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(54) **Title**: MOTOR NEURON-SPECIFIC EXPRESSION VECTORS

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taaacgcccc aatttgctac ttatcaaata gtatacattt ttggctcaga aaaaaacctg 60
atgtctgtat attacttctc aactaaaatc cctcagtcct taactggcat gtgtattagt 120
caaggcatct ttgagaaggg cattatttcc ctacacttag gatggggaaa gagaaattaa 180
aaaggaatcc taaaataggt gcatttaatt ctccccaatt taaatgtaag tggtgcgtct 240
tttaggcaat aatgatatgc cttttagtcc tccattacaa acacttccat cgatgaattt 300
ccttaatgtt gatgatggtt agtgcagttt gagggaatct gtatttattc agaaaatgtt 360
cccatagaat gacctaccag atgggccacg taacaatgca tggagacatc aaaccaccac 420
agacatttgg tgcttagaat aataaaaaga ctataaaatt agattagttg agtctaattt 480
ggaattggta tattccctac gcaccctcac cgctcttggg cagataaagc cttgagattt 540
agcgctgtgt caaagccaag actgtaactt ccagtaaaag ggagccgagg gaggggggc 600
ttgctgggag gtcgcggagg gcagagcagt gacctccaat gatttacagg cctttagctt 660
aatgaaattg tttcagtgac atgacagtaa gagctcgtaa tggattggat gccctaatgt 720
aatgaaatta ctcccttctg cctaaaaaaa aaaaaatgcg caattaatat ttactgagac 780
ctgacagcct ttggtgcgct cgctcgcctg tgtagttccc tcagacagtc agagagaaga 840
gacagagcag cgtggcagac aggcgggctc tgcaggagct cctggcaggg acaagcagag 900
cctgcaag                                                        908
(SEQ ID NO:1).
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FIG. 1

(57) **Abstract**: The present disclosure relates to nucleic acid promoter sequences that are able to specifically express genes operatively linked to the promoter in brainstem and spinal motor neuron cells, and to methods for using such promoters to selectively express genes in motor neurons in vitro and in vivo. It is based, at least in part, on the discovery that the nucleic acid of SEQ ID NO: 1 functioned as a motor neuron- specific promoter and was successful in expressing transgenes in motor neuron cells in vivo. The present disclosure also relates to compositions that can increase the activity or expression level of miR-218 and to compositions that can decrease the expression of miR-218 target nucleic acids.

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MOTOR NEURON-SPECIFIC EXPRESSION VECTORS

CROSS REFERENCE TO RELATED APPLICATIONS

The present application claims priority to U.S. Provisional Application No. 62/166,677 filed May 26, 2015, U.S. Provisional Application No. 62/168,755 filed May 30, 2015, and U.S. Provisional Application No. 62/268,357 filed December 16, 2015, priority to each of which is claimed, and the contents of each of which are incorporated by reference in their entireties herein.

GRANT INFORMATION

This invention was made with government support under grant R37 NS037116 awarded by the National Institutes of Health. The government has certain rights in the invention.

1. INTRODUCTION

The present invention relates to motor neuron-specific expression vectors and their use in expressing genes specifically in motor neuron cells.

2. BACKGROUND OF THE INVENTION

2.1 Motor Neuron Disease

Motor neuron diseases are diseases which lead to impairment of motor nerve functions due to the loss of motor neuron cells, or reduction of motor neuron cell function, and degenerative changes in the motor pathways of the central nervous system. Such diseases are different from other neurodegenerative diseases such as Alzheimer's disease, which are caused by the destruction of neurons other than motor neurons. Typically, motor neuron diseases are developmental or progressive, degenerative disorders that affect nerves in the upper or lower parts of the body. Motor neuron diseases include, in addition to amyotrophic lateral sclerosis (also known as Lou Gehrig's disease), diseases which are classified into primary lateral sclerosis (PLS) affecting only motor neurons located in the cortex such as pyramidal neurons (*i.e.*, upper motor neurons), progressive muscular atrophy (PMA) affecting only lower motor neurons (*e.g.*, motor neurons located in spinal cord and brainstem), progressive bulbar palsy (PBP) affecting lower motor neurons of the brainstem, spinal muscular atrophy (SMA), and the like. Symptoms may include difficulty swallowing, limb weakness, slurred speech,

impaired gait, facial weakness and muscle cramps. Respiration may be affected in the later stages of these diseases, frequently resulting in death. The cause(s) of most motor neuron diseases are not known, but environmental, toxic, viral or genetic factors are all suspects.

Motor neurons regulate all muscles, stimulating them to contract. Motor neurons originating in the cerebral cortex (*e.g.,* upper motor neurons) send fibers through the brainstem and the spinal cord, and are involved in controlling lower motor neurons. Motor neurons located in spinal cord and brainstem (*e.g.,* lower motor neurons) send fibers out to muscles. Lower motor neuron diseases are diseases involving lower motor neuron degeneration. When a lower motor neuron degenerates, the muscle fibers it normally activates become disconnected from the neuron, and do not contract, causing muscle weakness and diminished reflexes. Loss of either type of neurons results in weakness. Muscle atrophy (wasting) and painless weakness are the clinical hallmarks of motor neuron diseases. Amyotrophic Lateral Sclerosis (ALS) is a fatal motor neuron disease characterized by a loss of pyramidal cells in the cerebral motor cortex (i.e., giant Betz cells), anterior spinal motor neurons and brainstem motor neurons. ALS shows, from a clinical aspect, both upper motor neuron and lower motor neuron signs, and shows rapid clinical deterioration after onset of the disease, thus leading to death within a few years.

A small percentage (about 10%-15%) of ALS is inherited. Genetic epidemiology of ALS has revealed at least six chromosome locations accountable for the inheritance of disease (ALS1 to ALS6). Among these, three genes have been identified. The first gene identified was the cytosolic Cu/Zn superoxide dismutase (*SOD-1*) gene that accounts for 20% of the autosomal dominant form of ALS (Rosen et al., 1993, Nature, 1993 Mar. 4; 362(6415):59-62). The second was named as Alsin (*ALS2*), a potential guanine-nucleotide exchange factor (GEF) responsible for the juvenile recessive form of ALS. The third is *ALS4* that encodes for a DNA/RNA helicase domain containing protein called Senataxin, identified to be linked to the autosomal dominant form of juvenile ALS. Mutations in several other genes, including the *C9orf72*, *TARDBP*, *FUS*, *ANG*, *SETX*, and *VAPB* genes, have been associated with causing familial ALS and contributing to the development of sporadic ALS.

The only safe drug used for treating ALS is riluzole, which has an antagonistic effect against glutamate, and has been approved by the US Food and Drug Administration (FDA) for commercialization. However, this drug shows unsatisfactory efficacy, which means currently there is no definite therapy for ALS that can improve

rapidly deteriorating clinical conditions. Accordingly, therapeutic agents that target motor neurons would be beneficial for treating ALS and any other motor neuron specific disease.

SMA is an inherited disorder, caused by mutations in the survival of motor neuron 1 gene, *SMN1*. This disease preferentially affects lower motor neurons, while sparing upper motor neurons. There are many types of SMA which range in severity and onset of symptoms, with the most severe forms affecting patients neonatally resulting in death in just a couple of years. Other less severe forms of SMA compromise motor function leaving many patients wheelchair bound.

2.2 microRNAs

microRNAs are appreciated as important post-transcriptional modifiers of gene expression in a variety of mammalian biological contexts. microRNAs target the 3' UTR of mRNA transcripts via complementary binding of their 'seed sequence' in complex with Argonaute proteins, thereby inducing mRNA degradation and translational repression. Many groups have profiled microRNA expression in specific tissues, including the developing mammalian central nervous system (CNS). However, it is not well understood if specific cell types (such as developmentally defined neuronal subtypes) have unique microRNA expression patterns. Identifying a microRNA that exhibits motor neuron cell-type specificity and contributes dramatically to motor neuron identity may provide a means to express therapeutic and diagnostic agents specifically in motor neurons.

3. SUMMARY OF THE INVENTION

The present disclosure relates to a brainstem and spinal motor neuron-specific nucleic acid promoter sequence, compositions comprising said promoter sequence, and methods of using such compositions for treating a motor neuron disease in a subject in need thereof. The motor neuron-specific nucleic acid promoter sequence is able to express genes specifically in culture and in vivo. The present disclosure is based at least in part, on the discovery that the motor neuron-specific nucleic acid promoter sequence described herein by SEQ ID NO:1 was successful in expressing genes in motor neurons in vivo.

In certain non-limiting embodiments, the present invention provides for a nucleic acid sequence of SEQ ID NO:1. In one embodiment, the nucleic acid further

comprises one or more additional nucleic acids that is operably linked to the nucleic acid of SEQ ID NO:1.

In one embodiment, the one or more additional nucleic acid is selectively expressible in motor neurons.

In certain non-limiting embodiments, the present invention provides for a recombinant expression vector comprising a nucleic acid sequence of SEQ ID NO:1, wherein the vector further comprises one or more additional nucleic acids operably linked to the nucleic acid of SEQ ID NO:1, and wherein the exogenous nucleic acids are selectively expressible in a motor neuron.

In one embodiment, the nucleic acid sequence of SEQ ID NO:1 is a fragment of SEQ ID NO:1 that is sufficient to express a nucleic acid operatively linked to the fragment in a motor neuron.

In one embodiment, the nucleic acid operatively linked to the nucleic acid sequence of SEQ ID NO:1, or fragment thereof, comprises a miR-218 nucleic acid sequence, or a nucleic acid comprising a miR-218 seed sequence. In one embodiment, the nucleic acid operatively linked to the nucleic acid sequence of SEQ ID NO:1, or fragment thereof, comprises a nucleic acid encoding a transcription factor protein selected from the group consisting of Isl1, Isl2, Lhx3, Isl-Lhx3 fusion proteins, Phox2a, and a combination thereof. In one embodiment, the nucleic acid operatively linked to the nucleic acid sequence of SEQ ID NO:1, or fragment thereof, comprises a nucleic acid encoding an RNAi molecule, shRNA molecule, antisense RNA, catalytic RNA, or catalytic DNA specific for a miR-218 target nucleic acid, for example, a nucleic acid described by Figures 38 and 40.

The present disclosure also provides for compositions that can increase miR-218 expression, for example, miR-218 expression in a motor neuron, and methods of using such compositions for treating a motor neuron disease in a subject in need thereof. In certain embodiments, the composition comprises a miR-218 nucleic acid, or a nucleic acid comprising a miR-218 seed sequence. In certain embodiments, the composition comprises a transcription factor protein selected from the group consisting of Isl1, Isl2, Lhx3, Isl-Lhx3 fusion proteins, Phox2a, and a combination thereof, or one or more nucleic acid encoding said proteins.

The present disclosure also provides for compositions that can decrease expression of a miR-218 target nucleic acid, for example, a nucleic acid described by Figures 38 and 40. In certain embodiments, a miR-218 target nucleic acid is a nucleic

acid that hybridizes to a miR-218 seed sequence. In certain embodiments, the composition comprises an RNAi molecule, shRNA molecule, antisense RNA, catalytic RNA, catalytic DNA, or antibody specific for the miR-218 target nucleic acid, or protein encoded therefrom.

In one embodiment, the motor neuron is a spinal motor neuron. In one embodiment, the motor neuron is a brainstem motor neuron.

In another embodiment, the nucleic acids and/or expression vectors comprising the nucleic acids described herein, are comprised in a viral vector, for example, an adenovirus.

In certain non-limiting embodiments, the present disclosure provides for a method of introducing a nucleic acid or composition described herein into a motor neuron cell comprising administering, to the motor neuron cell, a composition or nucleic acid described herein, for example, a nucleic acid sequence of SEQ ID NO:1, or a vector comprising said nucleic acid, wherein the nucleic acid or expression vector further comprises an expressible nucleic acid.

In certain non-limiting embodiments, the present disclosure provides for a method for treating a subject diagnosed with or at risk of having a motor neuron disease, and/or reducing the severity of a motor neuron disease, comprising administering, to a subject in need of such treatment, an effective amount of a composition or nucleic acid described herein, for example, a nucleic acid sequence of SEQ ID NO:1, or a vector comprising said nucleic acid, wherein the nucleic acid or expression vector further comprises a therapeutic nucleic acid or gene.

In certain embodiments, the present disclosure provides for a cell *in vitro* comprising the nucleic acid of SEQ ID NO:1. In other embodiments, the disclosure provides for a transgenic animal comprising the nucleic acid of SEQ ID NO:1 operably linked to a nucleic acid or gene, for example in a recombinant vector. In certain embodiments, the nucleic acid or gene expresses a detectable protein, such as a fluorescent protein.

The present disclosure further provides for kits comprising one or more of the compositions or nucleic acids described herein, for example, a nucleic acid of SEQ ID NO:1, and/or a recombinant vector comprising the nucleic acid of SEQ ID NO:1. In certain embodiments, the kit further comprises instructions for administering the compositions or nucleic acids described herein for treating a subject diagnosed with or at risk for having a motor neuron disease.

The foregoing has outlined rather broadly the features and technical advantages of the present application in order that the detailed description that follows may be better understood. Additional features and advantages of the application will be described hereinafter which form the subject of the claims of the application. It should be appreciated by those skilled in the art that the conception and specific embodiment disclosed may be readily utilized as a basis for modifying or designing other structures for carrying out the same purposes of the present application. It should also be realized by those skilled in the art that such equivalent constructions do not depart from the spirit and scope of the application as set forth in the appended claims. The novel features which are believed to be characteristic of the application, both as to its organization and method of operation, together with further objects and advantages will be better understood from the following description.

4. BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the nucleic acid sequence of the motor neuron-specific promoter (SEQ ID NO:1) of the present disclosure.

Figure 2A-B shows brightfield **(A)** and fluorescence **(B)** images of an E11.5 transgenic mouse embryo expressing the 908 bp miR-218-promoter:eGFP transgene (218p:eGFP short). eGFP was expressed in both the cell bodies (arrow) and axons (bracket) of motor neurons in the spinal cord. eGFP was also expressed in brainstem motor nuclei of the embryos (arrowhead).

Figure 3A-B shows brightfield and fluorescence images of an E12.5 transgenic mouse embryo expressing the 7.6 kb miR-218 promoter:eGFP transgene (218p:eGFP long), or a homeobox gene (Hb9) promoter:GFP transgene (Hb9:GFP). eGFP was expressed in motor neurons in the spinal cord from both transgenes **(A, B)**. GFP expression from the 218p:eGFP long transgene was stronger than from the Hb9:GFP transgene (A).

Figure 4A-C shows brightfield (BF) and fluorescence (GFP) images of an E18.5 transgenic mouse embryo expressing the 7.6 kb miR-218 promoter:eGFP transgene (218p:eGFP long). eGFP was expressed in the brain and spinal cord of the mouse central nervous system **(A)**. **(B)** shows expression of the eGFP construct in motor neurons in the cervical, thoracic and lumbar regions of the spinal cord. **(C)** shows expression of the eGFP transgene in brainstem motor neurons (MNs).

Figure 5 shows expression of GFP in cross sections of spinal cord from P5 transgenic mice expressing the 7.6 kb miR-218 promoter:eGFP transgene (218p:eGFP) or a homeobox gene (Hb9) promoter:GFP transgene (HB9). Expression of GFP in motor neurons of 218p:eGFP transgenic mice was greater than in HB9 transgenic mice.

Figure 6 shows expression of GFP in cranial and spinal motor neurons in E18.5 transgenic mice expressing the 7.6 kb miR-218 promoter:eGFP transgene (pre-miR-218-2::eGFP) as compared to expression of miR-218 mRNA. miR-218 mRNA was detected by *in situ* hybridization.

Figure 7 shows expression of GFP in brainstem motor neuron nuclei in E18.5 transgenic mice expressing the 7.6 kb miR-218 promoter:eGFP transgene (218p::eGFP) or homeobox gene (Hb9) promoter:GFP transgene (Hb9), as compared to expression of miR-218 mRNA. miR-218 mRNA was detected by *in situ* hybridization. miR-218 mRNA and 218p::eGFP were expressed in N10 and N12 brainstem motor neuron nuclei, while Hb9 was expressed in N12 brainstem motor neuron nuclei.

Figure 8 shows expression of GFP in posterior motor neurons of transgenic *Xenopus laevis* swimming tadpoles. The transgenic animals expressed the *Xenopus laevis* homolog of the 7.6 kb miR-218 promoter operatively linked to GFP.

Figure 9 shows the differentiation of mouse embryonic stem cells (mESCs) expressing a homeobox gene (Hb9) promoter:GFP transgene (Hb9:GFP) into ES-derived motor neurons, wherein miR-218 was enriched in the motor neurons expressing Hb9:GFP.

Figure 10 shows miR-218 mRNA expression in lumbar spinal cord region of E11.5 mouse embryos; in lumbar and thoracic regions of spinal cord, as well as in brainstem, of E12.5 embryos; in lumbar spinal cord region of P0 mice; and in lumbar spinal cord region of adult mice.

Figure 11 shows miR-218 mRNA expression in motor neurons of E11.5 mice wherein the mRNA was expressed in motor neuron cells that migrated into the ventro-lateral spinal cord. Cells migrating into the ventro-lateral spinal cord were identified by expression of a homeobox gene (Hb9) promoter:GFP transgene (Hb9:GFP).

Figure 12 shows miR-218 mRNA expression in brainstem and spinal motor neurons in the P0 mouse central nervous system.

Figure 13 Shows that miR-218 is encoded within intron 14, and upstream of exon 6, of the Slit2 and Slit3 genes, and that miR-218 expression is driven by a promoter independently of Slit2/3 expression.

Figure 14 shows that miR-218 promoter expression is motor neuron specific.

Figure 15 shows that expression of miR-218 is more specific than Slit 2/3 in motor neurons of E12.5 mouse embryos. miR-218 was also expressed in motor neurons of Slit2$^{-/-}$ mice.

Figure 16 shows that Isl-Lhx3 and Isl-Phox2a transcription factors bind to HxREs (HOX responsive elements) located within the proximal region of both miR-218-1 and miR-218-2 promoters to drive miR-218 expression.

Figure 17 shows sequence alignment of the 7.6 kb and 908 bp miR-218 promoters from various vertebrate species.

Figure 18 shows the *in vivo* CRISPR-mediated micro-deletion of miR-218. miR-218 knockout (KO) mice and embryonic stem (ES) cells were generated.

Figure 19 shows that miR-218 knockout mice exhibited decreased viability and motor weakness.

Figure 20 shows that gene expression profiling of miR-218 KO motor neurons (E12.5) reveals de-repression of miR-218 predicted targets.

Figure 21 shows that fifty-five miR-218 predicted targets are statistically misregulated in both *in vivo* KO mice (E12.5 mouse embryos) and *in vitro* KO cells (ES-derived motor neurons).

Figure 22 shows that miR-218 indirectly activates genes involved in synaptic transmission.

Figure 23 shows that miR-218 KO motor neuron transcriptome shifts towards a generic ventral interneuron identity.

Figure 24 shows proposed inter-relationships of alternative miR-218 (also referred to as pri-miR-218) processing in motor neurons.

Figure 25 shows microRNA profiling of Hb9:GFP+ motor neurons by RNA-seq.

Figure 26A-H shows 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 27A-J shows Loss of miR-218 results in systemic neuromuscular failure, and motoneuron 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 28A-E shows 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 29A-C shows 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 (*24*) 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 30A-G shows miR-218 is abundantly and specifically expressed across motoneuron subtypes. **(A)** miRNA sequencing was performed on 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. No other miRNAs show comparable enrichment and abundance as miR-218 in motoneurons. **(B and C)** Sectioning and miR-218 *in situ* hybridization of the spinal cord (B) and brainstem (C) at E18.5. 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 Figure 1H. **(D)** miR-218 is expressed in both α- (NeuN+,ChAT+) and γ- (NeuN-,ChAT+, black 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 (arrows) 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 spinal cord in ventrolateral motoneurons.

Figure 31A-C shows Alternative, motoneuron-specific promoters drive transcription of miR-218-1 and miR- 218-2. **(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 *(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-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 (shown here at E18.5).

Figure 32A-H shows 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 clearly identifiable reduction of *in situ* hybridization signal intensity. (H) However, complete signal loss is only seen when all four miR-218 alleles (218^{DKO}) are genetically ablated.

