole mount and transverse section at E11.5 (arrowheads identify motor columns). (C) miR-218 co-localizes with ChAT^{+} motoneurons at P10. (D, E) PolyA^{+} RNAseq reads from E12.5 floor plate and motoneurons and motoneuron-specifying transcription factor ChIP peaks and HxRE DNA binding motifs at the *Slit3* locus containing *pre-miR-218-2*. (F) Transcription of miR-218 in motoneurons by alternative promoters. (G, H) *tg(218-2::eGFP)* mice. (G) Expression of eGFP in spinal and brainstem motoneurons of the CNS, and (H) in miR-218^{+} motor nuclei nX and nXII. Scale bars: (C) 50µm (I) 200µm.

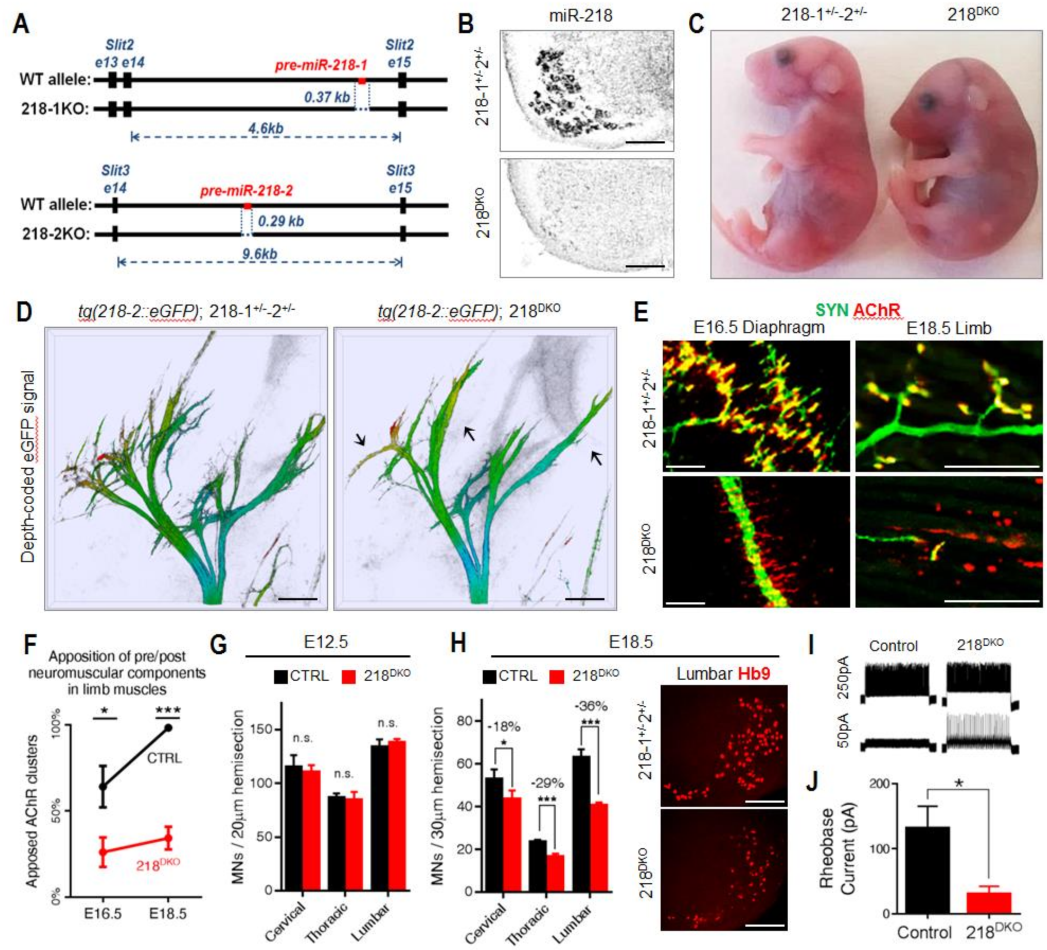


Figure 2 Loss of miR-218 results in systemic neuromuscular failure, motorneuron cell loss, and hyperexcitability. (A) CRISPR/Cas9-mediated multiplexed micro-deletions of *pre-miR-218-1* and *pre-miR-218-2* from the mouse genome. (B) miR-218 *in situ* hybridization signal in control and 218^{DKO} E18.5 spinal cords. (C) Cesarean-delivered 218^{DKO} E18.5 embryos exhibit flaccid paralysis and die within minutes. (D) Decreased intramuscular branching (arrows) of E14.5 motor nerves in tg(*218-2::eGFP*);218^{DKO} embryos (deep peroneal nerve). (E and F) In 218^{DKO} embryos, (E) NMJs exhibit abnormal morphology, and (F) most limb $AChR^{+}$ clusters are aneural (n= 3). (G and H) Motoneuron counts at E12.5 (n=4 and 3), and E18.5 (n=4) across spinal segments. (I and J) (I) Representative traces of control and 218^{DKO} motoneurons after intracellular current injection, and (J) rheobase quantification (n= 9 and 5). Statistics: (F, G, H) standard deviation and results of two-tailed t-test are shown. (J) SEM shown, non-parametric Mann-Whitney t-test results shown. * and *** denotes p-value <0.05 and p-value <0.001. n.s. denotes not significant. Scale bars: (B, D and H) 150µm, (E) 50µm.

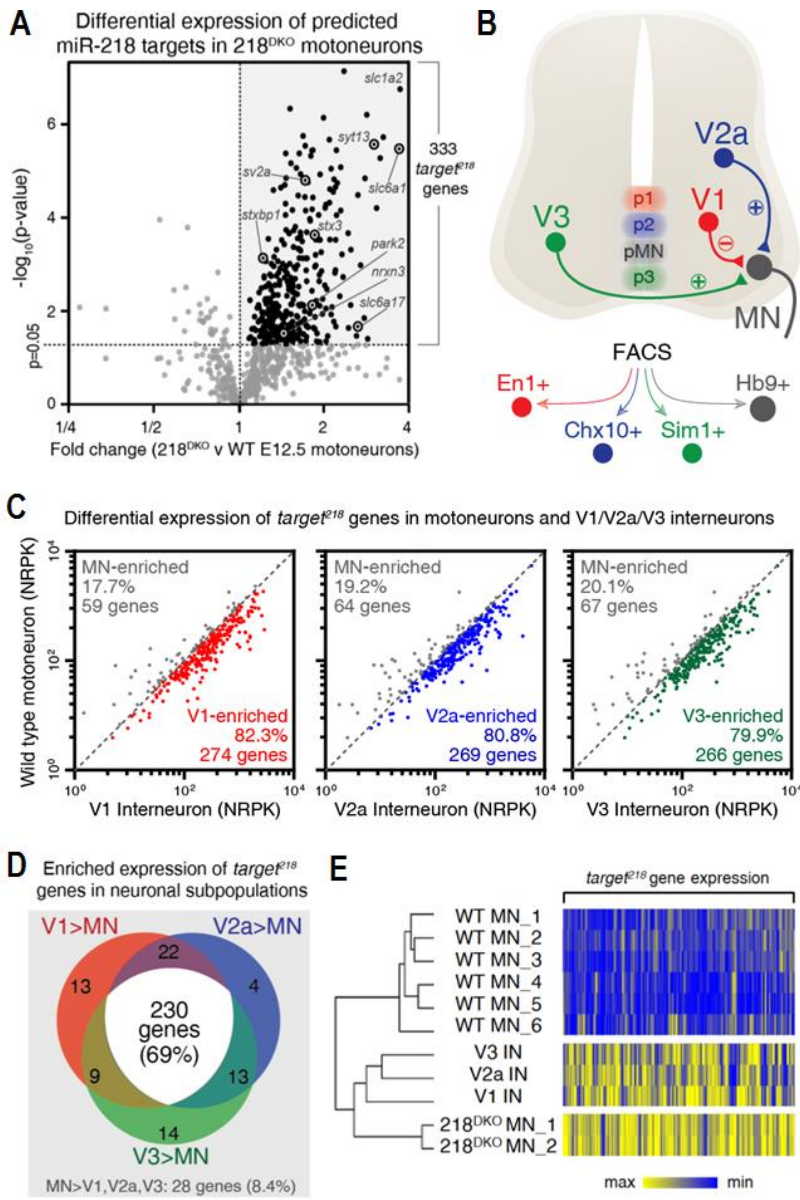


Figure 3 miR-218 represses an extensive genetic network in motoneurons. (A) Volcano plot (mRNA fold difference versus p-value) of 218^{DKO} versus wild type motoneurons of genes with predicted miR-218 binding sites (TargetScan6, n = 6 and 2). 333 of these genes (designated *TARGET*^{218} genes) are significantly de-repressed in 218^{DKO} motoneurons. *TARGET*^{218} genes involved in neurotransmitter transport are labelled. (B) Motoneurons and V1, V2a, and V3 interneuron subpopulations derive from adjacent progenitor domains (p1, p2, pMN, p3) and were labelled with transgenes or Cre-reporters for FACS-isolation and RNA sequencing. (C) *TARGET*^{218} genes are expressed at low levels in motoneurons relative to each of V1, V2a and V3 interneurons. (D) Most *TARGET*^{218} genes are expressed lower in motoneurons compared to all three of V1, V2a and V3 interneurons. (E) Hierarchical clustering of *TARGET*^{218} gene expression in wild type motoneuron (WT MN, six replicates), 218^{DKO} motoneuron (218^{DKO} MN, two replicates) and interneuron subpopulations (V1 IN, V2a IN, V3 IN).