Figure 33A-I shows Early motoneuron developmental stages are unaffected in 218^{DKO} mutants. **(A and B)** Representative sections of the thoracic (A) and lumbar (B) spinal cords of control and 218^{DKO} E12.5 embryos stained with transcription factor markers of motoneuron subpopulations. MMC (Lhx+,Hb9+), LMC (Lhx3-,Hb9+), MMCm (Lhx3+,Isl1/2-), and MMCl (Lhx3+,Isl1/2+) motoneurons were identified by immunolabelling in 20um cryosections across cervical, thoracic and lumbar segments. 218^{DKO} motoneuron cell bodies are positioned in the ventrolateral spinal cord indistinguishably from controls. dorsal root ganglion cells outside the spinal cord are Isl1/2+ (DRG). **(C-E)** Motoneurons in hemicords were manually counted in cryosections rostrocaudally spaced apart in 320um intervals across spinal segments. The number of each of these motoneuron subtypes is unaffected in 218^{DKO} embryos (n=4 and 3 animals, standard deviation is shown). **(F)** Average numbers of motoneurons of each identified subtype in 20um cryosections of control and 218^{DKO} hemi-spinal cords No significant change in motoneuron numbers is noted across each of these spinal cord regions at E12.5. **(G)** Hb9::gfp control and 218^{DKO} 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)** 218^{DKO} 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 miR-218.

Figure 34A-B shows 218DKO embryos are not viable and have defective motor axon innervation of muscle. **(A)** miR-218-1$^{-/-}$2$^{+/-}$ male and female mice were used for generating 218^{DKO} embryos. 218^{DKO} embryos were observed at Mendelian frequencies at E18.5, though all of these embryos showed no motor responses 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 branching within muscle. Axon bundle thickness is normal, but complexity of branching is significantly compromised across motor nerves in 218^{DKO} embryos. Boxed area denotes area of imaging of the deep peroneal nerve shown in (Fig. 27D).

Figure 35A-C shows neuromuscular junctions are abnormal in 218^{DKO} embryos. **(A-C)** Pre- (SYN+) and post-synaptic (AChR+) neuromuscular junction components were identified by synaptophysin antibody and α-bungarotoxin staining. (A) In E16.5 218^{DKO} 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 14.5 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) lack motor innervation.

Figure 36A-B shows reduced numbers of motoneurons in E18.5 218^{DKO} spinal cords. **(A)** Motoneurons were identified by Hb9 nuclear staining in control and 218^{DKO} E18.5 spinal cord sections from lumbar, cervical and thoracic regions at E18.5. Lumbar spinal cord staining is shown in (Fig. 27H). **(B)** Hb9^{+} motoneurons in 30uM cryosections of hemi-spinal cords were counted in 500um intervals across the rostrocaudal axis of cervical, thoracic, and lumbar regions. Significant reductions in motoneuron numbers were observed in 218^{DKO} spinal cords across all of these regions (n=4, standard deviation is shown).

Figure 37A-J shows 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)** I_h currents were slightly reduced in 218^{DKO} motoneurons. **(H and I)** Current ramps induced firing of 218^{DKO} 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 218^{DKO} embryos.

Figure 38A-G shows altered gene expression of miR-218 target genes in 218_{DKO} motoneurons. **(A)** NRPK gene expression in wild type versus 218^{DKO} motoneurons FACS-isolated from E12.5 spinal cords. Genes with robust miR-218 binding sites (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 6mer, 7mer, and 8mer 3'UTR complementary seed matches is shown. **(C-E)** Transcripts were ranked from upregulated to downregulated in 218^{DKO} 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 are specifically and significantly enriched for miR-218 binding sites. **(F)** Top ten biological process GO categories enriched in $TARGET^{218}$ genes as determined by the Gorilla platform. **(G)** Specific genes within the negative regulation of synaptic transport and neurotransmitter transport categories are listed.

Figure 39A-C shows FACS-isolated subpopulations specifically 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 transgenic neuronal progenitors by either FACS (pMN) or collecting whole neurospheres at day 4 of differentiation. **(B)** Representative FACS plots demonstrate clear 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, $Olig2^+$ progenitors were

captured before motoneurogenesis, as indicated by the expression of Olig2 but absence of Hb9, Isl1, and Isl2 expression.

Figure 40 shows the *TARGET*^{218} described by Example 2.

Figure 41 shows primer and sequence information for the primers described by Example 2.

Figure 42 shows that MicroRNA-218 (miR-218) expression is detected by in situ hybridization in adult human spinal cords from neurologically healthy controls. miR-218 specifically identifies motoneurons.

5. DETAILED DESCRIPTION OF THE INVENTION

The present disclosure relates to methods and compositions for treating and/or reducing the severity of a motor neuron disease. For purposes of clarity and not by way of limitation, the detailed description of the invention is divided into the following subsections:

(i) motor neuron-specific promoters;

(ii) therapeutic nucleic acids operably linked to a motor neuron-specific promoter;

(iii) therapeutic compositions;

(iv) pharmaceutical compositions;

(v) methods of use; and

(vi) kits.

The following are terms relevant to the present invention:

An "individual" or "subject" herein is a vertebrate, such as a human or non-human animal, for example, a mammal. Mammals include, but are not limited to, humans, primates, farm animals, sport animals, rodents and pets. Non-limiting examples of non-human animal subjects include rodents such as mice, rats, hamsters, and guinea pigs; rabbits; dogs; cats; sheep; pigs; goats; cattle; horses; and non-human primates such as apes and monkeys.

An "effective amount" of a substance as that term is used herein is that amount sufficient to effect beneficial or desired results, including clinical results, and, as such, an "effective amount" depends upon the context in which it is being applied. In the context of administering a composition to treat and/or reduce the severity of a motor neuron disease in a subject, an effective amount of a composition described herein is an

amount sufficient to treat and/or ameliorate a motor neuron disease, as well as decrease the severity and/or reduce the likelihood of a motor neuron disease. The decrease can be a 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98% or 99% decrease in severity of a motor neuron disease, or likelihood of developing a motor neuron disease. An effective amount can be administered in one or more administrations.

As used herein, and as well-understood in the art, "treatment" is an approach for obtaining beneficial or desired results, including clinical results. For purposes of this subject matter, beneficial or desired clinical results include, but are not limited to, alleviation or amelioration of one or more symptoms, diminishment of extent of disease, stabilized (i.e., not worsening) state of disease, prevention of disease, delay or slowing of disease progression, and/or amelioration or palliation of the disease state. The decrease can be a 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98% or 99% decrease in severity of complications or symptoms. "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment.

The term "expression vector" is used to denote a nucleic acid molecule that is either linear or circular, into which another nucleic acid sequence fragment of appropriate size can be integrated. Such nucleic acid fragment(s) can include additional segments that provide for transcription of a gene encoded by the nucleic acid sequence fragment. The additional segments can include and are not limited to: promoters, transcription terminators, enhancers, internal ribosome entry sites, untranslated regions, polyadenylation signals, selectable markers, origins of replication and such, as known in the art. Expression vectors are often derived from plasmids, cosmids, viral vectors and yeast artificial chromosomes; vectors are often recombinant molecules containing nucleic acid sequences from several sources.

The term "operably linked," when applied to nucleic acid sequences, for example in an expression vector, indicates that the sequences are arranged so that they function cooperatively in order to achieve their intended purposes, i.e., a promoter sequence allows for initiation of transcription that proceeds through a linked coding sequence as far as the termination signal.

A "nucleic acid molecule" is a single or double stranded covalently-linked sequence of nucleotides in which the 3' and 5' ends on each nucleotide are joined by phosphodiester bonds and/or non-phosphodiester bonds. In certain embodiments, the polynucleotide can be made up of deoxyribonucleotide bases, ribonucleotide bases or a combination of deoxyribonucleotide and ribonucleotide bases (for example a RNA:DNA

backbone hybrid). In certain embodiments, the polynucleotide can include other chemical moieties in addition to or in place of the deoxyribonucleotide and/or ribonucleotide bases. Polynucleotides include DNA and RNA, and can be manufactured synthetically in vitro or isolated from natural sources.

The term "promoter" as used herein denotes a region within a gene to which transcription factors and/or RNA polymerase can bind so as to control expression of an associated coding sequence. Promoters are commonly, but not always, located in the 5' non-coding regions of genes, upstream of the translation initiation codon. The promoter region of a gene can include one or more consensus sequences that act as recognizable binding sites for sequence specific nucleic acid binding domains of nucleic acid binding proteins. Nevertheless, such binding sites can also be located in regions outside of the promoter, for example in enhancer regions located in introns or downstream of the coding sequence.

A "regulatory gene" is a gene involved in controlling the expression of one or more other genes.

5.1 Motor neuron-specific promoters

The present disclosure provides for motor neuron-specific promoter nucleic acid sequences. In certain non-limiting embodiments the promoter specifically expresses a nucleic acid or gene operatively linked to the promoter in motor neurons. Thus, when said promoter, operatively linked to a gene in, for example, an expression vector, is injected or otherwise administered into a subject, the gene is expressed in motor neurons at much higher levels than it is expressed in other cells or tissues, such as, for example, liver, kidney, lung, or muscle.

In certain non-limiting embodiments, the promoter comprises the nucleic acid sequence:

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taaacgcccc aatttgctac ttatcaaata gtatacattt ttggctcaga aaaaaacctg  60
atgtctgtat attacttctc aactaaaatc cctcagtcct taactggcat gtgtattagt  120
caaggcatct ttgagaaggg cattatttcc ctacacttag gatggggaaa gagaaattaa  180
aaaggaatcc taaaataggt gcatttaatt ctccccaatt taaatgtaag tggtgcgtct  240
tttaggcaat aatgatatgc cttttagtcc tccattacaa acacttccat cgatgaattt  300
ccttaatgtt gatgatggtt agtgcagttt gagggaatct gtatttattc agaaaatgtt  360
cccatagaat gacctaccag atgggccacg taacaatgca tggagacatc aaaccaccac  420
agacatttgg tgcttagaat aataaaaaga ctataaaatt agattagttg agtctaattt  480
ggaattggta tattccctac gcaccctcac cgctcttggg cagataaagc cttgagattt  540
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agcgctgtgt caaagccaag actgtaactt ccagtaaaag ggagccgagg gaggggggagc  600
ttgctgggag gtcgcggagg gcagagcagt gacctccaat gatttacagg cctttagctt  660
aatgaaattg tttcagtgac atgacagtaa gagctcgtaa tggattggat gccctaatgt  720
aatgaaatta ctcccttctg cctaaaaaaa aaaaaatgcg caattaatat ttactgagac  780
ctgacagcct ttggtgcgct cgctcgcctg tgtagttccc tcagacagtc agagagaaga  840
gacagagcag cgtggcagac aggcgggctc tgcaggagct cctggcaggg acaagcagag  900
cctgcaag                                                          908
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(SEQ ID NO:1).

In a certain non-limiting embodiment, the promoter comprises the nucleic acid sequence of SEQ ID NO:1, or a nucleic acid having a sequence that is at least 60, 65, 70, 75, 80, 85, 90, 95, or at least 99 percent homologous thereto (where homology may be determined using standard software such as BLAST or FASTA).

In certain non-limiting embodiments, the promoter comprises a nucleic acid of SEQ ID NO:1 in which 1, 2, 3, 4, 5 or more nucleotides are either deleted, substituted by another nucleotide, or in which 1, 2, 3, 4, 5 or more nucleotides are added. In other embodiments, the nucleic acid of SEQ ID NO:1 comprises a conservative nucleotide substitution such that the promoter maintains its ability to express a nucleic acid operably linked to the promoter in motor neurons.

In certain non-limiting embodiments, the promoter comprises a nucleic acid of SEQ ID NO:1, or a fragment thereof, wherein the length of the nucleic acid is 10 kilobases (kb) or less, 9.5 kb or less, 9 kb or less, 8.5 kb or less, 8 kb or less, 7.5 kb or less, 7 kb or less, 6.5 kb or less, 6 kb or less, 5.5 kb or less, 5 kb or less, 4.5 kb or less, 4 kb or less, 3.5 kb or less, 3 kb or less, 2.5 kb or less, 2 kb or less, 1.5 kb or less, 1 kb or less, 500 base pairs (bp) or less, 400 bp or less, 300 bp or less, 200 bp or less, 100 bp or less, 50 bp or less, or 20 bp or less.

In certain non-limiting embodiments, the promoter comprises a nucleic acid of SEQ ID NO:1, or a fragment thereof, wherein the nucleic acid is not adjacent to another nucleic acid sequence present in an endogenous miR-218 promoter, or an endogenous Slit2 or Slit3 promoter. In certain embodiments, said nucleic acid sequence of the endogenous miR-218, Slit2 or Slit3 promoter is a nucleic acid having a length of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more nucleotide base pairs.

In certain embodiments, the miR-218 is a mouse miR-218 described by GenBank Accession No. NR_029798 or NR_029799, or a nucleic acid having a sequence that is at least 60, 65, 70, 75, 80, 85, 90, 95, or at least 99 percent homologous thereto

(where homology may be determined using standard software such as BLAST or FASTA).

In certain embodiments, the miR-218 is a human miR-218 described by GenBank Accession No. NR_029632 or NR_029631, or a nucleic acid having a sequence that is at least 60, 65, 70, 75, 80, 85, 90, 95, or at least 99 percent homologous thereto (where homology may be determined using standard software such as BLAST or FASTA).

In certain embodiments, the Slit2 is a mouse Slit2 described by GenBank Accession No. NM_001291227, NM_001291228, NM_178804, NR_111900, or NC_000071, or a nucleic acid having a sequence that is at least 60, 65, 70, 75, 80, 85, 90, 95, or at least 99 percent homologous thereto (where homology may be determined using standard software such as BLAST or FASTA).

In certain embodiments, the Slit2 is a human Slit2 described by GenBank Accession No. NM_001289135, NM_001289136, NM_004787, or NC_000004, or a nucleic acid having a sequence that is at least 60, 65, 70, 75, 80, 85, 90, 95, or at least 99 percent homologous thereto (where homology may be determined using standard software such as BLAST or FASTA).

In certain embodiments, the Slit3 is a mouse Slit3 described by GenBank Accession No. NM_011412 or NC_000077, or a nucleic acid having a sequence that is at least 60, 65, 70, 75, 80, 85, 90, 95, or at least 99 percent homologous thereto (where homology may be determined using standard software such as BLAST or FASTA).

In certain embodiments, the Slit3 is a human Slit3 described by GenBank Accession No. NM_001271946, NM_003062, NG_033081, or NC_000005, or a nucleic acid having a sequence that is at least 60, 65, 70, 75, 80, 85, 90, 95, or at least 99 percent homologous thereto (where homology may be determined using standard software such as BLAST or FASTA).

In certain non-limiting embodiments, the promoter comprises one or more nucleic acid sequence, or fragment thereof, described in Table 1.

Table 1. Nucleic acid promoter sequences

taaacgcccc aatttgctac (SEQ ID N0:2)	agattagttg agtctaattt (SEQ ID N0:25)
ttatcaaata gtatacattt (SEQ ID N0:3)	ggaattggta tattccctac (SEQ ID N0:26)
ttggctcaga aaaaaacctg (SEQ ID N0:4)	gcaccctcac cgctcttggg (SEQ ID N0:27)

atgtctgtat attacttctc (SEQ ID N0:5)	cagataaagc cttgagattt (SEQ ID N0:28)
aactaaaatc cctcagtcct (SEQ ID N0:6)	agcgctgtgt caaagccaag (SEQ ID N0:29)
taactggcat gtgtattagt (SEQ ID N0:7)	actgtaactt ccagtaaaag (SEQ ID N0:30)
caaggcatct ttgagaaggg (SEQ ID N0:8)	ggagccgagg gaggggggagc (SEQ ID N0:31)
cattatttcc ctacacttag (SEQ ID N0:9)	ttgctgggag gtcgcggagg (SEQ ID N0:32)
gatggggaaa gagaaattaa (SEQ ID N0:10)	gcagagcagt gacctccaat (SEQ ID N0:33)
aaaggaatcc taaaataggt (SEQ ID N0:11)	gatttacagg cctttagctt (SEQ ID N0:34)
gcatttaatt ctccccaatt (SEQ ID N0:12)	aatgaaattg tttcagtgac (SEQ ID N0:35)
taaatgtaag tggtgcgtct (SEQ ID N0:13)	atgacagtaa gagctcgtaa (SEQ ID N0:36)
tttaggcaat aatgatatgc (SEQ ID N0:14)	tggattggat gccctaatgt (SEQ ID N0:37)
cttttagtcc tccattacaa (SEQ ID N0:15)	aatgaaatta ctcccttctg (SEQ ID N0:38)
acacttccat cgatgaattt (SEQ ID N0:16)	cctaaaaaaa aaaaaatgcg (SEQ ID N0:39)
ccttaatgtt gatgatggtt (SEQ ID N0:17)	caattaatat ttactgagac (SEQ ID N0:40)
agtgcagttt gagggaatct (SEQ ID N0:18)	ctgacagcct ttggtgcgct (SEQ ID N0:41)
gtatttattc agaaaatgtt (SEQ ID N0:19)	cgctcgcctg tgtagttccc (SEQ ID N0:42)
cccatagaat gacctaccag (SEQ ID N0:20)	tcagacagtc agagagaaga (SEQ ID N0:43)
atgggccacg taacaatgca (SEQ ID N0:21)	gacagagcag cgtggcagac (SEQ ID N0:44)
tggagacatc aaaccaccac (SEQ ID N0:22)	aggcgggctc tgcaggagct (SEQ ID N0:45)
agacatttgg tgcttagaat (SEQ ID N0:23)	cctggcaggg acaagcagag (SEQ ID N0:46)
aataaaaaga ctataaaatt (SEQ ID N0:24)	cctgcaag (SEQ ID N0:47)

In one embodiment, the promoter specifically expresses an operatively linked nucleic acid or gene in a brainstem and/or spinal motor neuron. Such motor neurons include, for example, motor neurons having an axon that exits the central nervous system.

In certain embodiments, the motor neuron is a sympathetic and/or parasympathetic motor neuron.

In other embodiments, the promoter specifically expresses the operatively linked nucleic acid or gene in a cranial motor neuron.

In certain embodiments, the motor neuron is a lower motor neuron, for example, an efferent neuron that innervates a muscle.

In certain embodiments, the motor neuron is a somatic motor neuron that innervates skeletal muscle, for example, muscles of the limbs, abdomen, and intercostal muscles.

In other embodiments, the motor neuron is a visceral motor neuron, for example, motor neurons that innervate the branchial muscles of the head and neck, or motor neurons that innervate cardiac muscle and smooth muscles of the viscera, or that synapse onto neurons of the autonomic nervous system.

In certain embodiments, compositions disclosed herein include a promoter nucleic acid sequence, wherein said nucleic acid sequence is part of an expression vector, wherein the expression vector further comprises a second nucleic acid that is expressed in a host cell, for example in a motor neuron cell.

In certain embodiments, the compositions described herein are administered to treat or prevent a motor neuron disease, by way of gene therapy. Gene therapy refers to therapy performed by the administration to a subject of an expressed or expressible nucleic acid.

Any of the methods for gene therapy available in the art can be used according to the present disclosure. Exemplary methods are described below. For general reviews of the methods of gene therapy, see Kron and Kreppel, Curr Gene Ther 12(5):362-73 (2012); Yi *et al.* Curr Gene Ther 11(3):218-28 (2011); Goldspiel *et al.*, Clinical Pharmacy 12:488-505 (1993); Wu and Wu, Biotherapy 3:87-95 (1991); Tolstoshev, Ann. Rev. Pharmacol. Toxicol. 32:573-596 (1993); Mulligan, Science 260:926-932 (1993); Morgan and Anderson, Ann. Rev. Biochem. 62:191-217 (1993); and May, TIBTECH 11(5):155-215 (1993). Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel et al. (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, NY (1993); and Kriegler, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY (1990).

Delivery of nucleic acid into a subject or cell, e.g., in an *in vitro* cell culture, can be either direct, in which case the subject or cell is directly exposed to the

nucleic acid or nucleic acid-carrying vectors, or indirect, in which case cells are first transformed with the nucleic acids *in vitro*, then transplanted into the subject.

In certain embodiments, the nucleic acid may be directly administered *in vivo*, where it is expressed to produce the encoded product. This can be accomplished by any of numerous methods known in the art, *e.g.*, by constructing them as part of an appropriate nucleic acid expression vector and administering it so that they become intracellular, *e.g.*, by infection using defective or attenuated retroviral or other viral vectors (see U.S. Pat. No. 4,980,286), or by direct injection of naked DNA, or by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, encapsulation in liposomes, microparticles, or microcapsules, or by administering them in linkage to a peptide which is known to enter the nucleus, by administering it in linkage to a ligand subject to receptor-mediated endocytosis (*see, e.g.*, Wu and Wu, *J. Biol. Chem.* (1987);262:4429-4432). The nucleic acid-ligand complexes can also be formed in which the ligand includes a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In addition, the nucleic acid can be targeted *in vivo* for cell specific uptake and expression, by targeting a specific receptor (see, *e.g.*, PCT Publications WO 92/06180; WO 92/22635; WO92/20316; WO93/14188, WO 93/20221). Alternatively, the nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination (Koller and Smithies, Proc. Natl. Acad. Sci. USA (1989);86:8932-8935; Zijlstra *et al.*, Nature (1989);342:435-438).

In certain embodiments, a viral vector that contains a promoter nucleic acid described herein operatively linked to an expressible nucleic acid or gene can be used. For example, a retroviral vector can be used (*see* Miller *et al.*, Meth. Enzymol. (1993);217:581-599). These retroviral vectors contain the components necessary for the correct packaging of the viral genome and integration into the host cell DNA. More detail about retroviral vectors can be found in Boesen *et al.*, Biotherapy (1994);6:291-302. Other references illustrating the use of retroviral vectors in gene therapy are: Anson, Genet Vaccines Ther 13;2(1):9 (2004); Clowes *et al.*, *J. Clin. Invest.* (1994);93:644-651; Kiem *et al.*, Blood (1994);83:1467-1473; Salmons and Gunzberg, Human Gene Therapy (1993);4:129-141; and Grossman and Wilson, Curr. Opin. in Genetics and Devel. (1993);3:110-114.

In certain embodiments, the vehicle for delivering the nucleic acids of the present disclosure is an adenovirus. Adenoviruses have the advantage of being capable of

infecting non-dividing cells. Kron and Kreppel, Curr Gene Ther 12(5):362-73 (2012) and Kozarsky and Wilson, Current Opinion in Genetics and Development 3:499-503 (1993) present a review of adenovirus-based gene therapy. Bout *et al.*, Human Gene Therapy 5:3-10 (1994) demonstrated the use of adenovirus vectors to transfer genes to the respiratory epithelia of rhesus monkeys. Other instances of the use of adenoviruses in gene therapy can be found in Rosenfeld *et al.*, Science 252:431-434 (1991); Rosenfeld *et al.*, Cell 68:143-155 (1992); Mastrangeli et al., J. Clin. Invest. 91:225-234 (1993); PCT Publication WO94/12649; and Wang, *et al.*, Gene Therapy 2:775-783 (1995).

In certain embodiments, an adeno-associated virus (AAV) can be used (Zhong et al. J Genet Syndr Gene Ther. 2012 Jan 10;S1. pii: 008; High, KA, Blood, 120(23):4482-7 (2012); Walsh *et al.*, Proc. Soc. Exp. Biol. Med. 204:289-300 (1993); U.S. Pat. No. 5,436,146). In certain embodiments, AAV vectors are used. Vectors that can be used in gene therapy are discussed in detail below.

Recombinant cells can also be used in the methods of treatment described herein, where nucleic acid sequences encoding a promoter nucleic acid operatively linked to an expressible nucleic acid or gene, are introduced into the cells such that they are expressible by the cells or their progeny, and the recombinant cells are then administered *in vivo* for therapeutic effect.

In certain non-limiting examples, the methods of the present invention involve transferring a nucleic acid of the present disclosure, for example an expression vector comprising a promoter sequence as described herein and one or more expressible nucleic acids, to a host cell in a cell culture by methods such as transfection, electroporation, microinjection, infection with a viral or bacteriophage vector containing the nucleic acid sequences, lipofection, calcium phosphate mediated transfection, cell fusion, chromosome-mediated gene transfer, microcell-mediated gene transfer, spheroplast fusion, etc. Usually, the method of transfer includes the transfer of a selectable marker to the host cells. The cells are then placed under selection to isolate those host cells that have taken up and are expressing the transferred gene. Those host cells are then delivered to a patient. Numerous techniques are known in the art for the introduction of foreign genes into cells (see, *e.g.*, Loeffler and Behr, Meth. Enzymol. 217:599-618 (1993); Cohen *et al.*, Meth. Enzymol. 217:618-644 (1993); Cline, Pharmac. Ther. 29:69-92m (1985)), and can be used in accordance with the present disclosure, provided that the necessary developmental and physiological functions of the recipient cells are not disrupted.

In certain embodiments, the nucleic acid can be introduced into motor neuron cells and/or motor neuron stem or progenitor cells, and/or embryonic stem cells (ESCs), and/or induced pluripotent stem cells (iPSCs), prior to administration *in vivo* of the resulting recombinant cell by any method known in the art. Any stem and/or progenitor cells which can be isolated and maintained *in vitro* can be used (see *e.g.* PCT Publication WO 94/08598; Porada and Porada, J. Genet Syndr Gene Ther., May 25;S1. p11:011 (2012); Stemple and Anderson, Cell 71:973-985 (1992); Rheinwald, Meth. Cell Bio. 21A:229 (1980); and Pittelkow and Scott, Mayo Clinic Proc. 61:771 (1986)).

The resulting recombinant cells can be delivered to a patient by various methods known in the art. The amount of cells envisioned for use depends on the desired effect, patient state, etc., and can be determined by one skilled in the art.

In certain embodiments, the terms "vector" and "expression vector" mean the vehicle by which a nucleic acid such as a DNA or RNA sequence (e.g., a foreign gene) can be introduced into a host cell, so as to transform the host and promote expression (e.g., transcription and translation) of the introduced sequence. Vectors include plasmids, phages, viruses, etc.; they are discussed in greater detail below. A "therapeutic vector" as used herein refers to a vector which is acceptable for administration to an animal, and particularly to a human.

Vectors typically include the DNA of a transmissible agent, into which foreign DNA is inserted. A common way to insert one segment of DNA into another segment of DNA involves the use of enzymes called restriction enzymes that cleave DNA at specific sites (specific groups of nucleotides) called restriction sites. Generally, foreign DNA is inserted at one or more restriction sites of the vector DNA, and then is carried by the vector into a host cell along with the transmissible vector DNA. A segment or sequence of DNA having inserted or added DNA, such as an expression vector, can also be called a "DNA construct." A common type of vector is a "plasmid", which generally is a self-contained molecule of double-stranded DNA, usually of bacterial origin, that can readily accept additional (foreign) DNA and which can readily introduced into a suitable host cell. A plasmid vector often contains coding DNA and promoter DNA and has one or more restriction sites suitable for inserting foreign DNA. Coding DNA is a DNA sequence that encodes a particular amino acid sequence for a particular protein or enzyme. Promoter DNA is a DNA sequence which initiates, regulates, or otherwise mediates or controls the expression of the coding DNA. Promoter DNA and coding DNA can be from the same or different organisms. In certain embodiments the promoter

comprises a nucleic acid comprising a sequence of SEQ ID NO:1 as described herein, wherein the promoter is included on an expression vector with an expressible nucleic acid or gene, wherein the expressible nucleic acid or gene is specifically expressed in a motor neuron cell.

A large number of vectors, including plasmid and fungal vectors, have been described for replication and/or expression in a variety of eukaryotic and prokaryotic hosts. Non-limiting examples include pKK plasmids (Clonetech), pUC plasmids, pET plasmids (Novagen, Inc., Madison, Wis.), pRSET plasmids (Invitrogen, San Diego, Calif.), pCDNA3 plasmids (Invitrogen), pREP plasmids (Invitrogen), or pMAL plasmids (New England Biolabs, Beverly, Mass.), and many appropriate host cells, using methods disclosed or cited herein or otherwise known to those skilled in the relevant art. Recombinant cloning vectors will often include one or more replication systems for cloning or expression, one or more markers for selection in the host, e.g., antibiotic resistance, and one or more expression cassettes.

Suitable vectors include viruses, such as adenoviruses, adeno-associated virus (AAV), vaccinia, herpesviruses, baculoviruses, retroviruses, parvovirus, lentivirus, bacteriophages, cosmids, plasmids, fungal vectors, naked DNA, DNA lipid complexes, and other recombination vehicles typically used in the art which have been described for expression in a variety of eukaryotic and prokaryotic hosts, and can be used for gene therapy as well as for simple protein expression.

Viral vectors, especially adenoviral vectors can be complexed with a cationic amphiphile, such as a cationic lipid, polyL-lysine (PLL), and diethylaminoethyldextran (DELAE-dextran), which provide increased efficiency of viral infection of target cells (See, *e.g.,* PCT/US97/21496 filed Nov. 20, 1997, incorporated herein by reference). AAV vectors, such as those disclosed in Zhong et al., *J. Genet Syndr Gene Therapy* 2012 Jan 10;S1. pii: 008, U.S. Pat. Nos. 5,139,941, 5,252,479 and 5,753,500 and PCT publication WO 97/09441, the disclosures of which are incorporated herein, are also useful since these vectors integrate into host chromosomes, with a minimal need for repeat administration of vector. For a review of viral vectors in gene therapy, see McConnell et al., 2004, Hum Gene Ther. 15(11):1022-33; Mccarty et al., 2004, Annu Rev Genet. 38:819-45; Mah et al., 2002, Clin. Pharmacokinet. 41(12):901-11; Scott *et al.*, 2002, Neuromuscul. Disord. 12(Suppl 1):S23-9.

In certain non-limiting embodiments, the nucleic acids described herein, for example, a motor neuron specific promoter that is operatively liked to a therapeutic

nucleic acid, can be integrated into a host cell chromosome or nucleic acid, for example, by using transcription activator-like effector nucleases (TALENs), Zinc-finger nucleases (ZFNs), clustered regularly interspaced short palindromic repeats (CRISPRs, *see, e.g.,* Jinek et al., *Science* (2012);337:816-821), or transgene expression (*e.g.*, using a natural or chemically modified DNA or RNA).

5.2 Therapeutic nucleic acids operably linked to a motor neuron-specific promoter

According to the present disclosure, in certain embodiments, the promoter nucleic acids described herein are operably linked with an expressible nucleic acid, such as a gene, wherein the expressible nucleic acid is selectively expressed in a motor neuron. The promoter and expressible nucleic acid can be comprised in an expression vector, as described herein.

The expressible nucleic acid may encode a protein, micro RNA (miRNA), interfering RNA (RNAi) molecule, shRNA molecule, antisense RNA, catalytic RNA, or catalytic DNA. According to an embodiment of the present disclosure, the expressible nucleic acid provides a therapeutic effect when expressed in a motor neuron of a subject diagnosed with or at risk of having a motor neuron disease. In certain non-limiting embodiments, the expressible nucleic acid does not comprise a nucleic acid encoding a miR-218 micro RNA.

In certain embodiments, the expressible nucleic acid comprises DNA. In other embodiments, the expressible nucleic acid comprises RNA.

In certain non-limiting embodiments, the expressible nucleic acid encodes a protein. In certain non-limiting embodiments, the protein is selected from the group consisting of a cytokine, a growth factor, an enzyme, an ion channel, an anti-inflammatory peptide, and an antioxidant.

Non-limiting examples of expressible nucleic acids that can be operatively linked to the promoter nucleic acid sequences described herein include neurotrophic factors such as brain-derived neurotrophic factor (*BDNF*), glial cell-derived neurotrophic factor (*GDNF*), and/or neurotrophic factors (*NTF*) such as neurotrophins.

In certain embodiments, the compositions described herein can be used to replace the function of mutated proteins expressed by a subject. For example, patients with SMA have low levels of Survival of Motor Neuron (*SMN*) protein. A nucleic acid encoding a functional SMN protein can be operably linked to the promoter nucleic acids described herein such that functional protein is expressed in motor neurons of the subject.

Other functional proteins that can be expressed using the compositions described herein include proteins encoded by the genes superoxide dismutase 1 (*SOD1*); alsin (amyotrophic lateral sclerosis 2 (juvenile); *ALS2*); amyotrophic lateral sclerosis 4 (*ALS4*); angiogenin, ribonuclease, RNase A family, 5 (*ANG*); chromosome 9 open reading frame 72 (*C9orf72*); FUS RNA binding protein (*FUS*); senataxin (*SETX*); TAR DNA binding protein (*TARDBP*); VAMP (vesicle-associated membrane protein)-associated protein B and C (*VAPN*); dynactin 1 (*DCTN1*); neurofilament, heavy polypeptide (*NEFH*); and/or peripherin (*PRPH*).

In certain embodiments, the nucleic acid operably linked to the promoter nucleic acids described herein can be a regulatory non-coding RNA gene such as a microRNA, for example, miR-218, or a nucleic acid comprising a miR-218 seed sequence, for example, a 6mer, 7mer, or 8mer miR-218 seed sequence selected from the group consisting of 5' UUGUGCUU 3'; 5' UGUGCUU 3'; 5' UUGUGCU 3'; 5' UGUGCU 3'; 5' UUGUGC 3'; 5' GUGCUU 3'; 5' TTGTGCTT 3'; 5' TGTGCTT 3'; 5' TTGTGCT 3'; 5' TGTGCT 3'; 5' TTGTGC 3'; and 5' GTGCTT 3.

In certain embodiments, miR-218 comprises the nucleic acid sequence 5' UUGUGCUUGAUCUAACCAUGU 3'.

In certain embodiments, miR-218 comprises the nucleic acid sequence 5' UUGUGCUUGAUCUAACCAUGUG 3'.

In certain embodiments, miR-218 comprises the nucleic acid sequence 5' TTGTGCTTGATCTAACCATGT 3'.

In certain embodiments, miR-218 comprises the nucleic acid sequence 5' TTGTGCTTGATCTAACCATGTG 3'.

In certain embodiments, miR-218 comprises the nucleic acid sequence 5' GACCAGTCGC TGCGGGGCTT TCCTTTGTGC TTGATCTAAC CATGTGGTGG AACGATGGAA ACGGAACATG GTTCTGTCAA GCACCGCGGA AAGCACCGTG CTCTCCTGCA 3'.

In certain embodiments, miR-218 comprises the nucleic acid sequence 5' GACCAGUCGC UGCGGGGCUU UCCUUUGUGC UUGAUCUAAC CAUGUGGUGG AACGAUGGAA ACGGAACAUG GUUCUGUCAA GCACCGCGGA AAGCACCGUG CUCUCCUGCA 3'.

In certain embodiments, miR-218 comprises the nucleic acid sequence 5' GTGATAATGT AGCGAGATTT TCTGTTGTGC TTGATCTAAC CATGTGGTTG

CGAGGTATGA GTAAAACATG GTTCCGTCAA GCACCATGGA ACGTCACGCA GCTTTCTACA 3'.

In certain embodiments, miR-218 comprises the nucleic acid sequence 5' GUGAUAAUGU AGCGAGAUUU UCUGUUGUGC UUGAUCUAAC CAUGUGGUUG CGAGGUAUGA GUAAAACAUG GUUCCGUCAA GCACCAUGGA ACGUCACGCA GCUUUCUACA 3'.

In certain embodiments, miR-218 comprises a sequence that is complimentary to one or more nucleic acid sequences described herein.

In certain embodiments, the expressible nucleic acid operably linked to the promoter nucleic acids described herein encodes a protein that increases miR-218 expression level in motor neurons. In certain embodiments, the expressible nucleic acid encodes a transcription factor protein, such as but not limited to, a transcription factor selected from the group consisting of Isl1, Isl2, Lhx3, Isl-Lhx3 fusion proteins, Phox2a, and a combination thereof.

In certain embodiments, the expressible nucleic acid operably linked to the promoter nucleic acids described herein encodes a compound that inhibits or reduces expression of a miR-218 target nucleic acid. Such a compound can be, for example, an RNAi molecule, shRNA molecule, antisense RNA, catalytic RNA, catalytic DNA, protein, or antibody or fragment thereof, specific for a miR-218 target nucleic acid, or protein encoded therefrom. In certain embodiments, a miR-218 target nucleic acid is a nucleic acid that hybridizes to a miR-218 seed sequence. In certain embodiments, the miR-218 target nucleic acid is selected from the genes described by Figures 38 and 40. In certain embodiments, the miR-218 target nucleic acid comprises a nucleic acid sequence selected from the group consisting of 5'AAGCACAA 3'; 5' AAGCACA 3'; 5' AGCACAA 3'; 5' AGCACA 3'; 5' GCACAA 3'; and 5' AAGCAC 3'.

In one set of non-limiting examples, the expressible nucleic acid is an apoptosis inhibitor, such as one of the so-called inhibitors of apoptosis ("IAPs"), for example, the human IAPs c-IAP1, c-IAP2, and XIAP.

In certain non-limiting embodiments, the expressible nucleic acid is a detectable DNA, RNA or protein. Non-limiting examples include fluorescent proteins such as green fluorescent protein (GFP), blue fluorescent protein (EBFP, EBFP2, Azurite, mKalama1), cyan fluorescent protein (ECFP, Cerulean, CyPet, mTurquoise2), and yellow fluorescent protein derivatives (YFP, Citrine, Venus, YPet); β-galactosidase (LacZ);

chloramphenicol acetyltransferase (cat); neomycin phosphotransferase (neo); enzymes such as oxidases and peroxidases; antibodies; and/or antigenic molecules.

In certain embodiments, the expressible nucleic acid can be used for analysis of motor neuron viability.

5

5.3 Therapeutic compositions

The present disclosure provides for compositions that can increase the activity or level of miR-218 in a cell, for example, a motor neuron. In certain embodiments, the compositions described by the present disclosure can decrease the expression level of a miR-218 target nucleic acid, or the expression level or activity of a protein encoded by a miR-218 target nucleic acid.

In certain embodiments, the composition comprises one or more expressible nucleic acids operably linked to a motor neuron-specific promoter, as described herein.

In certain embodiments, the composition comprises a mir-218 nucleic acid, or a nucleic acid comprising a miR-218 seed sequence, for example, a 6mer, 7mer, or 8mer miR-218 seed sequence selected from the group consisting of 5' UUGUGCUU 3'; 5' UGUGCUU 3'; 5' UUGUGCU 3'; 5' UGUGCU 3'; 5' UUGUGC 3'; 5' GUGCUU 3'; 5' TTGTGCTT 3'; 5' TGTGCTT 3'; 5' TTGTGCT 3'; 5' TGTGCT 3'; 5' TTGTGC 3'; and 5' GTGCTT 3'.

In certain embodiments, the composition comprises a mir-218 nucleic acid comprising the nucleic acid sequence 5' UUGUGCUUGAUCUAACCAUGU 3'.

In certain embodiments, the composition comprises a mir-218 nucleic acid comprising the nucleic acid sequence 5' UUGUGCUUGAUCUAACCAUGUG 3'.

In certain embodiments, the composition comprises a mir-218 nucleic acid comprising the nucleic acid sequence 5' TTGTGCTTGATCTAACCATGT 3'.

In certain embodiments, the composition comprises a mir-218 nucleic acid comprising the nucleic acid sequence 5' TTGTGCTTGATCTAACCATGTG 3'.

In certain embodiments, miR-218 comprises the nucleic acid sequence 5' GACCAGTCGC TGCGGGGCTT TCCTTTGTGC TTGATCTAAC CATGTGGTGG AACGATGGAA ACGGAACATG GTTCTGTCAA GCACCGCGGA AAGCACCGTG CTCTCCTGCA 3'.

In certain embodiments, miR-218 comprises the nucleic acid sequence 5' GACCAGUCGC UGCGGGGCUU UCCUUUGUGC UUGAUCUAAC

CAUGUGGUGG AACGAUGGAA ACGGAACAUG GUUCUGUCAA GCACCGCGGA AAGCACCGUG CUCUCCUGCA 3'.

In certain embodiments, miR-218 comprises the nucleic acid sequence 5' GTGATAATGT AGCGAGATTT TCTGTTGTGC TTGATCTAAC CATGTGGTTG CGAGGTATGA GTAAAACATG GTTCCGTCAA GCACCATGGA ACGTCACGCA GCTTTCTACA 3'.

In certain embodiments, miR-218 comprises the nucleic acid sequence 5' GUGAUAAUGU AGCGAGAUUU UCUGUUGUGC UUGAUCUAAC CAUGUGGUUG CGAGGUAUGA GUAAAACAUG GUUCCGUCAA GCACCAUGGA ACGUCACGCA GCUUUCUACA 3'.

In certain embodiments, miR-218 comprises a sequence that is complimentary to one or more nucleic acid sequences described herein.

In certain embodiments, the composition comprises a nucleic acid encoding a protein that increases miR-218 expression level in motor neurons, for example, a transcription factor protein, such as but not limited to, a transcription factor selected from the group consisting of Isl1, Isl2, Lhx3, Isl-Lhx3 fusion proteins, Phox2a, and a combination thereof. In certain embodiments, the nucleic acid is operably linked to a promoter and is constitutively expressed. In certain embodiments, expression of the nucleic acid is inducible or transient.