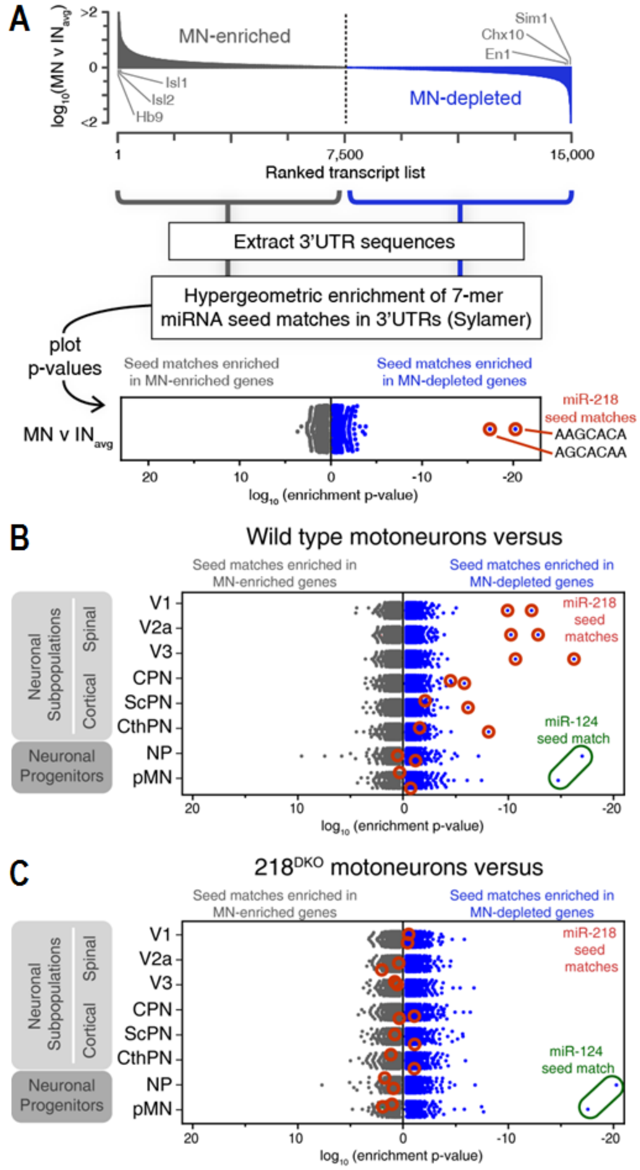


Figure 4 miR-218 represses a neuronal-gene network in motoneurons. (A) 7mer seed matches for miR-218 (AGCACAA and AAGCACA, red circles), but not those of other microRNAs, are significantly and specifically enriched in the 3'UTRs of genes expressed low in motoneurons relative to an average of V1/V2a/V3 interneuron populations (IN_{avg}). (B) Genes expressed lower in wild type motoneurons versus individual spinal and cortical [102] neuronal subpopulations were most enriched for miR-218 seed matches. (C) miR-218 seed matches are not enriched in genes expressed higher or lower in 218^{DKO} motoneurons versus other neuronal populations. Genes expressed lower in wild type (B) or 218^{DKO} (C) motoneurons versus neuronal progenitors were most enriched for the seed match to miR-124 (GTGCCTT). CPN, callosal; ScPN, subcerebral; CthPN subplate neurons; NP, mES-derived neuronal progenitors; pMN, FACS-purified $Olig2^+$ mES-derived neuronal progenitors.

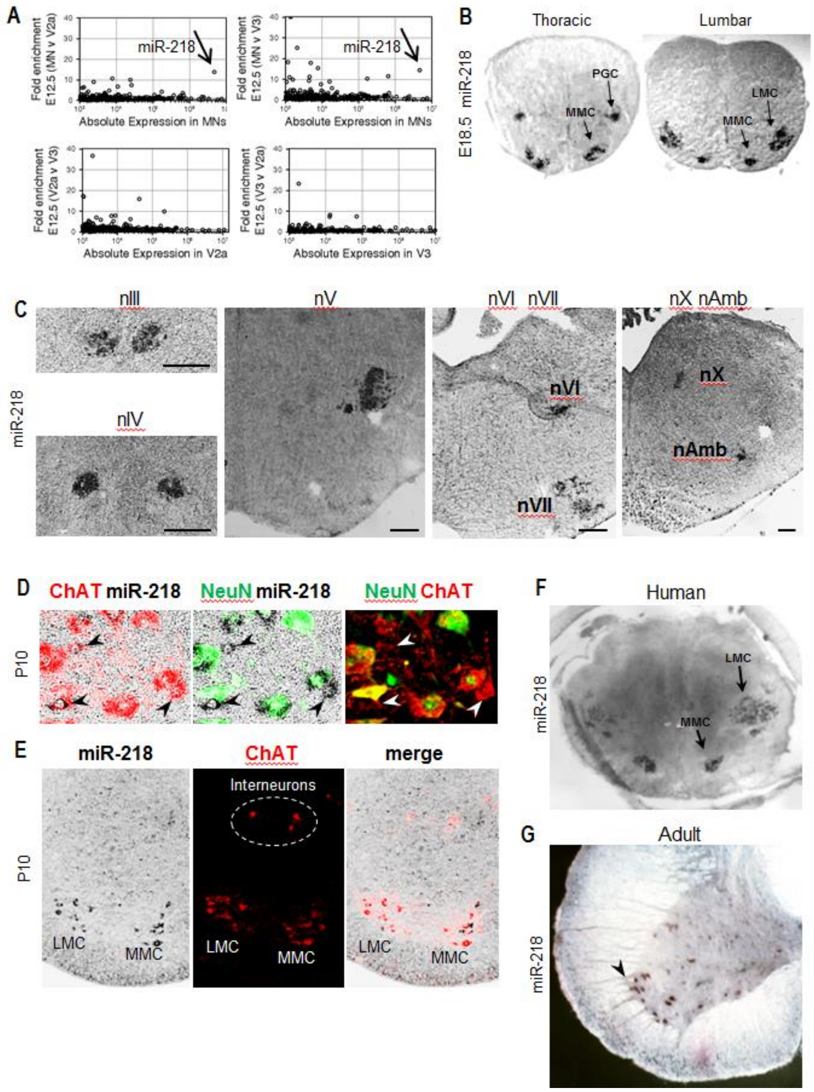


Figure S1 miR-218 is abundantly and specifically expressed across motoneurons. (A) miRNA sequencing reads from FACS-isolated motoneurons (Hb9:gfp), V2a interneurons (Chx10:Cre; Rosa:LNL:tdtomato) and V3 interneurons (Sim1:Cre; Rosa:LNL:tdtomato) from E12.5 dissected spinal cord tissue. (B and C) miR-218 in situ hybridization performed on E18.5 spinal cord (B) and brainstem (C) tissue. miR-218 was detected in the medial motor column (MMC), lateral motor column (LMC), preganglionic motor column (PGC), and all cranial motor nuclei: nuclei III (nIII), nuclei IV (nIV), nuclei V (nV), nuclei VI (nVI), nuclei VII (nVII), nuclei X (nX), and the ambiguous nucleus (nAmb). Staining of cranial nuclei XII (nXII) is displayed in (Fig. 1H). (D) miR-218 is expressed in both α- (NeuN+,ChAT+) and γ- (NeuN-,ChAT+, arrowheads) motoneurons located in the ventrolateral spinal cord at P10, as determined by dual immunohistochemistry and in situ hybridization. (E) miR-218 is not detected in ChAT+ interneurons (dotted circle) located in the dorsal spinal cord at P10. (F) miR-218 expression is detected in motoneurons of the human embryonic spinal cord (LMC and MMC shown here). (G) miR-218 expression is detected in the adult mouse spinal cord in ventrolateral motoneurons.