In certain embodiments, the composition comprises a transcription factor protein selected from the group consisting of Isl1, Isl2, Lhx3, Isl-Lhx3 fusion proteins, Phox2a, and a combination thereof.

In certain embodiments, the composition can decrease the expression level of a miR-218 target nucleic acid, or the expression level or activity of a protein encoded by a miR-218 target nucleic acid. In certain embodiments, a miR-218 target nucleic acid is a nucleic acid that hybridizes to a miR-218 seed sequence, as described herein. In certain embodiments, the composition comprises an RNAi molecule, shRNA molecule, antisense RNA, catalytic RNA, catalytic DNA, protein, or antibody specific for the miR-218 target nucleic acid, or protein encoded therefrom.

In certain embodiments, the miR-218 target nucleic acid comprises a nucleic acid encoding a gene that is a negative regulator of synaptic transmission or neurotransmitter transport. In certain embodiments, the miR-218 target nucleic acid comprises a nucleic acid encoding a gene that is selected from the genes described by Figures 38 and 40. In certain embodiments, the miR-218 target nucleic acid comprises a

nucleic acid encoding a gene selected from the group consisting of Slc1a2/GLT-1, Shank2, Grik2, Gnai2, Stxbp1, Park2, Slc6a1, Celf4, Syt13, Stx3, Sv2a, Nrxn3, Slc6a17, and combinations thereof.

In certain embodiments, the composition can be comprised in a vector as described herein.

In certain embodiments, the present disclosure provides for kits comprising one or more of the compositions and/or nucleic acids described herein.

5.4 Pharmaceutical compositions

In certain embodiments, the present disclosure provides for pharmaceutical compositions comprising the therapeutic compositions and nucleic acids described herein, for example, the motor neuron specific promoter nucleic acids described herein that are operably linked to an expressible nucleic acid. The pharmaceutical compositions can further comprise at least one other agent, such as a stabilizing compound or additional therapeutic agent, and can be administered in any sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline, buffered saline, dextrose, and water. The composition can be in a liquid or lyophilized form and include a diluent (Tris, citrate, acetate or phosphate buffers) having various pH values and ionic strengths, solubilizer such as Tween or Polysorbate, carriers such as human serum albumin or gelatin, preservatives such as thimerosal, parabens, benzylalconium chloride or benzyl alcohol, antioxidants such as ascorbic acid or sodium metabisulfite, and other components such as lysine or glycine. Selection of a particular composition will depend upon a number of factors, including the condition being treated, the route of administration and the pharmacokinetic parameters desired. A more extensive survey of components suitable for pharmaceutical compositions is found in Remington's Pharmaceutical Sciences, 18th ed. A. R. Gennaro, ed. Mack, Easton, PA (1980).

In certain embodiments, the methods and compositions of the present disclosure find use in treating a motor neuron disease. The compositions can be administered to the patient intravenously in a pharmaceutically acceptable carrier such as physiological saline. Standard methods for intracellular delivery can be used (e.g., delivery via liposome). Such methods are well known to those of ordinary skill in the art. The formulations of the present disclosure are useful for parenteral administration, such as intravenous, subcutaneous, intramuscular, and intraperitoneal administration. Therapeutic administration of a compound intracellularly can also be accomplished using

gene therapy. The route of administration eventually chosen will depend upon a number of factors and can be ascertained by one skilled in the art.

In certain embodiments, the pharmaceutical compositions of the present disclosure can be formulated using pharmaceutically acceptable carriers well known in the art in dosages suitable for oral administration. Such carriers enable the pharmaceutical compositions to be formulated as tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral or nasal ingestion by a patient to be treated.

Pharmaceutical compositions suitable for use in the present disclosure include, in certain embodiments, compositions where the active ingredients are contained in an effective amount to achieve the intended purpose. The amount will vary from one individual to another and will depend upon a number of factors, including the overall physical condition of the patient, e.g., severity and the underlying cause of the motor neuron disease.

In certain embodiments, the formulations of the present disclosure can be administered for prophylactic and/or therapeutic treatments. For example, in alternative embodiments, pharmaceutical compositions of the present disclosure are administered in an amount sufficient to treat, prevent and/or ameliorate a disease, e.g., a motor neuron disease. As is well known in the medical arts, dosages for any one patient depends upon many factors, including stage of the disease or condition, the severity of the disease or condition, the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and interaction with other drugs being concurrently administered.

Accordingly, in certain embodiments, the compositions described herein can be administered to a patient alone, or in combination with one or more other drugs, nucleotide sequences, lifestyle changes, etc. used in the treatment or prevention of disease, e.g., a motor neuron disease, or symptoms thereof or in pharmaceutical compositions where it is mixed with excipient(s) or other pharmaceutically acceptable carriers.

In certain embodiments, the pharmaceutically acceptable carrier is pharmaceutically inert. In certain embodiments of the present disclosure, the compositions described herein can be administered alone to a subject suffering from a disease, e.g., a motor neuron disease. The dosage regimen also takes into consideration pharmacokinetic parameters well known in the art, i.e., the active agents' rate of

absorption, bioavailability, metabolism, clearance, and the like (see, e.g., Hidalgo-Aragones (1996) J. Steroid Biochem. Mol. Biol. 58:611-617; Groning (1996) Pharmazie 51:337-341; Fotherby (1996) Contraception 54:59-69; Johnson (1995) J. Pharm. Sci. 84:1144-1146; Rohatagi (1995) Pharmazie 50:610-613; Brophy (1983) Eur. J. Clin. Pharmacol. 24:103-108; the latest Remington's, supra). The state of the art allows the clinician to determine the dosage regimen for each individual patient, active agent and disease or condition treated. Guidelines provided for similar compositions used as pharmaceuticals can be used as guidance to determine the dosage regiment, i.e., dose schedule and dosage levels, administered practicing the methods of the present disclosure are correct and appropriate.

Single or multiple administrations of formulations can be given depending on the dosage and frequency as required and tolerated by the patient. In certain embodiments, the formulations should provide a sufficient quantity of active agent to effectively treat, prevent or ameliorate the disease to be treated, e.g., a motor neuron disease, or symptoms or complications thereof as described herein.

In certain embodiments, the compositions of the present disclosure are administered once, twice, or three times per day; or once, twice, or three times per week, by intravenous (IV) or subcutaneous (SC) injection to reach a suggested target therapeutic endpoint. Once the target has been achieved, a maintenance dosing schedule is established which will vary depending upon the patient.

5.5 METHODS OF USE

In certain non-limiting embodiments, the present disclosure provides for a method of treating and/or reducing the severity of a motor neuron disease by administering to a subject in need thereof, a therapeutic composition and/or nucleic acid described herein, for example, the motor neuron specific promoter nucleic acids described herein that are operably linked to an expressible nucleic acid or gene, wherein the expressible nucleic acid or gene is selectively expressed in motor neurons. Accordingly, the present disclosure provides for a method of selectively delivering an expressible nucleic acid to a motor neuron in a subject.

Subjects in need of such treatment or compositions include subjects who have been diagnosed with, or are at risk for developing, a motor neuron disease. In certain embodiments, a motor neuron disease can be any progressive neurological disorder that destroys motor neurons, or disrupts motor neuron function, and thereby

disrupt involuntary or voluntary muscle activity such as speaking, walking, breathing, and swallowing. For example, a motor neuron disease is a disorder that disrupts the signaling from nerve cells in the brain (*i.e.,* upper motor neurons) to nerve cells in the brain stem and spinal cord (*i.e.,* lower motor neurons), to muscle tissue.

In certain embodiments, the motor neuron disease is amyotrophic lateral sclerosis (ALS), primary lateral sclerosis (PLS), progressive muscular atrophy, progressive bulbar palsy, pseudobulbar palsy, spinal muscular atrophy (SMA), post-polio syndrome (PPS), spinal and bulbar muscular atrophy (SBMA), Charcot–Marie–Tooth disease (CMT), Guillain–Barré syndrome (GBS), or any other motor neuron disease known in the art.

In certain embodiments, a subject who is at risk for developing a motor neuron disease is a subject who has or had family members diagnosed with a motor neuron disease.

The present disclosure provides for a method of treating a subject suffering from a motor neuron disease, comprising administering, to the subject, an effective amount of a composition comprising a promoter nucleic acid sequence that is operably linked to an expressible nucleic acid or gene, where the expressible nucleic acid or gene inhibits cell death, improves motor neuron cell function, lengthens subject survival, or a combination thereof.

In certain embodiments, an effective amount of a composition described herein is an amount which treats or reduces the severity of a motor neuron disease in a subject. For example, treating or reducing the severity of a motor neuron disease refers to an amelioration in the clinical symptoms or signs of a motor neuron disease, for example, but not by way of limitation, one or more of the following: reduction in the ability to make voluntary movements such as arm and leg movements; speech, swallowing and/or chewing impediments; muscle spasticity, spasms, cramps, and/or fasciculations; breathing impediments; jaw and/or facial muscle weakness; muscle tone loss; and muscle weakness.

5.6 KITS

The presently disclosed subject matter provides for kits for treating a subject diagnosed with, or at risk for having, a motor neuron disease. In certain embodiments, the kit comprises one or more of the compositions or nucleic acids described herein, for example, a nucleic acid of SEQ ID NO:1, and/or a recombinant vector comprising the nucleic acid of SEQ ID NO:1. In certain embodiments, the kit

further comprises instructions for administering the compositions or nucleic acids described herein for treating a subject diagnosed with or at risk for having a motor neuron disease.

In certain embodiments, the present disclosure provides for kits comprising one or more of the compositions or nucleic acids described herein in unit dosage form. In certain embodiments, the kit comprises a sterile container which contains the therapeutic composition; such containers can be boxes, ampules, bottles, vials, tubes, bags, pouches, blister-packs, or other suitable container forms known in the art. Such containers can be made of plastic, glass, laminated paper, metal foil, or other materials suitable for holding medicaments.

In certain embodiments, the kit comprises instructions for administering one or more of the compositions or nucleic acids described herein to a subject suffering from, or suspected of having, a motor neuron disease. The instructions can comprise information about the use of the compositions or nucleic acids for treating or preventing a motor neuron disease. In certain embodiments, the instructions comprise at least one of the following: description of the therapeutic agent; dosage schedule and administration for treating or preventing a motor neuron disease or symptoms thereof; precautions; warnings; indications; counter-indications; over dosage information; adverse reactions; animal pharmacology; clinical studies; and/or references. The instructions can be printed directly on the container (when present), or as a label applied to the container, or as a separate sheet, pamphlet, card, or folder supplied in or with the container.

6. EXAMPLES

The presently disclosed subject matter will be better understood by reference to the following Examples, which are provided as exemplary of the invention, and not by way of limitation.

6.1 EXAMPLE 1: Motor neuron-specific expression of a recombinant vector

The present example demonstrates that a 908 base pair nucleic acid sequence from the promoter region of the gene miR-218 is sufficient to specifically express a recombinant green fluorescent transgene in motor neuron cells *in vivo*. The miR-218 promoter is 7.6 kb and is also known as Pri-miR-218-2:promoter, miR218-promoter, MN-Slit3-promoter, Slit3-promoter, 218-promoter, 218p, and Slit3p.

Methods: A 908 bp nucleic acid was isolated from the 7.6 kb promoter region of a miR-218 gene that is specifically expressed in mouse motor neurons. The 908 bp nucleic acid is shown in FIG. 1 as SEQ ID NO:1. The 908 bp nucleic acid sequence of SEQ ID NO:1 was operatively linked to eGFP in a recombinant construct (218p:eGFP short). The full length 7.6 kb miR-218 promoter was also operatively linked to eGFP in a recombinant vector (218p:eGFP long).

Transgenic mouse lines were generated for both the 218p:eGFP short and 218p:eGFP long recombinant transgenes. Motor neuron expression levels of GFP in E11.5, E12.5 or E18.5 day mouse embryos were assessed for the transgenic mouse lines. Expression of GFP under the control of a homeobox gene (Hb9) promoter (Hb9:GFP) was used as a control.

A transgenic *Xenopus laevis* was generated using a recombinant transgene comprising the *Xenopus laevis* homolog of the 7.6 kb miR-218 promoter, which was operatively linked to GFP. GFP expression levels in spinal motor neurons of transgenic swimming tadpoles was assessed.

Results:

908 bp promoter sequence

As shown in FIG. 2, an eGFP reporter gene under control of the 908 bp promoter of SEQ ID NO:1 (218p:eGFP short) was expressed in both the cell bodies (FIG. 2B, arrow) and axons (FIG. 2B, bracket) of motor neurons in the spinal cord in E11.5 transgenic mouse embryos. eGFP was also expressed in brainstem motor nuclei of the embryos (FIG. 2B, arrowhead).

7.6 kb miR-218 promoter sequence

In transgenic mice expressing eGFP under the control of the 7.6 kb miR-218 promoter (218p:eGFP long), eGFP was expressed in motor neurons in E12.5 transgenic mouse embryos (FIG. 3A-B). eGFP expression from the 218p:eGFP long construct was stronger than eGFP expression under control of the homeobox gene (Hb9) promoter (Hb9:GFP) (FIG. 3A). eGFP expression under the control of the 7.6 kb miR-218 promoter (218p:eGFP long) was also detected in the central nervous system of E18.5 transgenic mouse embryos (FIG. 4A-C). eGFP expression was detected in cervical, thoracic and lumbar regions of the spinal cord (FIG. 4B), as well as in brainstem motor neurons (FIG. 4C). Cross sections of spinal cord from P5 mice show expression of GFP

in 218p:eGFP long transgenic mice as well as Hb9:GFP transgenic mice. GFP expression in motor neurons was greater in the 218p:eGFP transgenic mice (FIG. 5). FIG. 6 shows expression of 218p:eGFP long in cranial and spinal neurons, as compared to expression of miR-218 mRNA. As shown in FIG. 7, 218p:eGFP long was expressed in both N10 and N12 brainstem nuclei, while Hb9:GFP was only expressed in N12 brainstem nuclei.

In a transgenic *Xenopus laevis* expressing a recombinant transgene comprising the *Xenopus laevis* homolog of the 7.6 kb miR-218 promoter operatively linked to GFP, GFP was expressed in anterior and posterior spinal motor neurons of the swimming tadpole (FIG. 8).

miR-218 micro RNA is expressed in motor neurons

The micro RNA miR-218 was identified as being highly enriched in motor neurons. Mouse embryonic stem cells (mESCs) carrying a GFP reporter transgene under the control of a homeobox gene (Hb9) promoter (Hb9:GFP), which is expressed in motor neurons, were cultured with retinoic acid (RA) and Sonic Hedgehog (Shh) to differentiate the mESCs into motor neurons. miR-218 micro RNA was identified as being highly enriched in the motor neuron cells (FIG. 9).

miR-218 mRNA expression in mouse motor neurons was assessed by in situ hybridization in mouse embryos. miR-218 mRNA was detected in lumbar motor neurons of E11.5 mouse embryos, lumbar, thoracic and brainstem motor neurons of E12.5 mouse embryos, lumbar motor neurons of P0 mice, and lumbar motor neurons of adult mice (FIG. 10). miR-218 mRNA was detectable in motor neurons in E11.5 mouse embryos after the cells migrated to the ventro-lateral spinal cord (FIG. 11). miR-218 mRNA was also detected in all brainstem and spinal motor neurons in the P0 mouse central nervous system (FIG. 12).

Conclusion:

A single microRNA (miR-218) is abundantly expressed and specifically enriched in all brainstem and spinal motor neuron subtypes. miR-218 is encoded within the Slit2/3 genetic loci, however, novel motor neuron-specific alternative promoters drive pri-miR-218 expression independently of Slit2/3 promoters. miR-218 directly targets many transcriptional modifiers, and indirectly activates many genes involved in synaptic transmission. The miR-218-KO motor neuron transcriptome globally shifts towards that of 'generic' ventral spinal cord interneurons. Furthermore, the 908 bp miR-218 promoter

exhibited specificity and intensity that were comparable to the full 7.6 kb miR-218 promoter.

6.2 EXAMPLE 2: Loss of motoneuron-specific microRNA-218 causes systemic neuromuscular failure

Summary:

Dysfunction of microRNA metabolism is thought to underlie diseases affecting motoneurons. One microRNA, miR-218, is abundantly and selectively expressed by developing and mature motoneurons. Here, we show that mutant mice lacking miR-218 die neonatally and exhibit neuromuscular junction (NMJ) defects, motoneuron hyperexcitability, and progressive motoneuron cell loss – hallmarks of motoneuron diseases such as amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA). Gene profiling reveals that miR-218 modestly represses a cohort of hundreds of genes that are neuronally-enriched, but are not specific to a single neuron subpopulation. Thus, the set of mRNAs targeted by miR-218, designated *TARGET*^{218}, define a neuronal gene network that is selectively tuned-down in motoneurons to prevent neuromuscular failure and neurodegeneration.

Methods:

Mouse Lines

The following mouse lines were used: Hb9:gfp, Chx10:Cre *(29)*, Sim1:Cre *(30)*, 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 *(11)*. 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 (guide sequences available in Supplemental Table 2). 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 (see Supplemental

Table 2 for primer information) 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

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. miRNA *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 , 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.

Slice preparation for intracellular recording

Hb9:*gfp*+ E18.5 embryos were removed from the uterus under isoflurane anesthesia and spinal cords were quickly isolated in ice cold, oxygenated 95% O2/5% CO2, 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. 218DKO mutants were identified by postmortem PCR genotyping. Lumbar regions of the spinal cords were isolated and mounted in low melting point agarose (4% in a CSF) 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 NaH_2PO_4, 26 $NaHCO_3$, 25 D-Glucose, 1 $MgSO_4$, 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 $KMeSO_4$, 5 KCl, 0.5 $CaCl_2$, 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 NaHCO3; 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 (http://www.cs.cmu.edu/~ckingsf/software/sailfish/) 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

(28A) Normalized reads per kilobase (NRPK) values of replicates (n=6 (WT), n=2 (218^{DKO})) were averaged. Genes that were not expressed by at least 10 NRPKs in either the wild type or 218^{DKO} data sets were eliminated. Significance p-values were determined by a two-tailed heteroscedastic t-test in Excel.

TargetScan6 context+ scores are a predictor of efficacy of miRNA targeting with more negative scores denoting a greater predicted efficacy of repression. context+ values were obtained from http://www.targetscan.org/mmu_61/. A cutoff of <-0.15 for context+ scores to be designated a *TARGET*^{218} gene was empirically established.

(28C) Differential NRPK expression of *TARGET*^{218} genes in wild-type motoneurons versus each interneuron subtype (n=6 (WT), 1 (V1), 1 (V2a), 1 (V3)).

(28E) Heirarchical clustering was performed with GENE-E software, using city block distances.

(29A) 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 (as determined by maximum NRPK level in either data set) were used for Sylamer motif enrichment analysis. Sylamer software (available at https://www.ebi.ac.uk/research/enright/software/sylamer) was used to assess miRNA seed match enrichment p-values. A FASTA file of 3'UTRs masked and purged of low complexity and redundant sequences (http://www.ebi.ac.uk/enright-srv/sylarray/purged.html) was obtained from and 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 ranked transcript list for Sylamer analysis. Sylamer settings: 7bp, bin size 2, markov correction 4, known miRNA seeds only. Enrichment p-values were exported and plotted in one-dimension using Prism GraphPad.

(29B and C) For RNA sequencing data sets from cortical projection neurons, raw data sets were obtained from data sets associated with DeCoN (24) and were

aligned to the genome using the same methods as in-house generated data sets. NRPK values from cortical neuron duplicates (E15.5 data set only) were averaged before performing Sylamer analysis (as above).

Results:

Motoneurons are a specialized neuronal subpopulation within the central nervous system (CNS) that establish synaptic connections with muscles to regulate movement. Diseases such as ALS and SMA, which affect motoneurons, seem to share a common pathogenic mechanism based on defective RNA metabolism and biogenesis of microRNAs (*1-5*). Consistent with earlier descriptions of miR-218 expression in embryonic motoneurons (*6, 7*), we found that miR-218 was the most abundant motoneuron microRNA and was enriched ~27-fold, as determined by small RNA sequencing of FACS-purified *Hb9::gfp*$^+$ motoneurons from embryonic mouse spinal cords (**Fig. 26A**). The spinal cord is comprised of many interneuron subtypes (*8*). We found that V2a and V3 ventral spinal interneurons did not express a single characteristic microRNA (**fig. 30A**), indicating motoneurons may be distinctly reliant on cell type-specific microRNA expression.

We detected miR-218 within visceral and somatic spinal motoneurons (**Fig. 26B, fig. 30B**) and brainstem motor nuclei (**fig. 30C**). Robust miR-218 expression persisted postnatally in α- and γ- choline acetyltransferase (ChAT)-positive motoneurons (**Fig. 26C, fig. 30D**) but not ChAT+ interneurons (**fig. 30E**). Likewise, miR-218 was selectively expressed in human embryonic motoneurons (**fig. 30F**). Compared with the extensive catalog of protein markers that delineate motoneuron subtypes (*8*), miR-218 is remarkable for its expression spanning motoneuron classes from embryonic stages into adulthood (**fig. 30G**) and its undetectable expression in other tissues.

Two paralogs of miR-218 are present in mammals, *miR-218-1* and *miR-218-2*, embedded within homologous introns of the *Slit2* and *Slit3* genes, respectively. The motoneuron-specifying transcription factors Isl1 and Lhx3 have been suggested to upregulate miR-218 by increasing transcription of *Slit2* and *Slit3* (*6*). We investigated Slit2 and Slit3 mRNA expression in motoneurons by RNA sequencing and discovered that both genes were transcribed using start sites located upstream of exon 6 (**Fig. 26D, fig 31A and B**). This intronic start site was not used by adjacent floor plate glial cells expressing *Slit2* and *Slit3*. Previously mapped Isl1/2, Lhx3, and Phox2a ChIP peaks (*9*) were observed at conserved hexamer DNA response elements (HxREs) proximal to exon

6 (**Fig. 26E**), suggesting the presence of intragenic motoneuron-specific promoters (**Fig. 26F**). To test this hypothesis, we generated a transgenic mouse line, tg(*218-2::eGFP*) with a 7.4 kilobase (kb) sequence containing putative regulatory elements (**fig. 31C**). *In vivo*, eGFP was expressed in tg(*218-2::eGFP*) spinal and cranial motoneurons, reproducing miR-218's endogenous expression pattern (**Fig. 26G** and **H**, **fig. 31C**). These findings demonstrate that primary miR-218 transcripts are under independent activation in motoneurons by promoters distinct from those that generate *Slit2* and *Slit3* full-length transcripts in other tissues.

Studies *in vitro* and in chick embryos have suggested miR-218 may contribute to motoneuron specification (*6*), however, such microRNA knockdown and overexpression studies can yield non-physiological affects (*10*). To determine the biological impact of miR-218 disruption *in vivo*, we used CRISPR/Cas9 gene editing (*11*) to create microdeletions of *miR-218-1* and *miR-218-2* precursor sequences in mouse (**Fig. 27A, fig. 32A-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. 27B, fig. 32E-H**). We found that the number and spatial organization of motoneurons in E12.5 spinal cords of 218^{DKO} mutants were unchanged compared to controls (**fig. 33A-F**). Subsequent developmental events including axonal exit from the spinal cord, outgrowth, and peripheral pathfinding were indistinguishable between Hb9::gfp^{+} control and 218^{DKO} embryos (**fig. 33G**). 218^{DKO} mutants displayed none of the phenotypes characteristic of *Slit2* and *Slit3* disruption (**fig. 33H** and **I**)(*12, 13*), indicating *Slit2* and *Slit3* function is unaffected by the intronic microdeletions. We conclude that miR-218 is dispensable for *in vivo* motoneuron fate specification and gross early motoneuron development.

However, 218^{DKO} mice were never viable (**fig. 34A**). 218^{DKO} embryos exhibited akinesia, kyphosis, and weak or absent responses to pain stimulation after caesarean delivery at E18.5 and died within minutes due to an apparent lack of respiration (**Fig. 27C**) – a phenotype similar to mice carrying null alleles of Agrin, MuSK, choline acetyltransferase (ChAT) and other components required for neuromuscular transmission (*14*). Consequently, we investigated whether loss of miR-218 impacts neuromuscular synaptogenesis, an intricate process in which motor nerves first innervate muscle and subsequently form pre-synaptic specializations with post-synaptic acetylcholine receptors (AChRs) expressed by developing muscle (*15*). We examined glycerol-cleared tg(*218-2::eGFP*) embryos and found that the motor nerves of 218^{DKO} motoneurons reached

E14.5 limb tissue (**fig. 34B**). However, motor axons appeared to lack penetrating, fine intra-muscular branches across limb (**Fig. 27D**), diaphragm and intercostal muscle groups (**Fig. 27E**, **fig. 35A-B**). At E18.5, the majority of α-bungarotoxin-labelled AChR+ clusters lack motor innervation in 218^{DKO} limb muscles (**Fig. 27F, fig. 35C**), reflecting a gross failure of motoneurons to establish neuromuscular junctions needed to control body movements.

While 218^{DKO} mutants had normal numbers of motoneurons at E12.5 (**Fig. 27G**), 218^{DKO} mutants had 18 to 36% fewer motoneurons at cervical, thoracic, and lumbar spinal cord segments at E18.5, indicating neurodegenerative cell loss (**Fig. 27H; fig. 36A-B**). To examine whether the physiology of the remaining motoneurons was altered in mice lacking miR-218, we assessed fictive locomotion (*16*) and performed intracellular recordings of *Hb9::gfp*$^+$ LMC α-motoneurons from E18.5 lumbar spinal slices (**fig. 37A**). Left/right and flexor/extensor activation of motor roots were 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. 37B-J**). However, action potentials were elicited by a 4.4-fold lower rheobase current in 218^{DKO} motoneurons compared with controls (**Fig. 27I** and **J**), indicative of membrane hyperexcitability. Thus, miR-218 does not significantly contribute to early motoneuron development, but it is critical for the regulation of neuromuscular synapses, membrane excitability, and motoneuron survival.

These phenotypic defects suggested that motoneuron-specific gene regulation depends on the post-transcriptional repression of miR-218 target mRNAs. To extend beyond microRNA-target identification studies performed *in vitro* using cancer cell lines (*6, 17*), we identified miR-218 gene targets within motoneurons by performing polyA$^+$ RNA sequencing of FACS-isolated Hb9::*gfp*$^+$ motoneurons from wild type and 218^{DKO} E12.5 spinal cords, before the apparent onset of defects (**fig. 38A**). Using Sylamer (*18*), we determined that 6bp, 7bp and 8bp 3'UTR complementary seed matches to miR-218 were enriched within genes expressed higher in 218^{DKO} motoneurons than controls (**fig. 38B-E**), reflecting widespread de-repression of miR-218 target genes in 218^{DKO} motoneurons.

We identified 333 miR-218 target genes that met the following criteria: expression >10 normalized reads per kilobase (NRPK), possession of miR-218 binding sites (context+ score ≤ -0.15, TargetScan6)(*19*), and significant upregulation in 218^{DKO} versus wild type motoneurons (**Fig. 28A**). This cohort of 333 genes is likely to be under

direct miR-218 mediated repression, and we named this coordinately regulated gene set *TARGET*^{218}. *TARGET*^{218} genes are enriched for neurotransmission and neurotransmitter transport biological processes (**fig. 38F** and **G**). The most highly upregulated *TARGET*^{218} gene, Slc1a2/GLT-1 (266% increase), is a glutamate reuptake transporter known to be modulated by riluzole – the only medication approved for the treatment of ALS (*20*). On average, *TARGET*^{218} genes were expressed 61.1% higher in 218^{DKO} motoneurons, and 47 of these genes were increased by at least 2-fold (**Fig. 40**). The wide breadth of target genes affected in 218^{DKO} motoneurons suggests miR-218 shapes expression of an extensive genetic network, rather than merely modulating a small group of individual genes within a single molecular pathway.

Other microRNA-gene regulatory networks have been shown to reinforce the repression of differentiation programs to confer robustness to cell-fate decisions (*21-23*), however, the lack of cell specification errors in 218^{DKO} embryos suggested that miR-218 might have a novel regulatory role. 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. 28B, fig. 39A-C**). We found that ~80% *TARGET*^{218} genes are expressed lower in wild type motoneurons versus each of V1, V2a, and V3 interneurons (**Fig. 28C**). Moreover, the majority (69.1%) of *TARGET*^{218} genes are expressed lower in wild type motoneurons versus all three of the spinal interneurons subpopulations profiled (**Fig. 28D**). 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. 28E**). Thus, rather than reinforcing a preexisting 'low' target gene expression in motoneurons, miR-218 establishes the low *TARGET*^{218} expression observed in motoneurons relative to interneurons.

To evaluate microRNA-mediated repression in an unbiased manner, we bioinformatically evaluated the statistical enrichment of binding sites for all microRNAs across the transcriptome of investigated cell types. Using Sylamer (*18*), we determined the hypergeometric statistical enrichment of 7bp 3'UTR sequences complementary to known microRNA seed sequences (microRNA seed matches) in transcripts expressed higher or lower in motoneurons versus interneurons (**Fig. 29A**). We found that 3'UTR

seed matches to miR-218 are significantly enriched in transcripts expressed lower in wild type motoneurons versus averaged (**Fig. 29A**) and individual spinal interneuron subpopulations – and even distantly located cortical neuron subpopulations (**Fig. 29B**)(*24*). 3'UTR seed matches to miR-218 were no longer found to be enriched in genes expressed higher or lower in 218^{DKO} motoneurons (**Fig. 29C**). The 3'UTR seed match to miR-124 (a pan-neuronal microRNA abundantly expressed in motoneurons and other CNS neurons (*25*)), but not 3'UTR seed matches to miR-218, was overrepresented in transcripts expressed lower in motoneurons versus purified motoneuron progenitors differentiated from embryonic stem cells (**Fig. 29B** and **C**). We conclude: [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] although miR-124 and miR-218 are co-expressed in motoneurons, their regulatory roles differ – miR-124 represses a neuronal progenitor-associated gene network, while miR-218 represses a gene network coordinately expressed by other spinal and cortical neuronal subpopulations.

In summary, we have identified a neuronal gene network that is selectively repressed 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 (*1, 26-28*). The link between miR-218 and motoneuron diseases likely extends beyond phenotypic similarities. Patients suffering from motoneuron diseases carry genetic mutations in ubiquitously expressed RNA processing factors (e.g. TAR DNA binding protein-43, fused in sarcoma, survival of motor neuron) or expansion repeats in C9ORF72 that sequester RNA binding proteins (*1, 2*), but the biological mechanisms that magnify the effects of these changes on motoneurons are unclear. microRNA processing pathways, and specifically, the repression of miR-218's genetic network, might be particularly sensitive to defects in these RNA metabolic pathways thought to underlie motoneuron disease.

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30. Y. Zhang *et al.*, V3 spinal neurons establish a robust and balanced locomotor rhythm during walking. *Neuron* **60**, 84-96 (2008).

* * *

Although the presently disclosed subject matter and its advantages have been described in detail, it should be understood that various changes, substitutions and alterations can be made herein without departing from the spirit and scope of the invention as defined by the appended claims. Moreover, the scope of the present application is not intended to be limited to the particular embodiments of the process, machine, manufacture, composition of matter, means, methods and steps described in the specification. As one of ordinary skill in the art will readily appreciate from the disclosure of the presently disclosed subject matter, processes, machines, manufacture, compositions of matter, means, methods, or steps, presently existing or later to be developed that perform substantially the same function or achieve substantially the same result as the corresponding embodiments described herein may be utilized according to the presently disclosed subject matter. Accordingly, the appended claims are intended to include within their scope such processes, machines, manufacture, compositions of matter, means, methods, or steps.

Patents, patent applications, publications, product descriptions and protocols are cited throughout this application the disclosures of which are incorporated herein by reference in their entireties for all purposes.

WE CLAIM:

1. A composition comprising a recombinant nucleic acid comprising a motor neuron-specific promoter nucleic acid comprising SEQ ID NO:1, operatively linked to a second nucleic acid.

2. The composition of claim 1, wherein the second nucleic acid is selected from the group consisting of a nucleic acid encoding an anti-inflammatory peptide, a cytokine, a growth factor, an enzyme, an ion channel, an apoptosis inhibitor and an antioxidant.

3. The composition of claim 1, wherein the second nucleic acid encodes a miR-218.

4. The composition of claim 1, wherein the second nucleic acid comprises a nucleic acid sequence selected from the group consisting of 5' UUGUGCUU 3'; 5' UGUGCUU 3'; 5' UUGUGCU 3'; 5' UGUGCU 3'; 5' UUGUGC 3'; and 5' GUGCUU 3'.

5. The composition of claim 1, wherein the second nucleic acid is selected from the group consisting of a nucleic acid encoding a transcription factor selected from the group consisting of Isl1, Isl2, Lhx3, Isl-Lhx3 fusion proteins, Phox2a, and a combination thereof.

6. The composition of claim 1, wherein the second nucleic acid encodes a transcript that selectively hybridizes to a target comprising a nucleic acid sequence selected from the group consisting of 5'AAGCACAA 3'; 5' AAGCACA 3'; 5' AGCACAA 3'; 5' AGCACA 3'; 5' GCACAA 3'; and 5' AAGCAC 3'.

7. The composition of any one of claims 1-6, wherein the composition is comprised in a recombinant cell.

8. The composition of any one of claims 1-6, wherein the composition is comprised in a viral vector.

9. The composition of claim 8, wherein the viral vector is selected from the group consisting of adenovirus, adeno-associated virus, vaccinia, herpesviruses, baculoviruses, retroviruses, parvovirus, and lentivirus.

10. The composition of any preceding claim, wherein the composition is comprised in a pharmaceutical composition.

11. A method of introducing a nucleic acid into a motor neuron cell comprising administering, to the motor neuron cell, a composition according to any one of claims 1-10.

12. A method of treating a human subject diagnosed with or at risk of having a motor neuron disease, comprising administering to a subject in need thereof, a therapeutically effective amount of a composition according to any one of claims 1-10.

13. A method of treating a human subject diagnosed with or at risk of having a motor neuron disease, comprising administering to a subject in need thereof, a therapeutically effective amount of a composition comprising a nucleic acid encoding a miR-218, a nucleic acid encoding a compound that increases miR-218 expression, a compound that inhibits expression or activity of a miR-218 target nucleic acid or protein encoded therefrom, or a combination thereof.

14. The method of claim 13, wherein the miR-218 comprises a nucleic acid selected from the group consisting of 5' UUGUGCUU 3'; 5' UGUGCUU 3'; 5' UUGUGCU 3'; 5' UGUGCU 3'; 5' UUGUGC 3'; and 5' GUGCUU 3'.

15. The method of claim 13, wherein the nucleic acid encoding a compound that increases miR-218 expression encodes a transcription factor selected from the group consisting of Isl1, Isl2, Lux, Isl-Lhx3 fusion proteins, Phox2a, and a combination thereof.

16. The method of claim 13, wherein the miR-218 target nucleic acid comprises a nucleic acid that specifically hybridizes to a miR-218 seed sequence selected from the group consisting of 5' UUGUGCUU 3'; 5' UGUGCUU 3'; 5' UUGUGCU 3'; 5' UGUGCU 3'; 5' UUGUGC 3'; and 5' GUGCUU 3.

17. The method of claim 13, wherein the compound that inhibits expression or activity of a miR-218 target nucleic acid or protein encoded therefrom, is selected from the group consisting of an RNAi molecule, shRNA molecule, antisense RNA, catalytic RNA, catalytic DNA, protein, or antibody or fragment thereof, specific for a miR-218 target nucleic acid, or protein encoded therefrom.

18. The method of any one of claims 12-16, wherein the composition is administered in an amount effective to inhibit cell death, increase motor neuron cell function, lengthen subject survival, reduce one or more symptom or sign of a motor neuron disease, or a combination thereof.

19. The method of claim 18, wherein the one or more symptom or sign of a motor neuron disease is selected from the group consisting of a reduction in the ability to make voluntary movements; speech, swallowing and/or chewing impediments; muscle spasticity, spasms, cramps, and/or fasciculations; breathing impediments; jaw and/or facial muscle weakness; muscle tone loss; and muscle weakness.

20. The method of any one of claims 12-16, wherein the motor neuron disease is selected from the group consisting of amyotrophic lateral sclerosis (ALS), primary lateral sclerosis (PLS), progressive muscular atrophy, progressive bulbar palsy, pseudobulbar palsy, spinal muscular atrophy (SMA), post-polio syndrome (PPS), spinal and bulbar muscular atrophy (SBMA), Charcot–Marie–Tooth disease (CMT), and Guillain–Barré syndrome (GBS).

21. A kit comprising a composition according to any one of claims 1-10.

taaacgcccc aatttgctac ttatcaaata gtatacattt ttggctcaga aaaaaacctg 60
atgtctgtat attacttctc aactaaaatc cctcagtcct taactggcat gtgtattagt 120
caaggcatct ttgagaaggg cattatttcc ctacacttag gatggggaaa gagaaattaa 180
aaaggaatcc taaaataggt gcatttaatt ctccccaatt taaatgtaag tggtgcgtct 240
tttaggcaat aatgatatgc cttttagtcc tccattacaa acacttccat cgatgaattt 300
ccttaatgtt gatgatggtt agtgcagttt gagggaatct gtatttattc agaaaatgtt 360
cccatagaat gacctaccag atgggccacg taacaatgca tggagacatc aaaccaccac 420
agacatttgg tgcttagaat aataaaaaga ctataaaatt agattagttg agtctaattt 480
ggaattggta tattccctac gcaccctcac cgctcttggg cagataaagc cttgagattt 540
agcgctgtgt caaagccaag actgtaactt ccagtaaaag ggagccgagg gaggggggagc 600
ttgctgggag gtcgcggagg gcagagcagt gacctccaat gatttacagg cctttagctt 660
aatgaaattg tttcagtgac atgacagtaa gagctcgtaa tggattggat gccctaatgt 720
aatgaaatta ctcccttctg cctaaaaaaa aaaaaatgcg caattaatat ttactgagac 780
ctgacagcct ttggtgcgct cgctcgcctg tgtagttccc tcagacagtc agagagaaga 840
gacagagcag cgtggcagac aggcgggctc tgcaggagct cctggcaggg acaagcagag 900
cctgcaag 908
(SEQ ID NO:1).