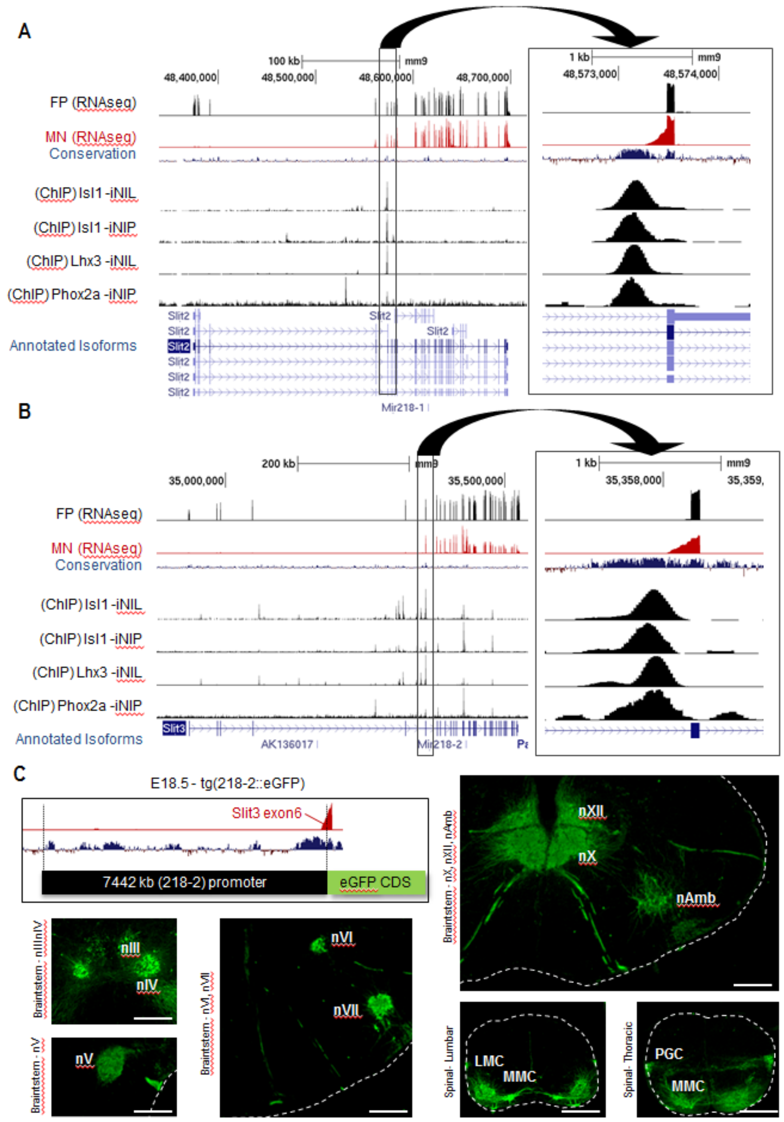


Figure S2 Alternative, motoneuron-specific promoters drive transcription of miR-218-1 and miR218-2. (A and B) UCSC genome browser views of Slit2 (A) and Slit3 (B) genomic loci showing (sequentially from top to bottom): RNA sequencing reads from the floor plate and motoneurons; evolutionary conservation; ChIP sequencing data (previously reported (9)) of Isl1, Lhx3 and Phox2a in ES-derived cranial (iNIP) and spinal (iNIL) motoneurons; and annotated gene isoforms. Large ChIP sequencing peaks for Isl1, Lhx3, and Phox2a are found upstream of exon 6 of both Slit2 and Slit3. (C) The 7.6kb genomic region upstream of Slit3's exon 6 contains many evolutionarily conserved regions which are putative Isl1/Lhx3/Phox2-responsive enhancer and promoter elements. This segment was cloned into a promoter-less vector upstream of the coding sequence of eGFP. A transgenic mouse line generated from this construct tg(218-2::eGFP) specifically expresses eGFP in cranial motor nuclei and spinal motor columns (E18.5).

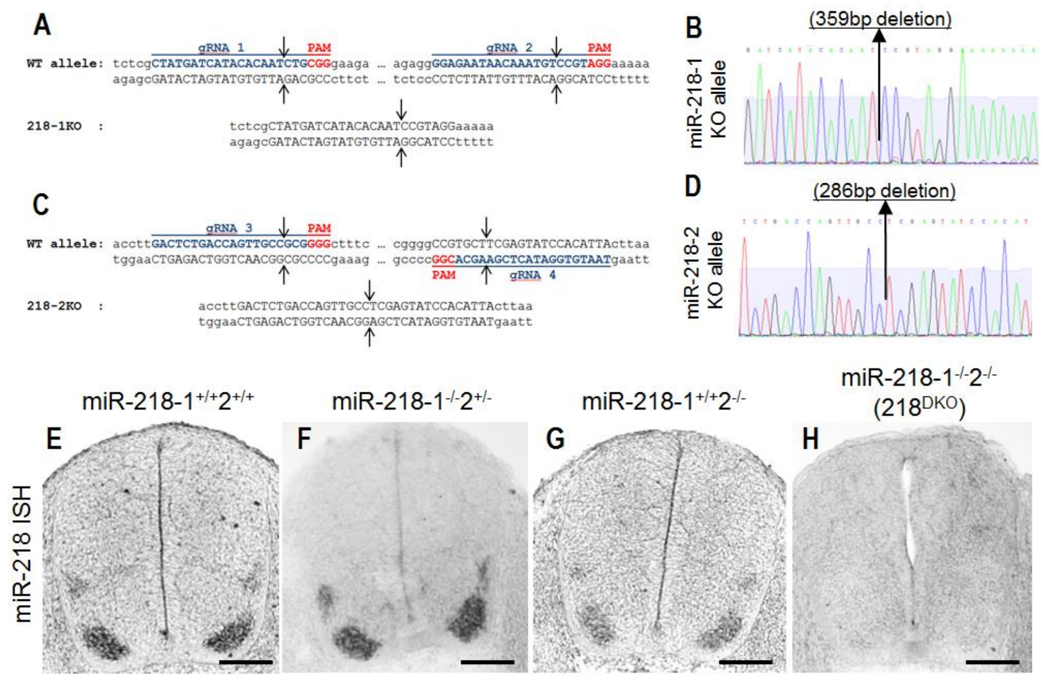


Figure S3 CRISPR/Cas9-mediated knockout of miR-218. (A-D) Design and validation of knockout mice. Guide RNA (gRNA) sequences (blue) and PAM sequences (red) used to generate deletions of miR-218-1 (A) and miR-218-2 (C) are highlighted. Induced double stranded break points are indicated with arrows and multiplexed deletions resulted in end joining. (B, D) CRISPR/Cas9 mediated genomic deletions were screened by PCR and validated by Sanger sequencing. (E-H) In situ hybridization was performed on miR-218 mutants. While deletion of both miR-218-1 alleles and one miR-218-2 allele (F) has little to no effect on signal intensity, deletion of both miR-218-2 alleles (G) results in a qualitative reduction of in situ hybridization signal intensity. (H) However, complete signal loss is only observed when all four miR-218 alleles (218DKO) are genetically ablated.