FIG. 1

E11.5 transgenic mouse line expressing 908bp miR-218-promoter:eGFP

Brightfield image

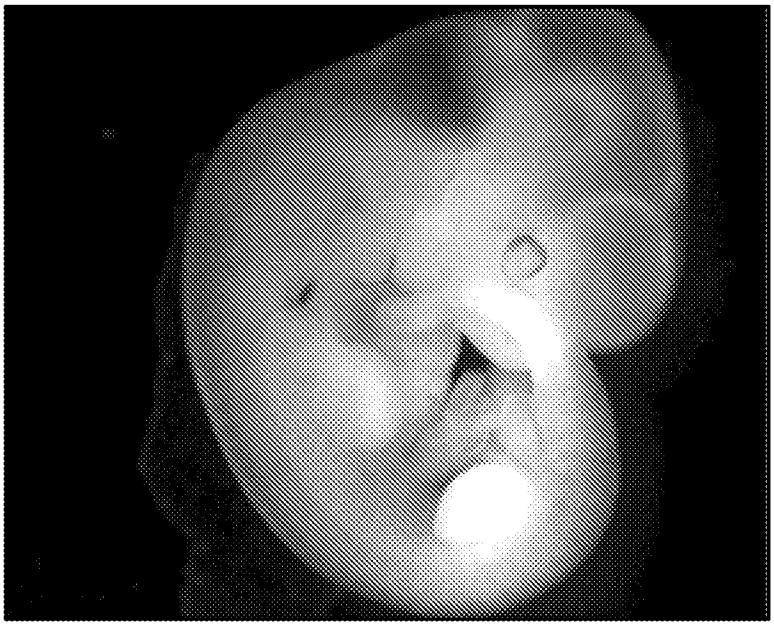
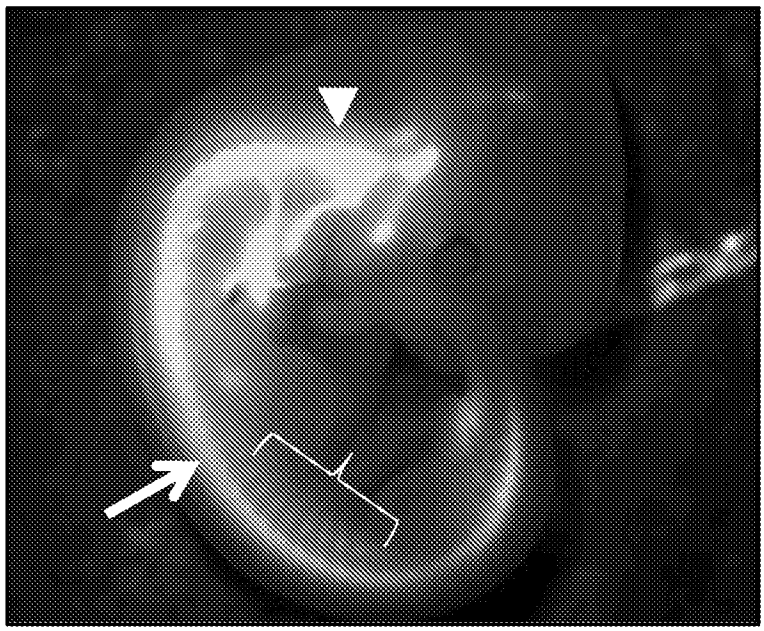


FIG. 2A

eGFP fluorescence



- Motor columns/cell bodies (arrow)
- Motor axons (bracket)
- Brainstem motor nuclei (arrowhead)

FIG. 2B

E12.5 transgenic mouse line expressing 7.6 kb miR-218-promoter:eGFP or homeobox gene (Hb9) promoter:eGFP

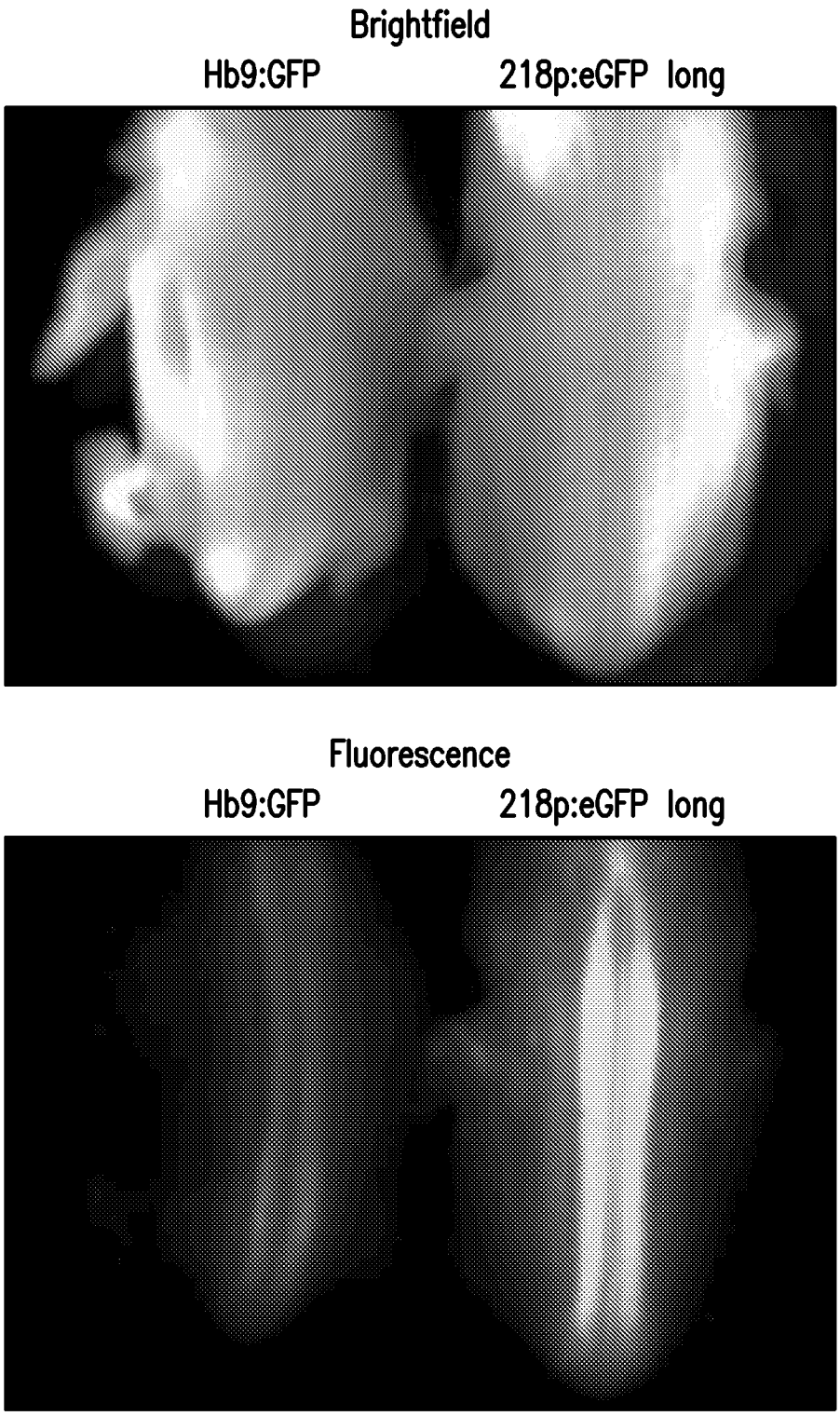


FIG. 3A

E12.5 transgenic mouse line expressing 7.6 kb miR-218-promoter:eGFP

Brightfield



Fluorescence

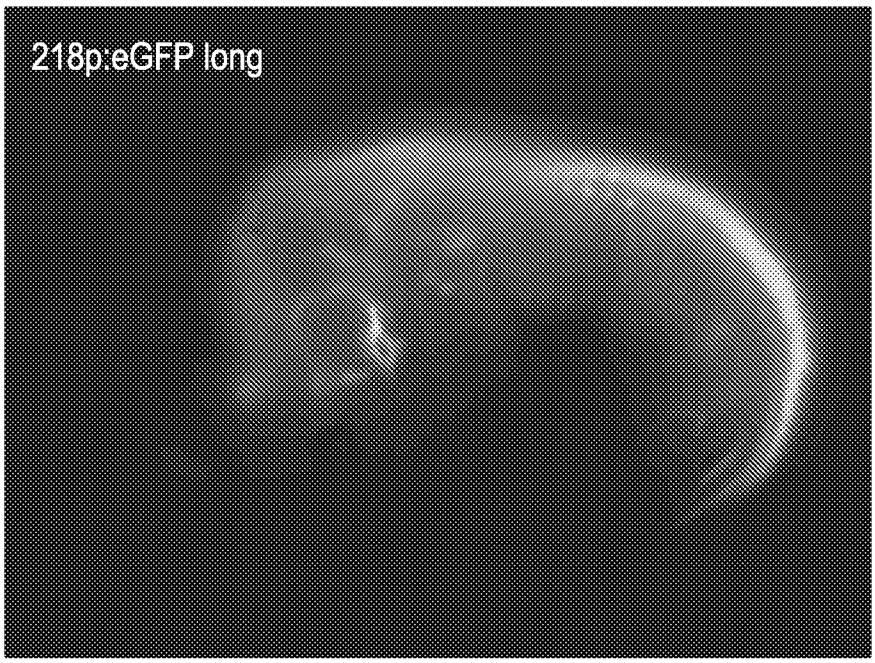


FIG. 3B

E18.5 transgenic mouse line expressing 7.6 kb miR-218-promoter:eGFP

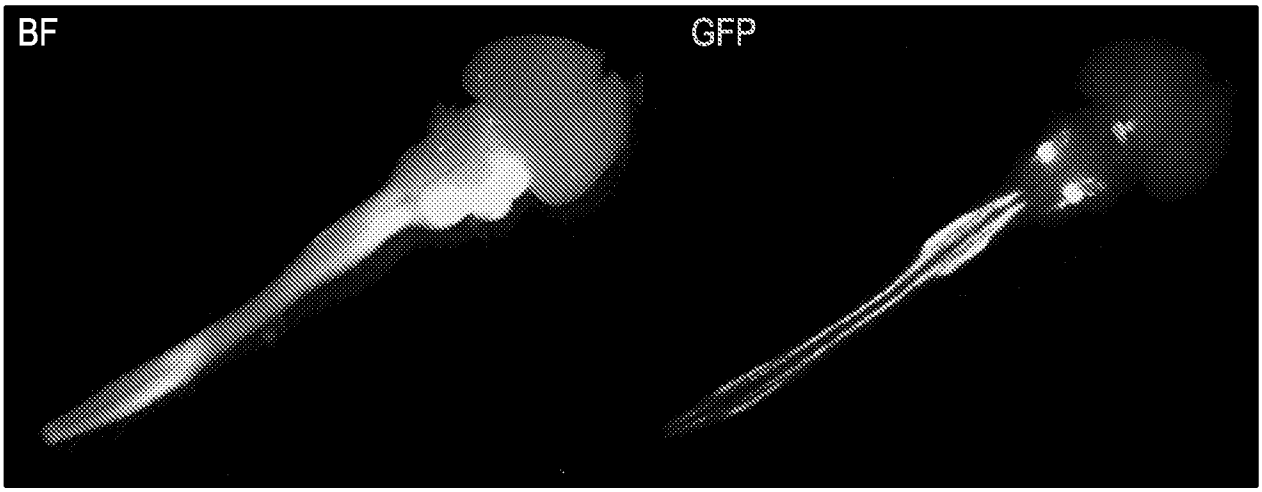


FIG. 4A

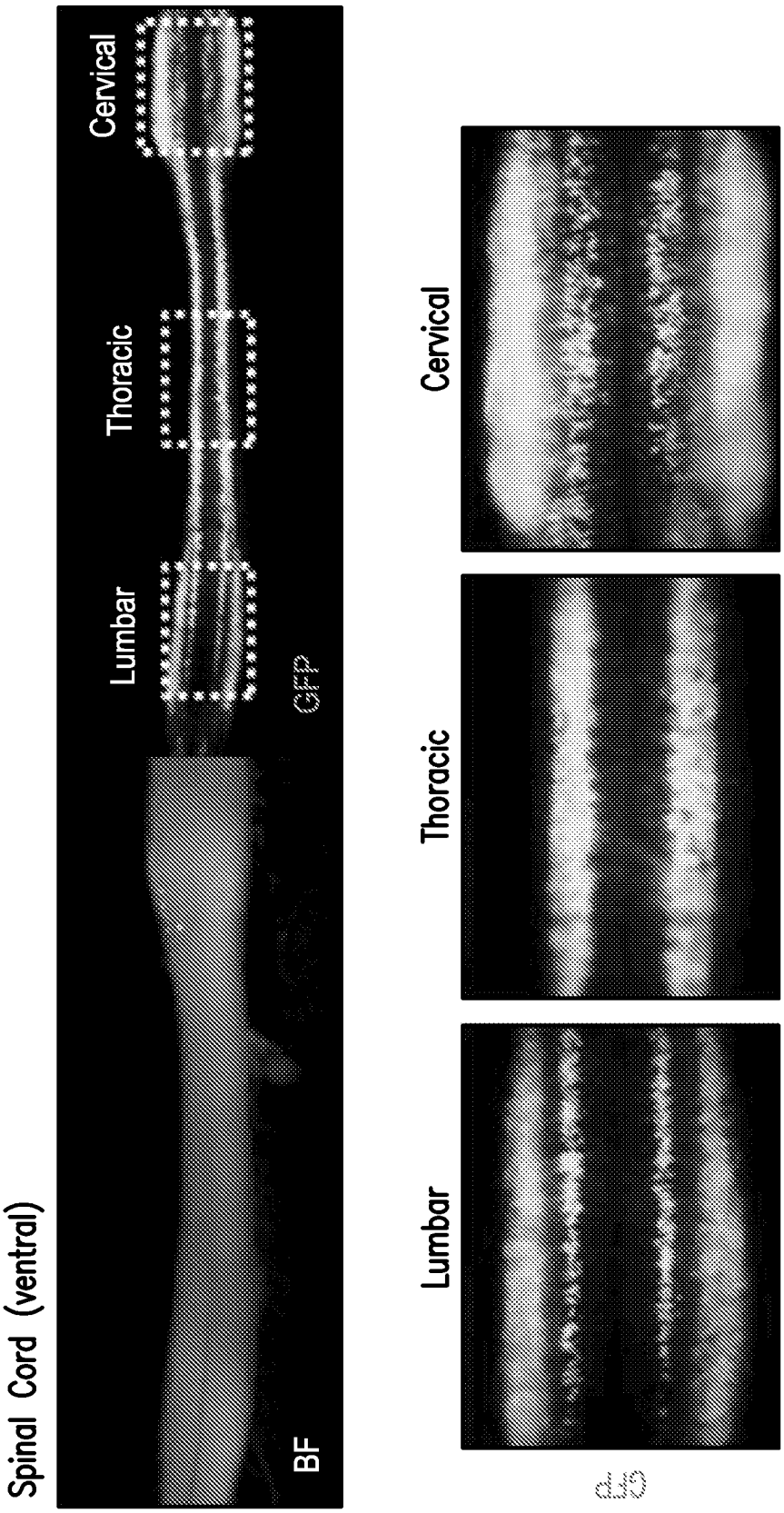

Spinal Cord (ventral)
BF
GFP
Lumbar
Thoracic
Cervical
Lumbar
Thoracic
Cervical
GFP

FIG. 4B

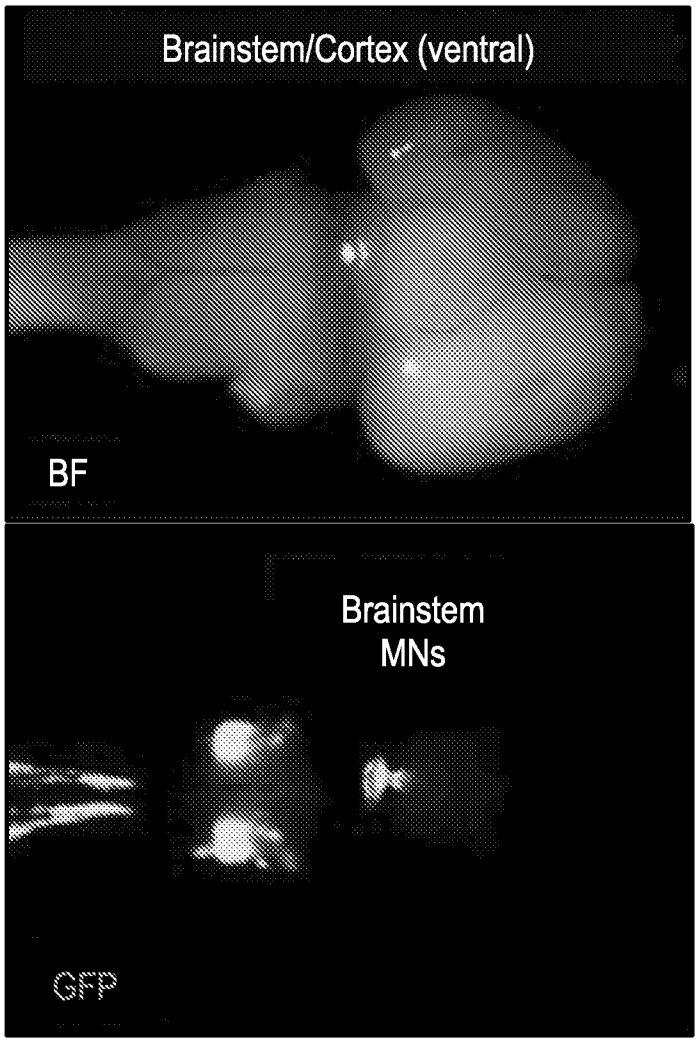


FIG. 4C

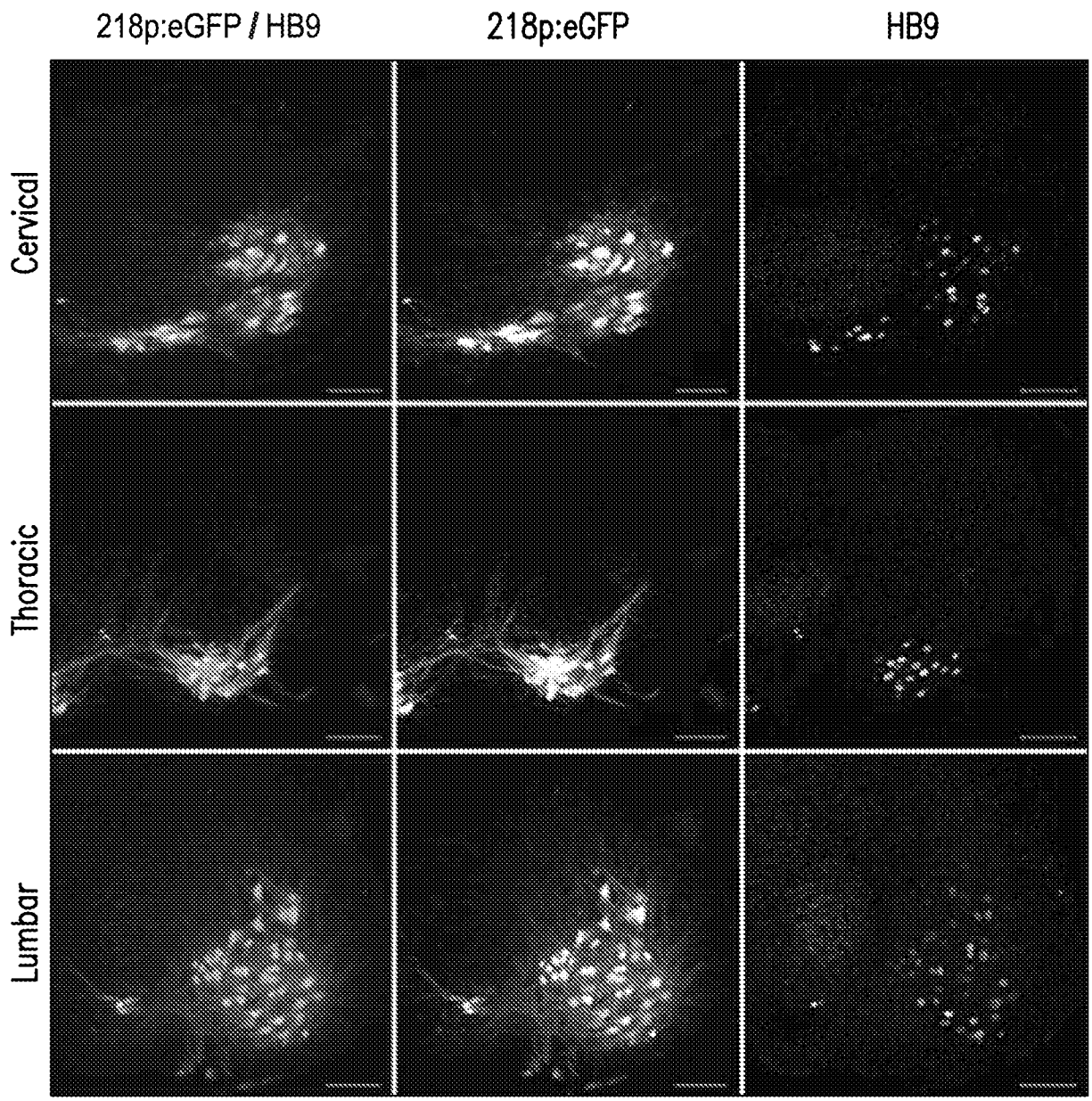


FIG. 5

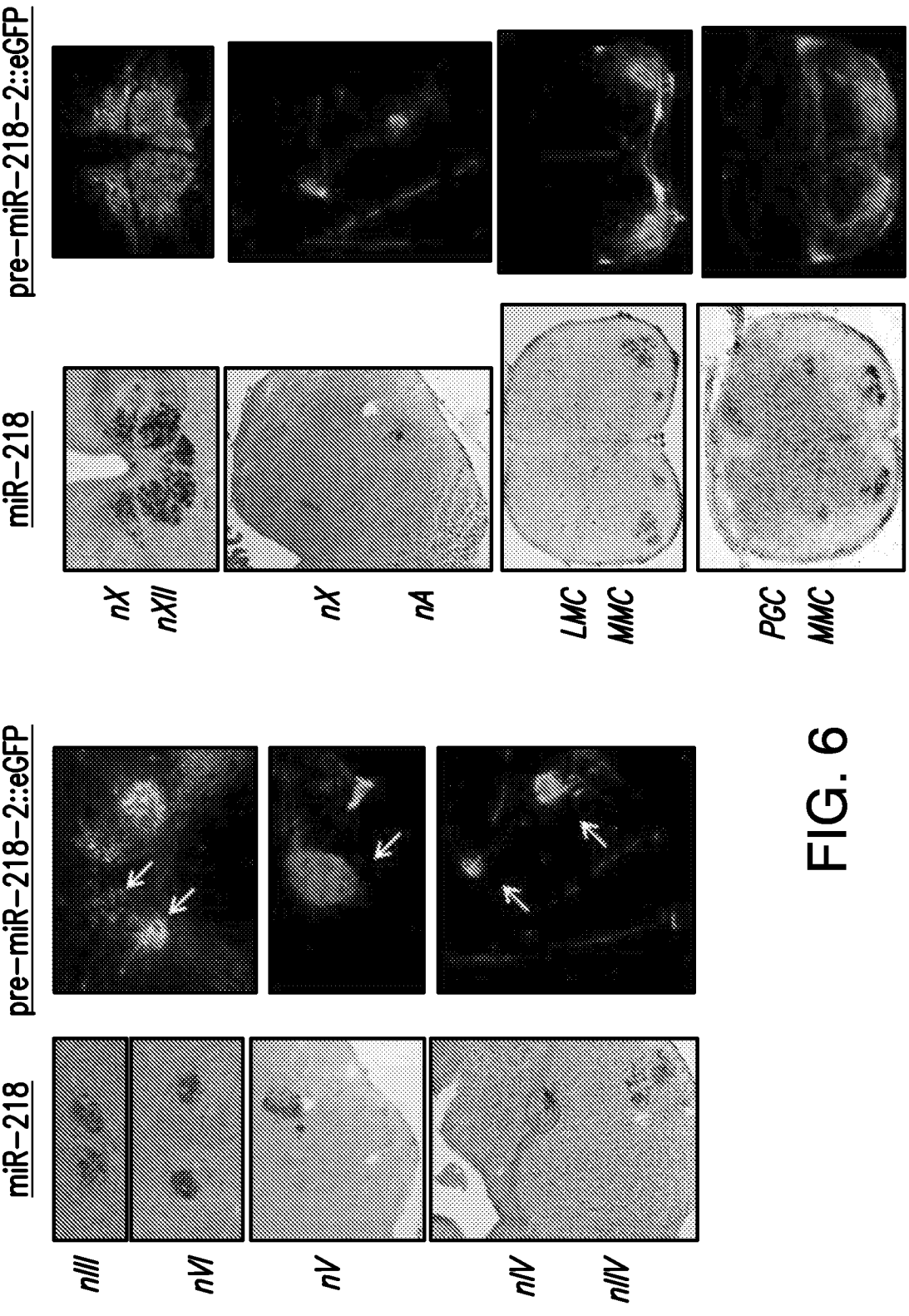

miR-218
pre-miR-218-2::eGFP
nIII
nVI
nV
nIV
nIIV
miR-218
pre-miR-218-2::eGFP
nX
nXII
nX
nA
LMC
MMC
PGC
MMC
E18.5

FIG. 6

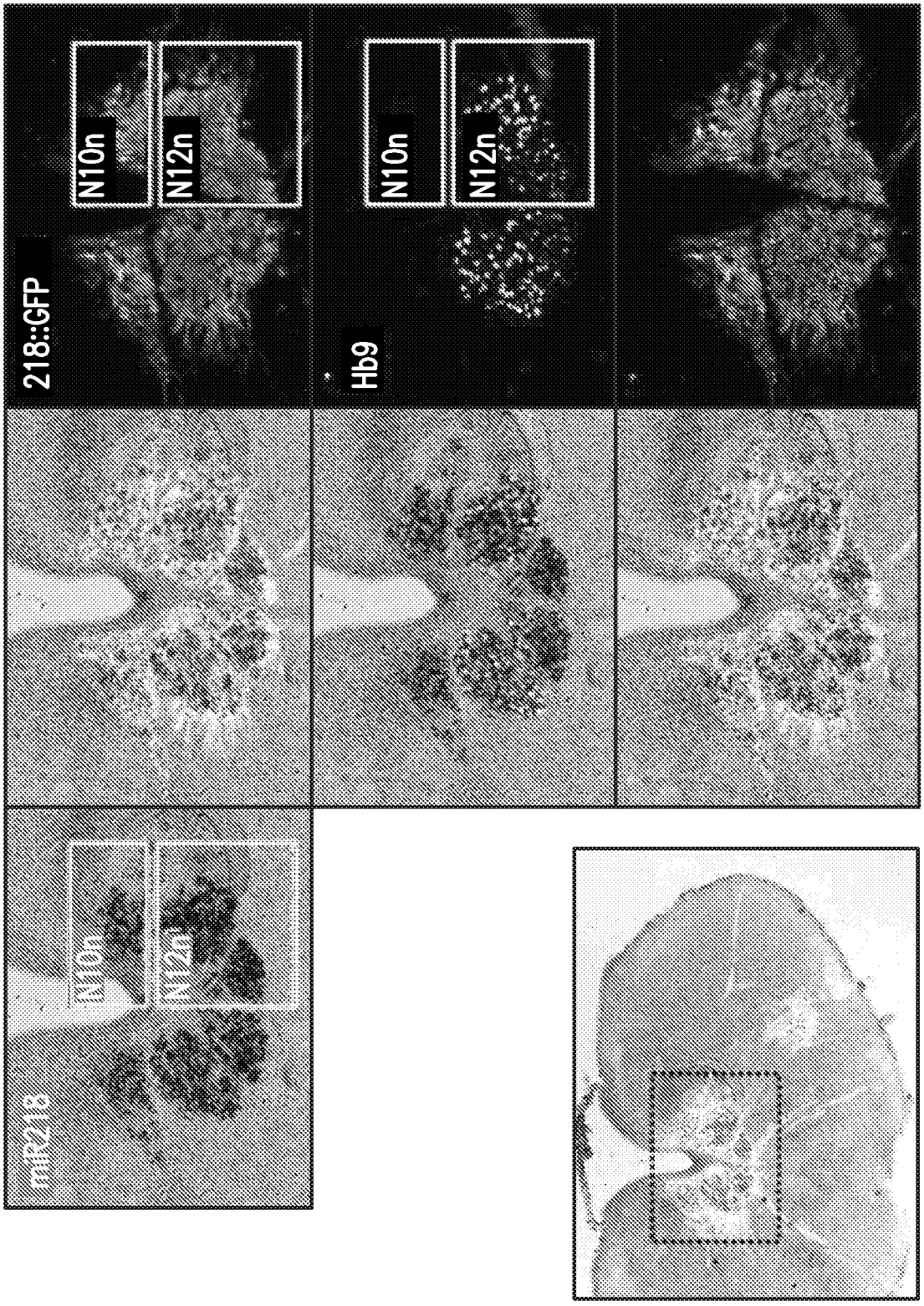


FIG. 7

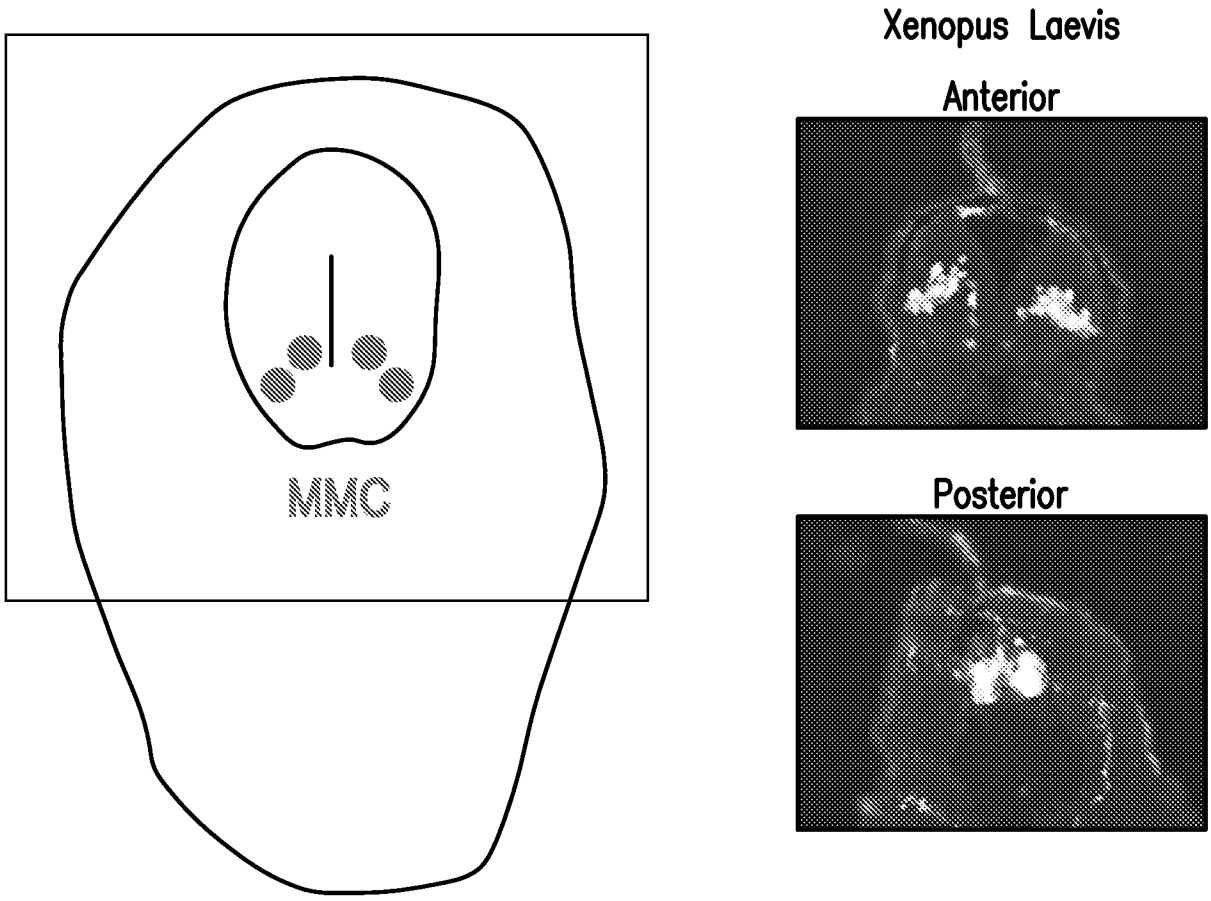


FIG. 8

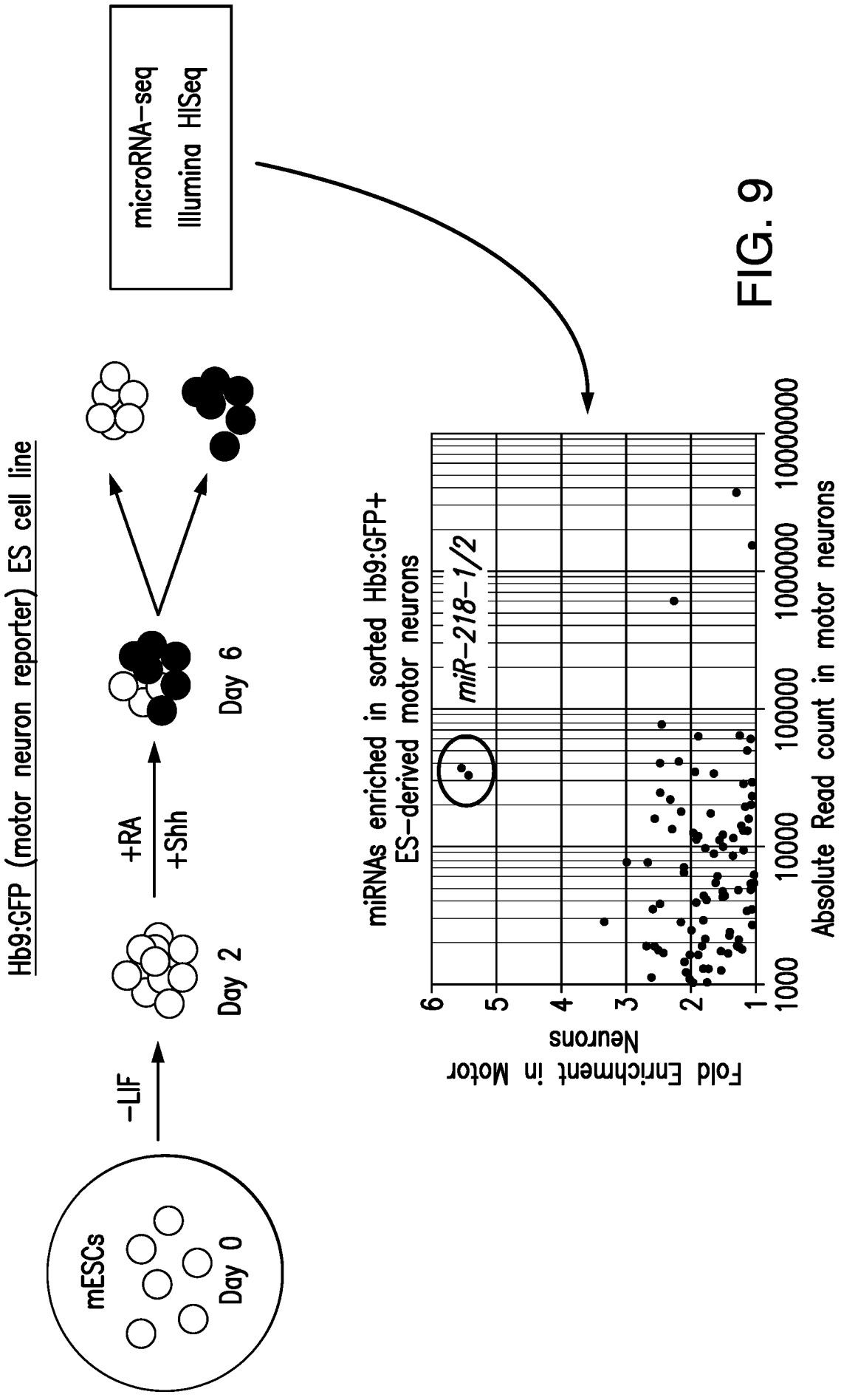

microRNA-seq
Illumina HISeq
Hb9:GFP (motor neuron reporter) ES cell line
mESCs
Day 0
-LIF
Day 2
+RA
+Shh
Day 6
miRNAs enriched in sorted Hb9:GFP+
ES-derived motor neurons
miR-218-1/2
Fold Enrichment in Motor Neurons
Absolute Read count in motor neurons
1
2
3
4
5
6
1000
10000
100000
1000000
10000000

FIG. 9

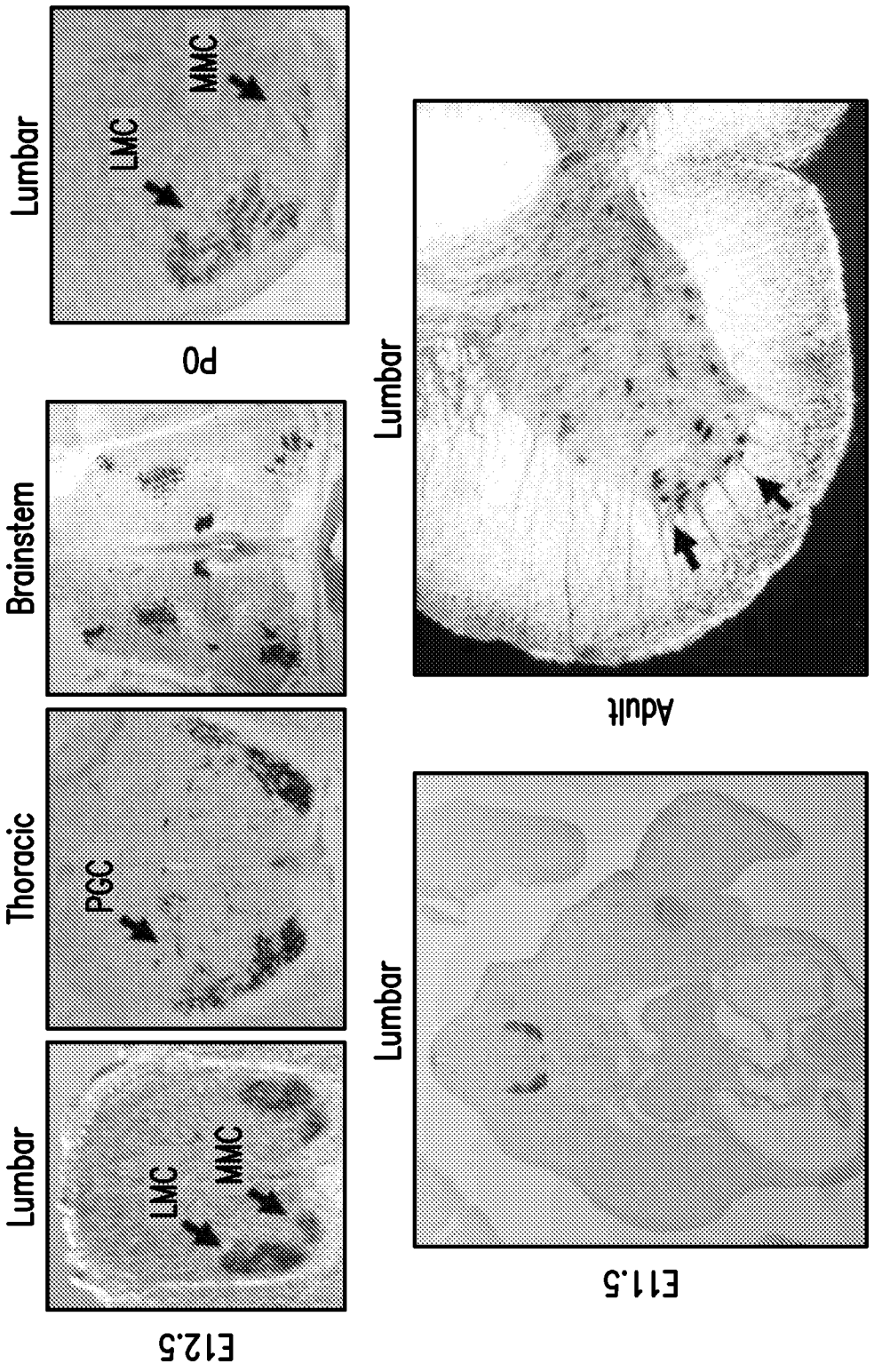

Lumbar
LMC
MMC
P0
Brainstem
Thoracic
PGC
Lumbar
LMC
MMC
E12.5
Lumbar
Adult
Lumbar
E11.5