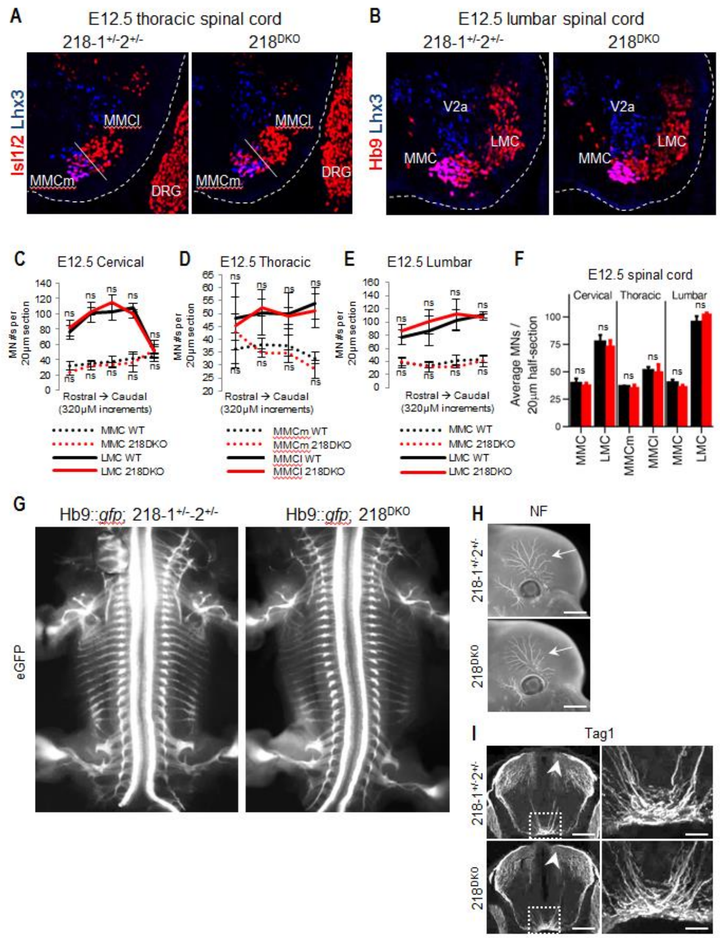


Figure S4 Early motoneuron developmental stages are unaffected in 218DKO mutants. (A-F) MMC (Lhx+,Hb9+), LMC (Lhx3-,Hb9+), MMCm (Lhx3+,Isl1/2low), and MMCl (Lhx3+,Isl1/2+) motoneurons were identified by immunolabelling 20um thick spinal sections across cervical, thoracic and lumbar segments. Dorsal root ganglion sensory neurons outside the spinal cord are Isl1/2+ (DRG). Representative sections of the thoracic (A) and lumbar (B) spinal cords of control and 218DKO E12.5 embryos. 218DKO motoneuron cell bodies are positioned in the ventrolateral spinal cord indistinguishably from controls. (C-E) Motoneurons in hemicords were identified by transcription factor staining and manually counted in cryosections rostrocaudally spaced apart in 320um intervals across indicated spinal segments. The number of each of these motoneuron subtypes is unaffected in 218DKO embryos (n=4 and 3 animals, standard deviation is shown). (F) Average numbers of motoneurons of each identified subtype in 20um cryosections of control (black) and 218DKO (red) hemi-spinal cords. No significant change in motoneuron numbers was found across each of these spinal cord regions at E12.5. (G) Hb9::gfp control and 218DKO E12.5 embryos were glycerol cleared and flat mounted to observe motor axon projection patterns. However, no differences in motor outgrowth or patterning could be observed at E12.5. (H and I) 218DKO mice do not exhibit neuronal defects associated with Slit2 and Slit3 ablation. Projection of the ophthalmic nerve (H, arrow), sensory neuron spinal cord innervation (I, left panels, arrowhead), and commissural axon guidance (I, inset) appear unaffected by the genetic ablation of miR218.

A

miR-218-1$^{-/-}$2$^{+/-}$ x miR-218-1$^{-/-}$2$^{+/-}$				
	# pups observed	observed frequency	expected frequency	response rate to tail pinch 20min after C-section
miR-218-1$^{-/-}$2$^{+/+}$	13	0.29	0.25	13 / 13
miR-218-1$^{-/-}$2$^{+/-}$	20	0.44	0.50	20 / 20
miR-218-1$^{-/-}$2$^{-/-}$ (218^{DKO})	12	0.27	0.25	0 / 12
		Chi Square Test, p= 0.993		

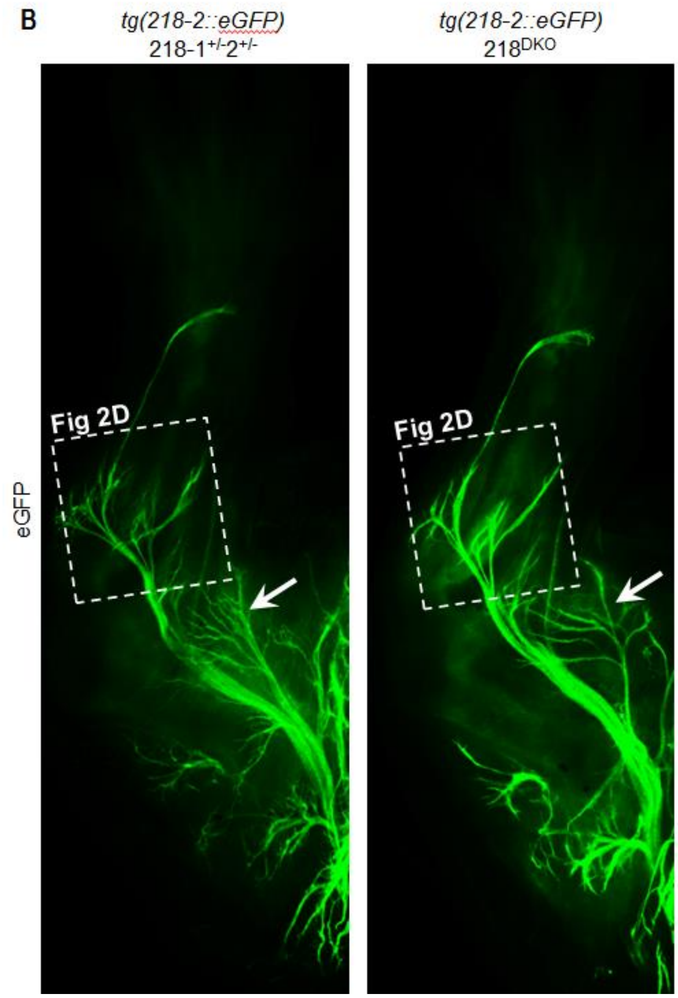


Figure S5 218DKO embryos are not viable and have defective motor axon innervation of muscle. (A) miR-218-1-/-2+/- male and female mice were bred to generate 218DKO embryos. 218DKO embryos were observed at Mendelian frequencies at E18.5, though these embryos consistently lacked motor responses when assessed 20 minutes after caesarean-section. (B) Glycerol cleared lower limbs of tg(218-2:eGFP) E14.5 embryos were deskinned, glycerol cleared, flat-mounted between glass coverslips, and imaged to observe motoneuron axonal branching within muscle. Axon bundle thickness is grossly unaffected, but complexity of branching is qualitatively reduced across motor nerves in 218DKO embryos. Boxed area denotes area of imaging of the deep peroneal nerve shown in (Fig. 2D).

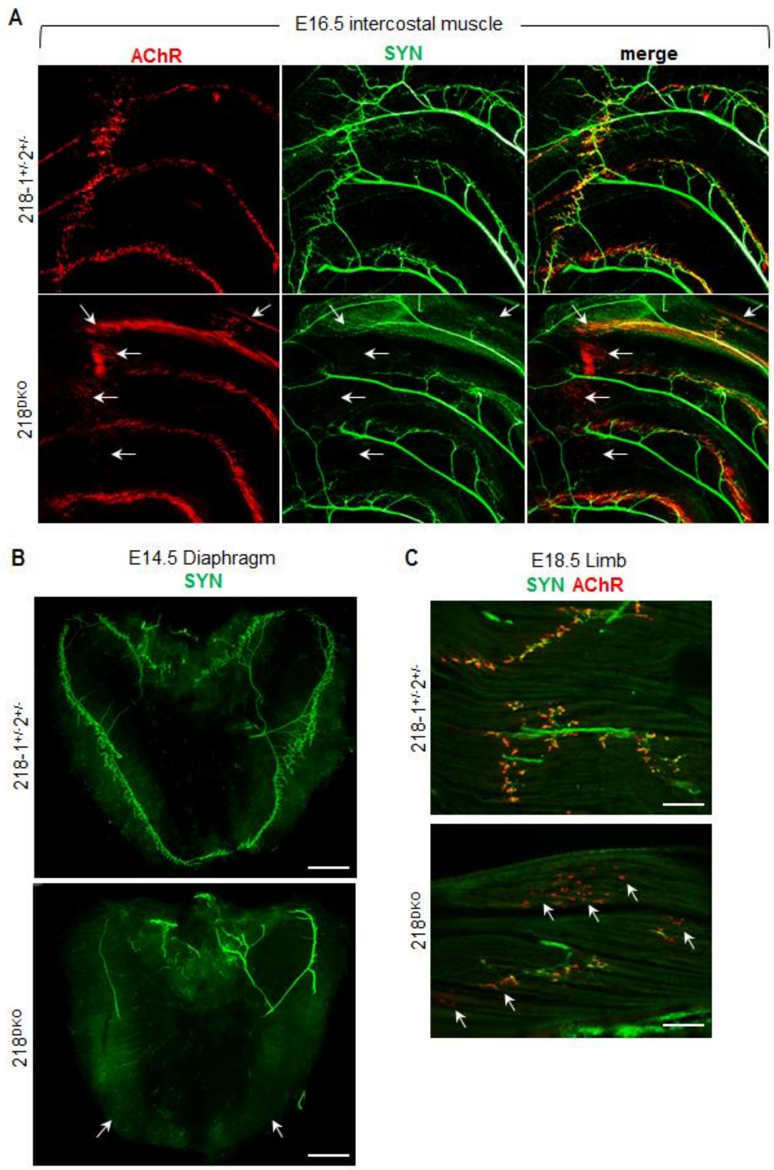


Figure S6 Neuromuscular junctions. (A-C) Pre- (SYN+) and post-synaptic (AChR+) neuromuscular junction components were identified by synaptophysin antibody and α-bungarotoxin staining. (A) In E16.5 218DKO intercostal muscles, motor nerves innervate muscle but fail to appropriately induce clustering of post-synaptic AChRs, exhibit less branching, and do not completely innervate the muscle (white arrows) compared with controls. (B) In E14.5 dissected diaphragms, motor axons only partially innervate the circumference of the muscle in mutants, with large areas of the diaphragm (white arrows) lacking motor innervation. (C) A representative section of E18.5 limb tissue demonstrating significant regions of limb AChR clusters (white arrows) that lack motor innervation.

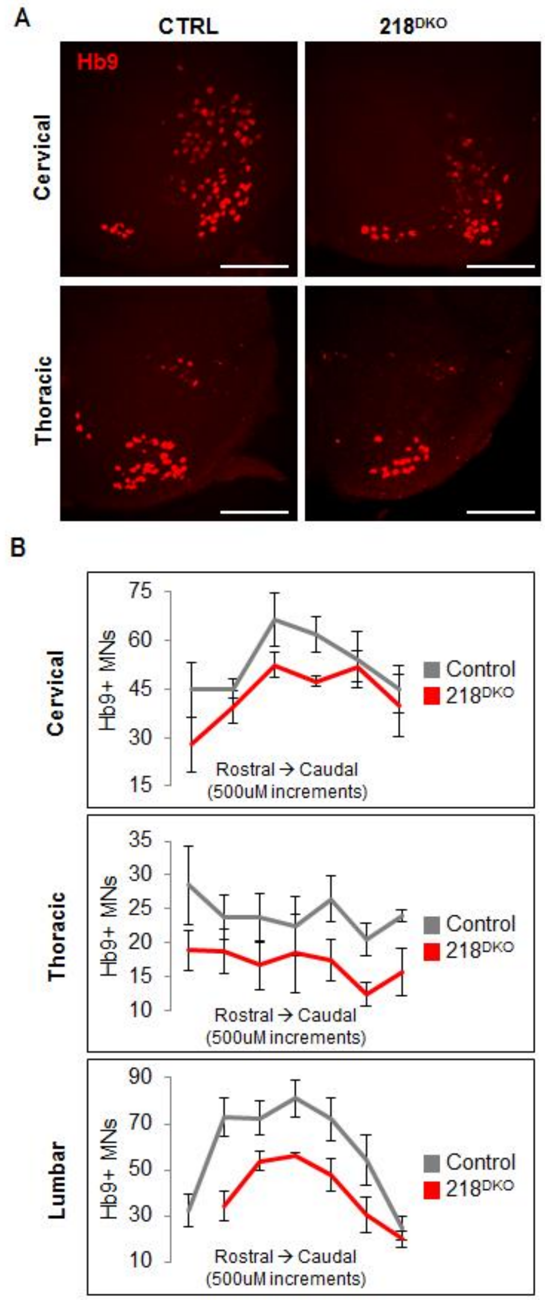


Figure S7 Reduced numbers of motoneurons in E18.5 218DKO spinal cords. (A) Motoneurons were identified by Hb9 nuclear staining in control and 218DKO E18.5 spinal cord sections from lumbar, cervical and thoracic regions at E18.5. Lumbar spinal cord staining is shown in (Fig. 2H). (B) Hb9+ motoneurons in 30uM cryosections of hemi-spinal cords were counted in 500um intervals across the rostrocaudal axis of cervical, thoracic, and lumbar spinal regions. Significant reductions in motoneuron numbers were observed in 218DKO spinal cords across all of these regions (n=4, standard deviation is shown).

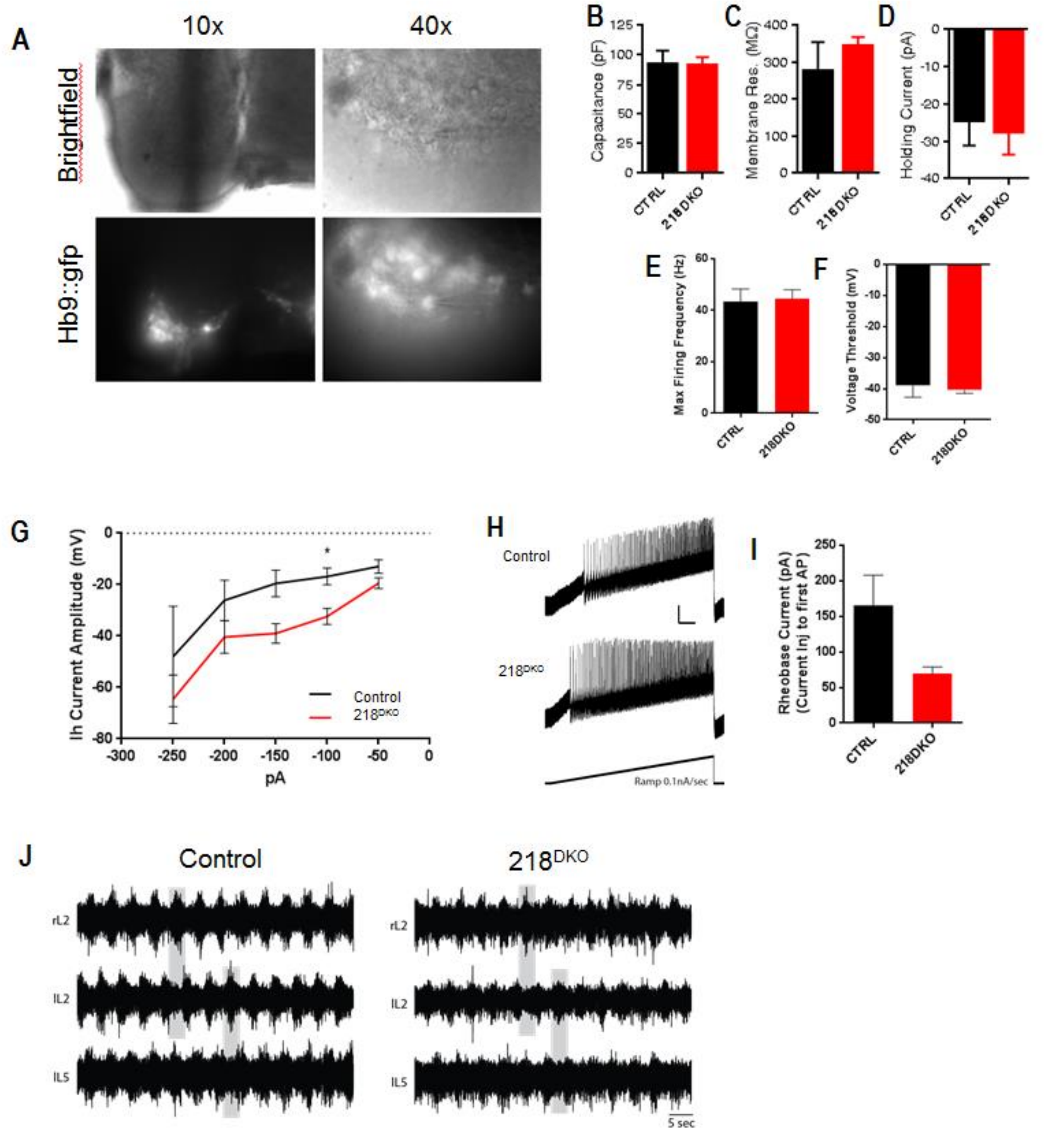
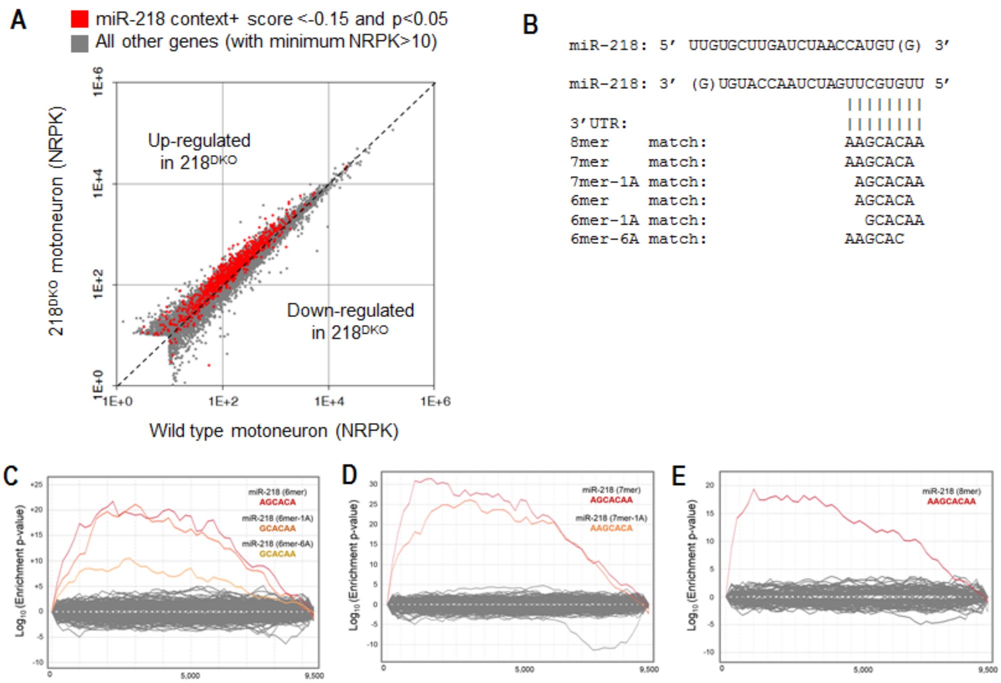


Figure S8 Motoneuron electrophysiology. (A) Large LMC α-motoneurons from lumbar spinal cords were identified by Hb9::gfp expression. Fine pulled glass electrodes were patched onto fluorescent cells to record intracellular electrophysiological properties. (B-F) Capacitances, membrane resistances, holding currents (at -70mV), max firing frequencies, and voltage thresholds were indistinguishable between controls and mutants (n=9, 5). (G) Ih currents were reduced in 218DKO motoneurons. (H and I) Current ramps induced firing of 218DKO mutant motor neurons with ~3-fold lower currents than required in control motor neurons. (J) Intraspinal motoneuron connectivity was assessed by chemical stimulation of the central pattern generator in an in vitro spinal cord preparation. Recording electrodes were placed on the ventral roots at L2 on ipsilateral and contralateral sides and ipsilaterally on L5. Alternating L/R activity and alternating flexor/extensor activity was observed in both controls and 218DKO embryos.



F Enriched gene ontology terms in target[218] genes

Description	P-value	Enrichment	# of genes
regulation of cellular process	1.1E-04	1.22	187
regulation of biological process	1.8E-04	1.2	193
negative regulation of synaptic transmission	1.9E-04	5.76	7
biological regulation	2.1E-04	1.19	199
regulation of primary metabolic process	2.9E-04	1.32	119
regulation of cellular metabolic process	3.3E-04	1.3	122
neurotransmitter transport	5.7E-04	4.24	8
regulation of macromolecule metabolic process	6.0E-04	1.3	118
regulation of signaling	6.2E-04	1.48	66
Kit signaling pathway	6.7E-04	38.69	2

G

negative regulation of synaptic transmission	
Shank2	sh3/ankyrin domain gene 2
Grik2	glutamate receptor, ionotropic, kainate 2 (beta 2)
Gnai2	guanine nucleotide binding protein (g protein), alpha inhibiting 2
Stxbp1	syntaxin binding protein 1
Park2	parkinson disease (autosomal recessive, juvenile) 2, parkin
Slc6a1	solute carrier family 6 (neurotransmitter transporter, gaba), member 1
Celf4	cugbp, elav like family member 4
neurotransmitter transport	
Syt13	synaptotagmin xiii
Stx3	syntaxin 3
Stxbp1	syntaxin binding protein 1
Park2	parkinson disease (autosomal recessive, juvenile) 2, parkin
Slc6a1	solute carrier family 6 (neurotransmitter transporter, gaba), member 1
Sv2a	synaptic vesicle glycoprotein 2 a
Nrxn3	neurexin iii
Slc6a17	solute carrier family 6 (neurotransmitter transporter), member 17

Figure S9 Gene expression of miR-218 target genes in 218DKO motoneurons. (A) NRPK gene expression in wild type versus 218DKO motoneurons FACS-isolated from E12.5 spinal cords. Genes with >10 NRPK, with robust miR-218 binding sites (TargetScan6 context+ score <-0.15) and that pass statistical significance are in red, and all other genes with NRPK>10 are in gray. (B) The nucleotide sequence of miR-218 and each of its canonical 6mer, 7mer, and 8mer 3'UTR complementary seed matches is shown. (C-E) Transcripts were ranked from upregulated to downregulated in 218DKO motoneurons versus wild type motoneurons, and Sylamer was used to determine the statistical enrichment of 6bp (C), 7bp (D), and 8bp (E) 3'UTR miRNA seed matches. Upregulated (de-repressed) genes in 218DKO motoneurons are specifically and significantly enriched for miR-218 binding sites. (F) Top ten biological process GO categories enriched in TARGET218 genes as determined by the Gorilla platform. (G) Specific genes within the negative regulation of synaptic transport and neurotransmitter transport categories are listed

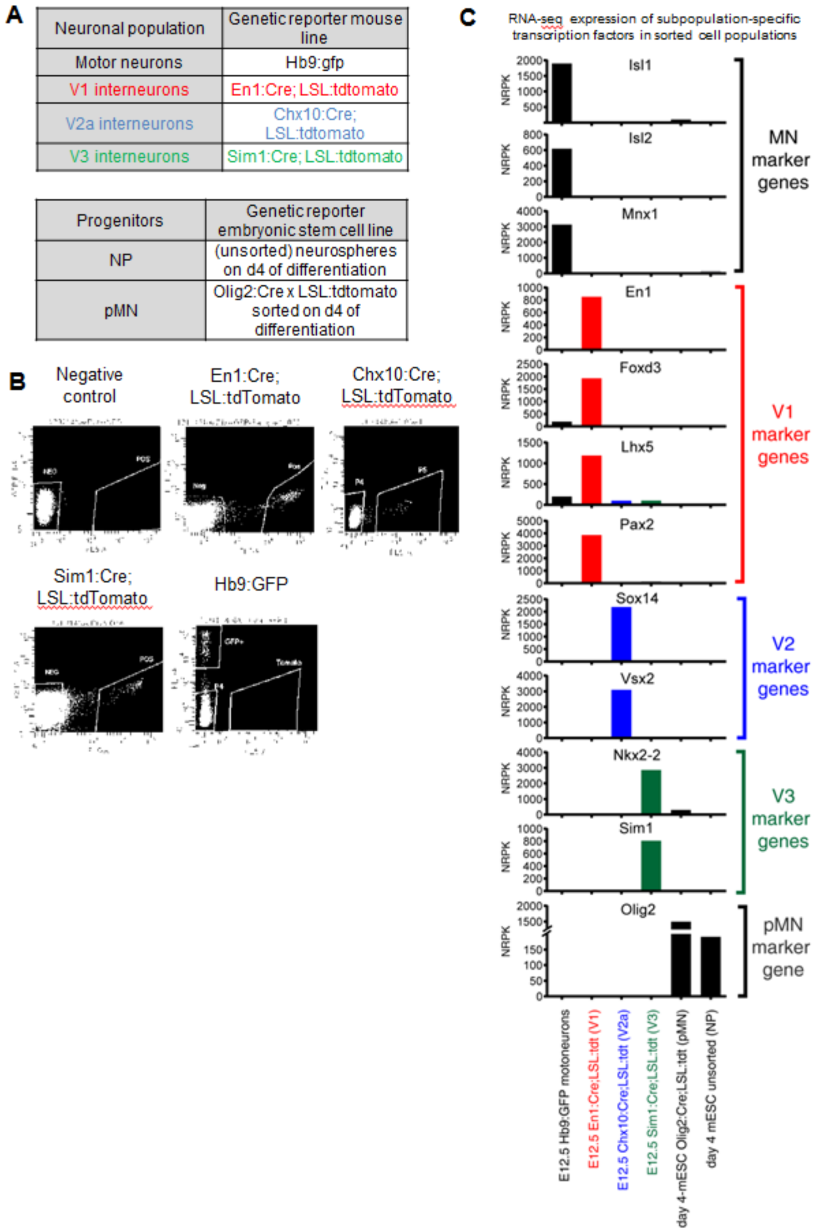


Figure S10 FACS-isolated subpopulations express known marker genes. (A) V1, V2a, V3 interneurons, and motoneurons were genetically labelled in E12.5 spinal cords using the reported mouse lines and motoneuron progenitors were isolated from mES-derived neuronal progenitors by either FACS (pMN) or collecting whole neurospheres at day 4 of differentiation. (B) Representative FACS plots demonstrate separation of fluorescently labelled cell populations. (C) Normalized reads per kilobase (NRPK) for known cellular marker genes are plotted for each dataset to validate the purity of cells. Each known marker gene is specifically and abundantly expressed in the respective dataset. Importantly, motoneuron progenitors were captured before motoneurogenesis, as indicated by the expression of Olig2 but relative absence of Hb9, Isl1, and Isl2 expression.

Chapter 3

Conclusion

The identification of miR-218 as a critical regulator of motor neuron function raises many new questions which will be outlined in this concluding chapter.

Are motor neurons the only neuronal subtype with the expression of a dedicated microRNA?

With the exception of miR-182/183/96 expression in sensory neurons including the dorsal root ganglion, cochlea, taste buds, olfactory neurons, and in the pineal gland [71], I have not yet identified another microRNA or microRNA cluster that exhibits comparable specificity in expression as miR-218 by searching the literature. It is yet to be observed whether microRNA profiling by microarray or small RNA sequencing of other neuronal subsets either by FACS-isolation or other methods might yet identify such neuronal subtype specific microRNAs. However, if none other are discovered, it raises a curious question of why the input and output cells of the entire CNS, motor and sensory neurons, are the only two neuronal subtypes with dedicated microRNA expression.

Though miR-218 is generated from pri-miR-218-1 and pri-miR-218-2 transcript isoforms, are they still able to produce functional protein?

Interestingly, my identification of novel promoters for Slit2 and Slit3 represent a surprising development in the field of axon guidance in that these new RNA isoforms may give rise to novel protein isoforms. Decades of work have uncovered important roles of Slit proteins in midline crossing, in neuronal migration, and in functions in diverse tissues [83]. The presence of novel Slit isoforms specifically expressed in motor neurons is a tantalizing possibility. Despite the decades of research on Slit proteins, antibodies able to detect endogenous Slit2 and Slit3 isoforms are generally thought to be of extremely poor quality, and in my hands, these antibodies have failed to produce results that are of sufficient quality to be trusted. Thus, proving that novel Slit protein isoforms are being produced is more difficult than performing a western blot, and other methods, such as mass spectroscopy might provide further insights into the presence of absence of new Slit protein isoforms that might harbor new and interesting functions.

How is the exquisite specificity of miR-218 expression achieved?

My work has identified alternative promoters driving the expression of alternative Slit2 and Slit3 isoforms specifically in motor neurons, and these transcripts are the precursors to miR-218. However, with both classical and alternative promoters, the precursor sequence to miR-218 is transcribed, though only in motor neurons is mature miR-218 abundant. This raises the discrepancy between transcription of miR-218 precursors and the generation of mature miR-218, a process known as microRNA biogenesis [34]. This process is under the regulation of the Drosha microprocessor complex and Dicer protein, and several studies with other microRNAs have shown that accessory proteins can modify the activity of these proteins for specific microRNAs [105-109]. These accessory modulators of microRNA biogenesis function in a similar manner to proteins that influence alternative splicing, and it is likely that such factors are regulating the alternative biogenesis of miR-218 from Slit2 and Slit3 transcripts in motor neurons versus other Slit2 and Slit3-expressing tissues and cell types. One possible model for such an interaction would be a protein factor that would repress miR-218 biogenesis in other tissues and that is absent from motor neurons. Conversely, a specific RNA binding protein accessory factor may be specifically expressed in motor neurons and thus will enhance miR-218 biogenesis with similar specificity. These possibilities will need to be evaluated by further studies that pay special attention to factors influencing the microRNA processing pathway.

Is miR-218 dysregulated in disease states?

Though some studies have investigated the expression of microRNAs in the post-mortem spinal cords of human patients that were suffering from motor neuron disease, a review or meta-analysis of such data might provide further insights into whether certain microRNAs (such as miR-218) are consistently dysregulated in disease. Other approaches might include the analysis of microRNA expression in mouse models of disease, and evaluating whether the over-expression of miR-218 might enhance motor neuron function with respect to neuromuscular function. These studies will also be important in the evaluation of miR-218 as a target for potential therapeutics that aim to enhance the neuromuscular junction in humans suffering from various motor neuron diseases.

Final thoughts

The work described herein highlights the importance of single microRNA on motor neuron development and function. However, microRNA mediated repression is just one of many forms of post-transcriptional regulatory modalities that have the potential to influence neuronal subtype specific gene regulation. In the past decade, intense effort has focused on transcriptional mechanisms of gene regulation such as the combinatorial expression of transcription factors that specify neuronal diversity during embryonic development. In the future, studies of mechanisms of RNA processing (spanning alternative microRNA biogenesis, splicing, and polyadenylation) within neuronal subtypes may make the greatest advances in our understanding of how neuronal subtype identity is governed.

Methods

Chapter 1 Methods

Chapter 1 does not contain experimental data.

Chapter 2 Methods

All experiments presented in this dissertation were performed in accordance with the Salk Institute Institutional Animal Care and Use Committee guidelines

ES cell derivation and culture

To differentiate mES cells into motor neuron progenitors: 10^6 mES cells were passaged onto 10cm dishes in ADFNK media (1:1 Advanced DMEM [Invitrogen] : Neurobasal Media [Invitrogen], 10% KOSR [Invitrogen], L-Glutamine 2mM, 14.3mM 2-mercaptoethanol, 1% Antibiotic/Antimycotic [Invitrogen]) for 30 minutes to allow carried-over MEF to adhere. Unattached cells were transferred to a new 10cm dish and media was replaced every two days. Smoothened agonist [Millipore] and all-trans retinoic acid were present in ADFNK media at 100nM and 1uM, respectively, from day 2 to day 4 to induce ventral spinal progenitors.

Mouse Lines

The following mouse lines were used: Hb9:gfp [110], Chx10:Cre [111], Sim1:Cre [112], En1:Cre (Jax 007916), Wnt1:Cre (Jax 003829), and Rosa:LSL:tdtomato (Jax 007905). En1:Cre, Sim1:Cre, Wnt1:Cre, and Chx10:Cre males were crossed with Rosa:LSL:tdtomato females to generate embryos in which specific the respective neuronal populations express tdTomato.

miR-218-1 and miR-218-2 knockout mice were generated using CRISPR/Cas9 targeting, as described [86]. Briefly, Cas9 mRNA was in vitro transcribed, capped and polyadenylated using the Invitrogen mMachine kit. Guide RNAs were designed using crispr.mit.edu to decrease the likelihood of off-target effects and were *in vitro* transcribed using the New England Biolabs High Yeild In Vitro Transcription Kit. Mouse oocytes were microinjected with Cas9 mRNA:gRNA:gRNA mixtures (at concentrations of 30ng/uL:15ng/uL:15ng/uL) and were reimplanted into B6D2F1pseudopregnant females. Successful multiplexed deletions were detected by PCR genotyping and confirmed by Sanger sequencing, and positive founders were maintained and used for breeding. miR-218-1$^{+/-}$, miR-218-2$^{+/-}$, and Hb9:*gfp* mice were bred to generate 218^{DKO};Hb9:*gfp* embryos.

Immunohistochemistry and in situ hybridizations

Immunohistochemistry was performed as previously described [110]. Briefly, tissue was fixed in 4% PFA for 2 hours at 4C, washed in PBS o/n, cryoprotected in 30% sucrose for 30 minutes before mounting and freezing in OCT. microRNA *in situ* hybridizations were performed on whole mount embryos and tissue sections according to standard protocols (Exiqon) using a 5'/3'-DIG pre-labelled miR-218 LNA probe (cat: 18111-15; Exiqon). For dual *in situ* hybridization and immunofluorescence, *in situ* hybridization was performed first followed by the incubation of tissue sections in primary and secondary antibodies for immunohistochemistry.

Human spinal cords were obtained from NIH Tissue Bank. Microdissections of mouse tissues were performed under a Zeiss Stemi SV6 microscope, and imaging was performed with a Leica confocal CTR6500 (TCS SPE) microscope or Zeiss Lumar V12 stereomicroscope.

Antibodies: goat anti-ChAT (Millipore; AB144P), Rabbit anti-Neurofilament (Chemicon AB1987), mouse anti-Neurofilament (Developmental Studies Hybridoma Bank(DSHB), 2H3 for whole embryo staining), guinea pig anti-Lhx3 (#718), rabbit anti-Isl1/2 [113], rabbit anti-Hb9 (#6055), mouse anti-Tag1 (DSHB, 3.1C12), rabbit anti-TrkA (Millipore; 06-574), rabbit anti-NeuN (Millipore; ABN78), alpha-bungarotoxin-tetramethylrhodamine for AChR labelling (Life Technologies T-1175), rabbit anti-Synaptophysin (Santa Cruz: sc-9116), rabbit anti-GFP (Invitrogen).

Flat mounts

E12.5 flat mounts were prepared by decapitating and eviscerating Hb9:*gfp*+ embryos, fixing in 4% PFA for 2 hours at 4C, washing with PBS 3x, and sequentially transferring the tissue from 30%, 50%, 80% glycerol every 2 hours. Cleared flat mount tissue was mounted between two glass coverslips before imaging with Zeiss Lumar V12 stereomicroscope.

E14.5 flat mounts were prepared by removing the limbs of *tg(218-2::eGFP)* embryos, dissecting skin from muscle tissue, fixing in 4% PFA for 2 hours at 4C, washing with PBS 3x, and sequentially transferring the tissue from 30%, 50%, 80% glycerol every 2 hours. Cleared flat mount tissue was mounted between two glass coverslips before imaging with Zeiss Lumar V12 stereomicroscope. The *tg(218-2::eGFP)* transgene was used for these experiments due to the significantly brighter expression of fluorescence compared with the Hb9:gfp transgene allowing for more detailed imaging.

<u>Slice preparation for intracellular recording</u>

Hb9:*gfp*+ E18.5 embryos were removed from the uterus under isoflurane anesthesia and spinal cords were quickly isolated in ice cold, oxygenated 95% O_2/5% CO_2, ACSF containing (in mM): 128 NaCl, 2.5 KCl, 0.5 NaH_2PO_4, 21 $NaHCO_3$, 30 D-Glucose, 3 $MgSO_4$, and 1 $CaCl_2$ at pH=7.4 and 300-305mOsm. 218^{DKO} mutants were identified by postmortem PCR genotyping. Lumbar regions of the spinal cords were isolated and mounted in low melting point agarose (4% in aCSF) held at 37C in plastic molds. After mounting, molds were immediately placed on ice until agarose solidified, and spinal cords were sliced coronally (300µm) on a Leica VT1000S vibratome in an ice cold, oxygenated bath of aCSF. Spinal slices were transferred to a holding chamber and allowed to recover for a half hour at 32C and then transferred to an oxygenated holding chamber containing ACSF (in mM): 128 NaCl, 2.5 KCl, 0.5 NaH2PO4, 26 NaHCO3, 25 D-Glucose, 1 MgSO4, 2 CaCl, 0.4 ascorbic acid, and 2 Na-Pyruvate at pH=7.4 and 300-305mOsm at 28C.

Whole-cell Current Clamp Recordings

Following an hour of recovery a spinal slice was transferred to a recording chamber (Warner) which was continuously perfused with ACSF at a rate of 1-2mL/min heated with an inline heater (Warner) to 28C. Pulled thin-wall glass electrodes (WPI) with a tip resistance of 3.5-4.5 MΩ were filled with a potassium methanesulfonate based intracellular recording solution (in mM): 135 KMeSO4, 5 KCl, 0.5 CaCl2, 5 HEPES, 5 EGTA, 2 Mg-ATP, and 0.3 Na-GTP at pH=7.3 and 285-290mOsm. MultiClamp 700A amplifier and Digidata 1322a Digitizer (Molecular Devices) was used for data acquisition. Whole-cell recordings were filtered at 2kHz and digitized at 10kHz and monitored using pClamp 9 software. Liquid junction potential was not corrected for. Whole-cell current clamp experiments targeted large, Hb9:*gfp*+ motoneurons located in the lateral motor column of the ventral horn under 40X DIC magnification with a high speed IR camera (QImaging). eGFP epifluorescence co-localization was confirmed prior to break-in. Following 5 minutes post break-in whole-cell configuration membrane properties were collected at a holding potential of -70mV. Series resistance ranged between 8-20mOhms and any cells with changes >20% over the duration of the recording were discarded. Resting membrane potential was calculated 5 minutes following the transition to current clamp mode from the average of ten consecutive sweeps. The rheobase current was determined from a series of 5s square pulses (-250pA and up, 50pA steps) given at 20 second intervals to allow slow conductances to recover to their initial state. The first sweep to elicit an action potential was considered the rheobase current. For a more precise measure of rheobase current, the recruitment current coinciding with the first action potential on 0.1nA/sec current ramps (5 sec duration) repeated 10 times (20s interval) were measured. Voltage spiking threshold on the first spike was measured by finding the voltage first derivative value greater than 10mV/ms. Input conductance was measured as the slope of the current-voltage relationship by determining the steady state current at negative current injections (-100 to -10pA, 30pA steps). Ih current amplitude was measured during hyperpolarizing current injections as the peak current minus steady state current. AHP amplitude was measured by single action potentials elicited by 1ms square pulses (2-4nA) and AHP decay tau was fit with a single exponential.

Ventral Root Recordings

At E18.5, spinal cords from wild-type and 218^{DKO} animals were isolated in cold oxygenated dissection ACSF (128 mM NaCl; 4 mM KCl; 21 mM $NaHCO_3$; 0.5 mM NaH_2PO_4; 3 mM $MgSO_4$; 30 mM D-glucose; and 1 mM $CaCl_2$), and transferred to oxygenated room temperature recording ACSF (128 mM NaCl; 4 mM KCl; 21 mM $NaHCO_3$; 0.5 mM NaH_2PO_4; 1 mM $MgSO_4$; 30 mM D-glucose; and 2 mM $CaCl_2$). Suction electrodes were attached to the L2 and L4 or L5 ventral roots, and cords were then allowed to recover and equilibrate to room temperature for ~20 min. Pharmacologic induction of fictive locomotor activity was performed by bath application of 10µM *N*-methyl-D,L-aspartate and 20µM serotonin. Motoneuron activity was recorded, amplified 1000x, and filtered from 100Hz-3Hz. Analysis of fictive locomotor activity phase and cycles was conducted offline with custom written scripts in R.

FACS and RNA isolation

Spinal cords from E12.5 mice were micro-dissected using a Leica stereomicroscope and dissociated with papain (papain dissociation kit, Worthington Biochemical) for 45 minutes. Dissociated spinal tissue was triturated and centrifuged at 1000rpm for 5 minutes. Cells were resuspended in 1:1 Neurobasal:DMEM/F12 (without phenol red) with 3% Horse Serum (Invitrogen) and DNase (Worthingon Biochemical) and passed through a

35µm cell strainer (BD Falcon 08-771-23). Cells were sorted on a Becton Dickinson FACS Vantage SE DiVa using Coherent Sapphire 488nm and 568nm solid state lasers (200mW) and collected directly into miRvana RNA lysis buffer.

Collected cells were stored at -80C until RNA was collected using the miRvana miRNA isolation kit (Ambion AM1560). Samples were genotyped by PCR prior to RNA isolation. For both small RNA and polyA+ RNA sequencing experiments, the protocol for total RNA collection was used. RNA collected from cells isolated from one to (at most) three spinal cords were combined before sequencing to obtain at least 100ng RNA (determined by Agilent TapeStation) for library preparation.

RNA sequencing and gene expression quantification

mRNA sequencing libraries were prepared using the TruSeq RNA Library Preparation Kit (v2) according to the manufacturer's instructions (Illumina). Briefly, RNA with polyA$^+$ tails was selected using oligo-dT beads. mRNA was then fragmented and reverse-transcribed into cDNA. cDNA was end-repaired, index adapter-ligated and PCR amplified. AMPure XP beads (Beckman Coulter) were used to purify nucleic acids after each step.

Small RNA-sequencing libraries were prepared using NEBNext Small RNA Library Prep for Illumina. Briefly, 3' adapter was ligated to total RNA, any excess 3' adaptor were quenched by hybridization of reverse transcription primer to prevent primer dimers. RNA was then ligated to 5' adaptor, reverse transcribed and PCR amplified.

Libraries were then quantified, pooled and sequenced using either the Illumina HiSeq 2500 or Illumina HiSeq 2000 platforms at the Salk NGS Core and Beijing Genomics Institute. Raw sequencing data was demultiplexed and converted into FASTQ files using CASAVA (v1.8.2). A total of 50-base pair (bp) single-end reads or 100-bp paired-end reads were aligned to the mouse genome using Bowtie, allowing up to three mismatches per alignment and up to 20 alignments per read, filtering out any read aligning in more than 20 locations. For consistency in comparing some data sets, read lengths were cut down to 50 bp (from the 3′ end). All samples were filtered by removing reads with average base quality before 15.

Isoform gene expression quantification was performed using Sailfish [114] using the mm10 Refgene transcriptome database (available at the University of California, Santa Cruz Genome Browser). Isoform expressions were summed per gene locus to create gene-level expression for downstream fold change comparisons between groups.

Data analysis methods

Normalized reads per kilobase (NRPK) values of replicates were averaged and genes that were not expressed by at least 10 NRPKs in either data set were eliminated. P-values were determined by a two-tailed heteroscedastic t-test.

Context^{+} scores are a predictor of efficacy of microRNA targeting with more negative scores denoting a greater predicted efficacy of repression and were obtained from http://www.targetscan.org/mmu_61/. A cutoff of <-0.15 for context+ scores was empirically established.

Differential NRPK expression of target[218] genes in wild-type motoneurons versus each interneuron subtype (n=6 (WT), 1 (V1), 1 (V2a), 1 (V3)). Heirarchical clustering was performed with GENE-E software, using city block distances. RNA sequencing reads were aligned on the transcript-specific level (mm10, transcript database obtained from UCSC genome browser). NRPKs of gene expression in wild-type motoneuron replicates were averaged, and NRPKs of gene expression in from V1, V2a, and V3 interneurons were averaged. The top 15,000 most highly expressed genes were used for enrichment analysis, as determined by maximum NRPK level in either data set.

Sylamer software (available at https://www.ebi.ac.uk/research/enright/software/sylamer) was used to assess microRNA seed match enrichment p-values. A FASTA file of 3’UTRs masked and purged of low complexity and redundant sequences was exported from Sylarray (http://www.ebi.ac.uk/enright-srv/sylarray/). Transcripts were ranked by differential expression (most enriched in motoneuron to most depleted in motoneurons) to generate a transcript list for Sylamer analysis. Sylamer settings: 7bp, bin size 2, markov correction 4. Enrichment p-values were exported and plotted in one-dimension using Prism GraphPad.

For RNA sequencing data sets from cortical projection neurons, raw data sets were downloaded from [102] and were aligned to the genome using the same methods as in-house generated data sets. NRPK values from cortical projection neuron duplicates (E15.5 data sets) were averaged before performing Sylamer analysis (as above).

Chapter 3 Methods

Chapter 3 does not contain experimental data.

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