FIG. 10

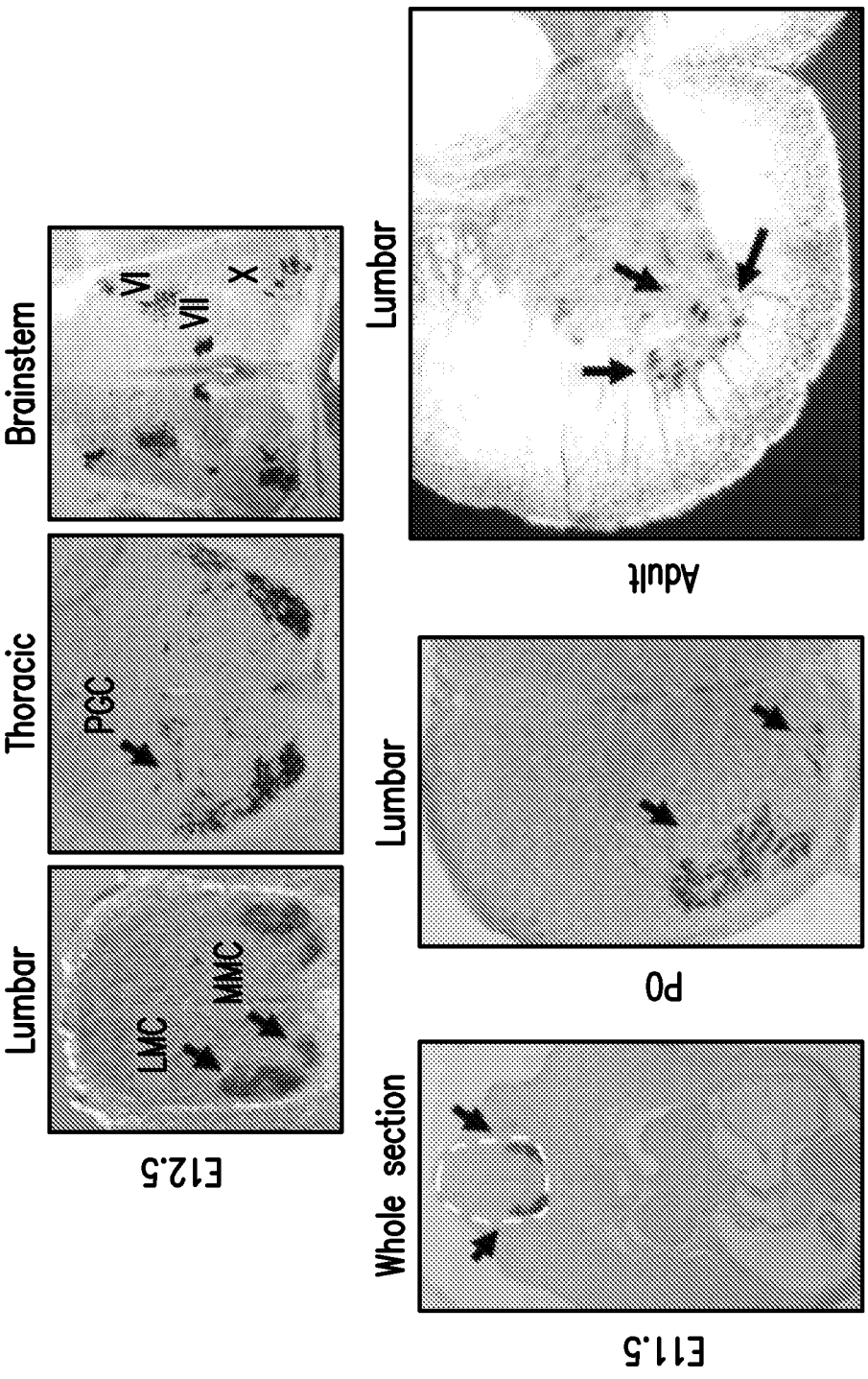


FIG. 10 Continued

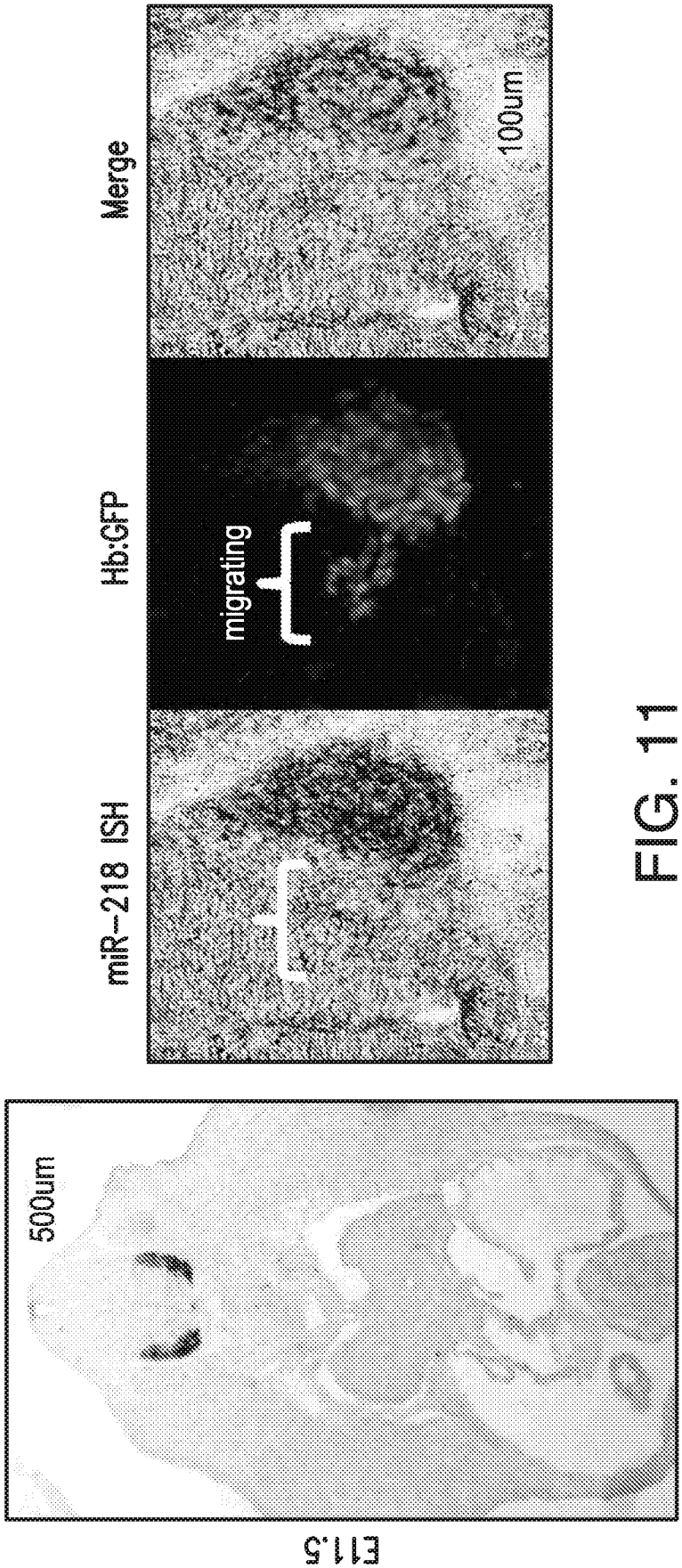


FIG. 11

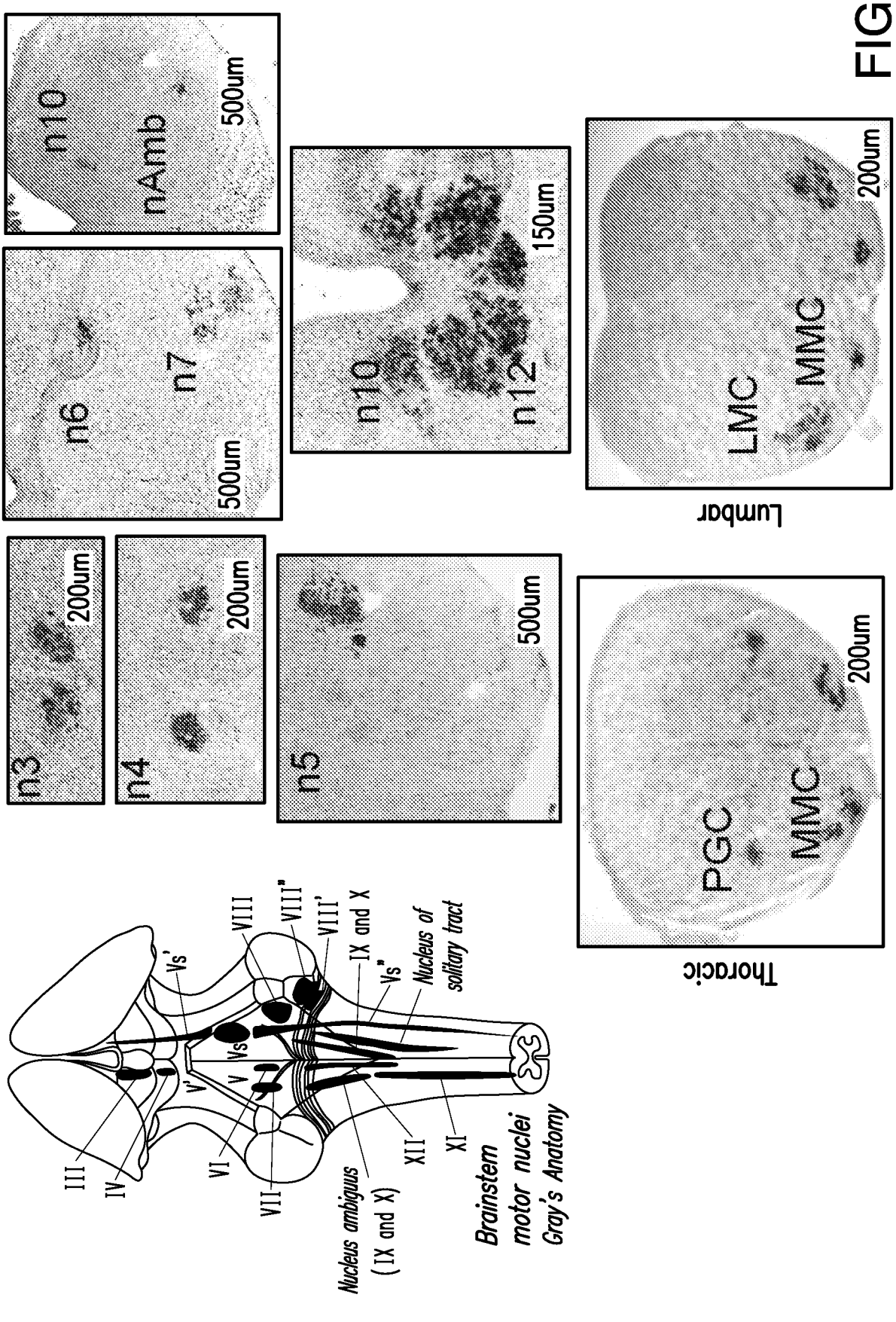

n3
200um
n4
200um
n5
500um
n6
n7
500um
n10
nAmb
500um
n10
n12
150um
LMC
MMC
200um
Lumbar
PGC
MMC
200um
Thoracic
III
IV
V'
V
Vs'
Vs
VI
VII
VIII
VIII'
VIII"
IX and X
Vs"
Nucleus of solitary tract
XII
XI
Nucleus ambiguus (IX and X)
Brainstem motor nuclei Gray's Anatomy

FIG. 12

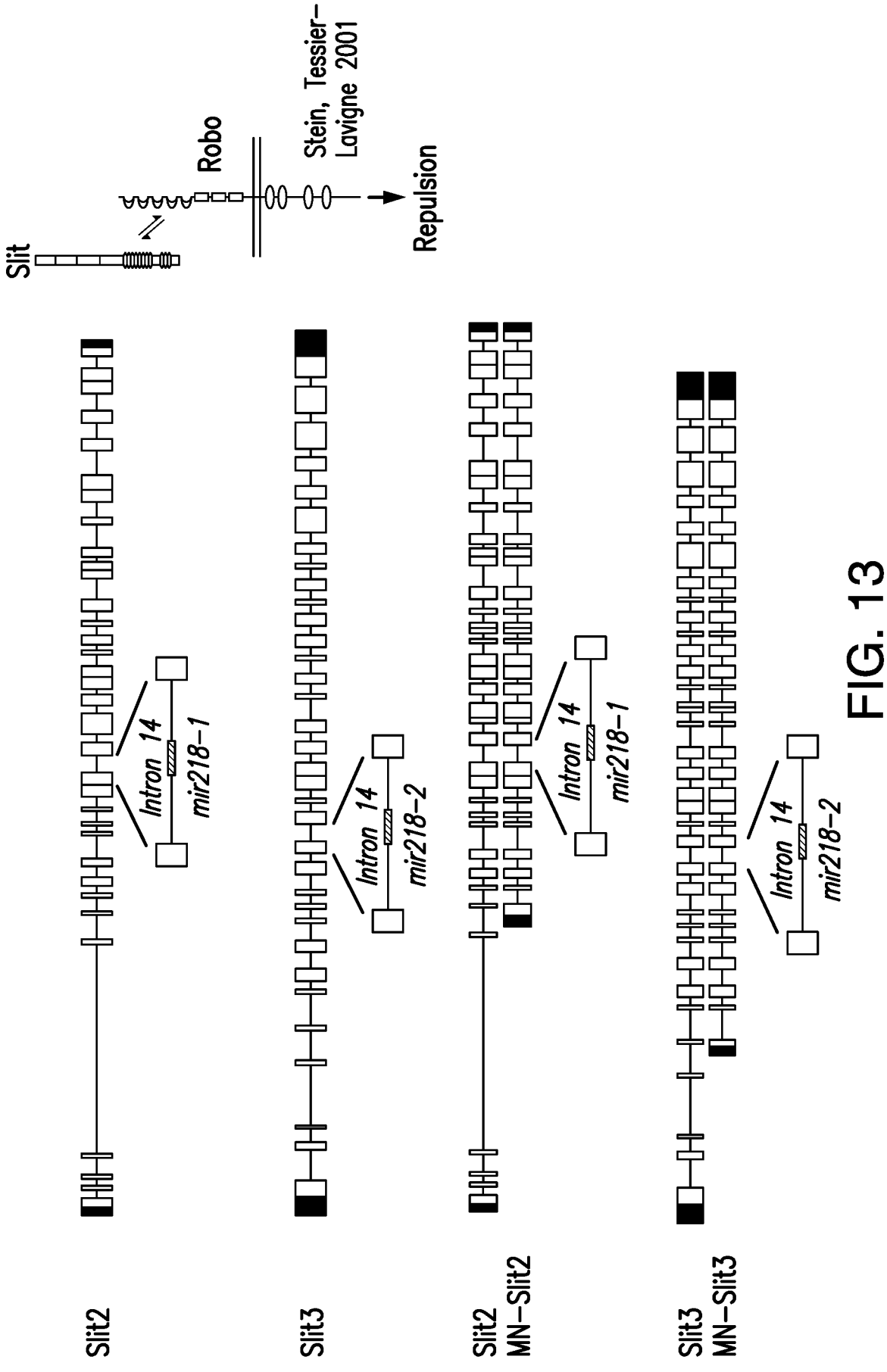
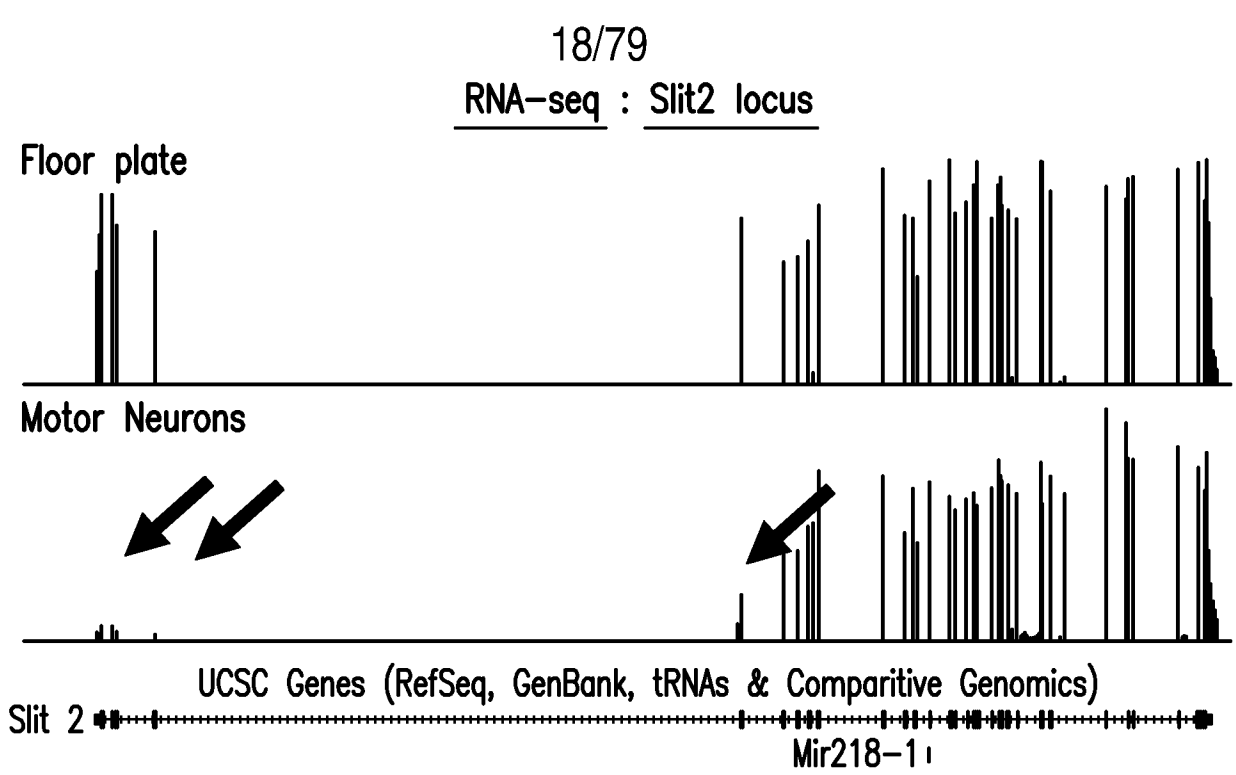
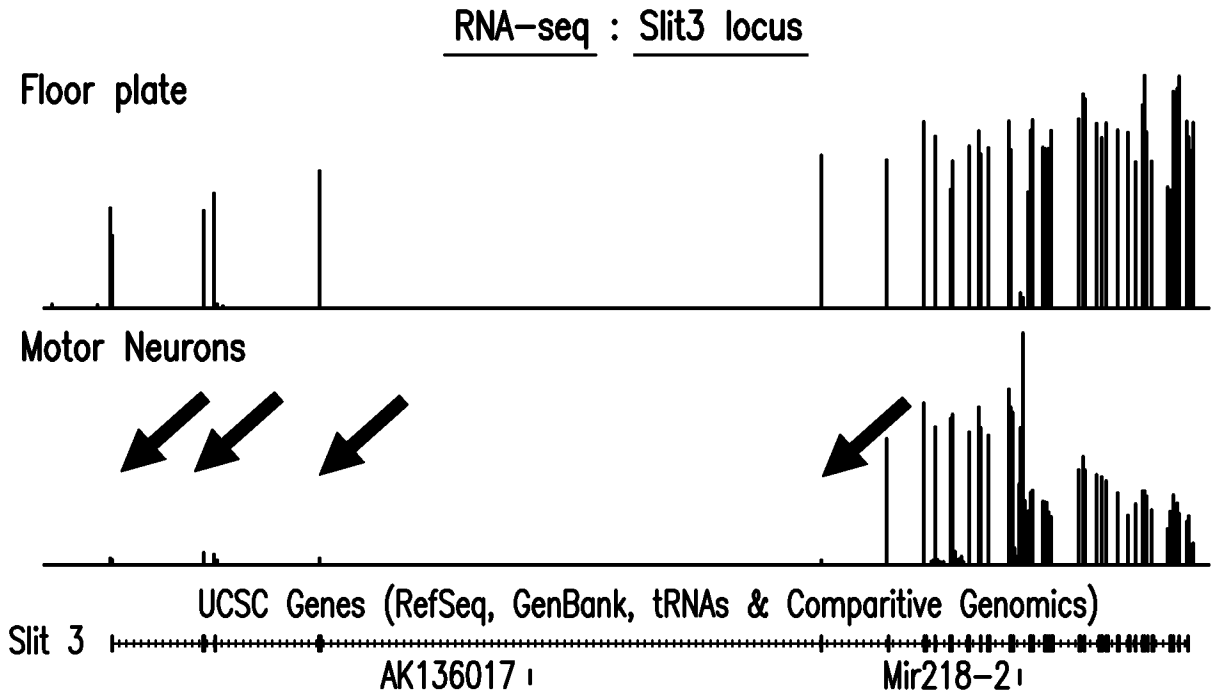

Slit
Robo
Stein, Tessier-Lavigne 2001
Repulsion
Slit2
Intron 14
mir218-1
Slit3
Intron 14
mir218-2
Slit2
MN-Slit2
Intron 14
mir218-1
Slit3
MN-Slit3
Intron 14
mir218-2

FIG. 13





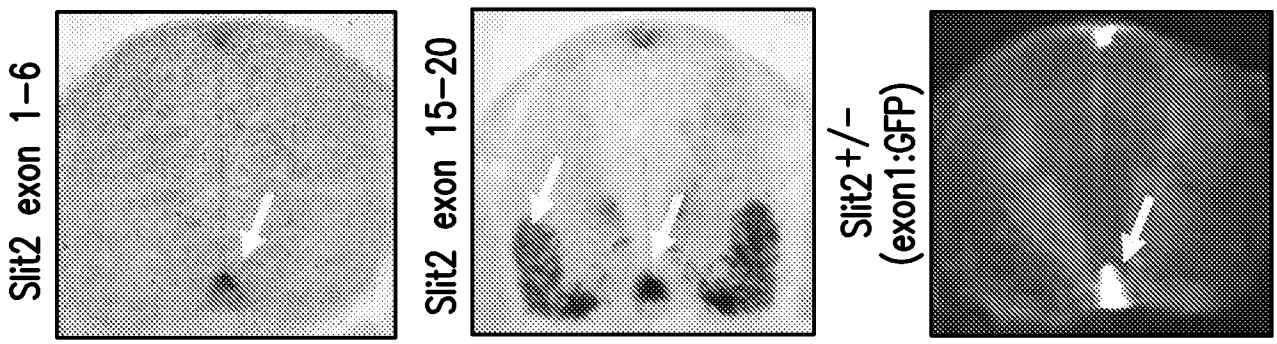


FIG. 13 Continued

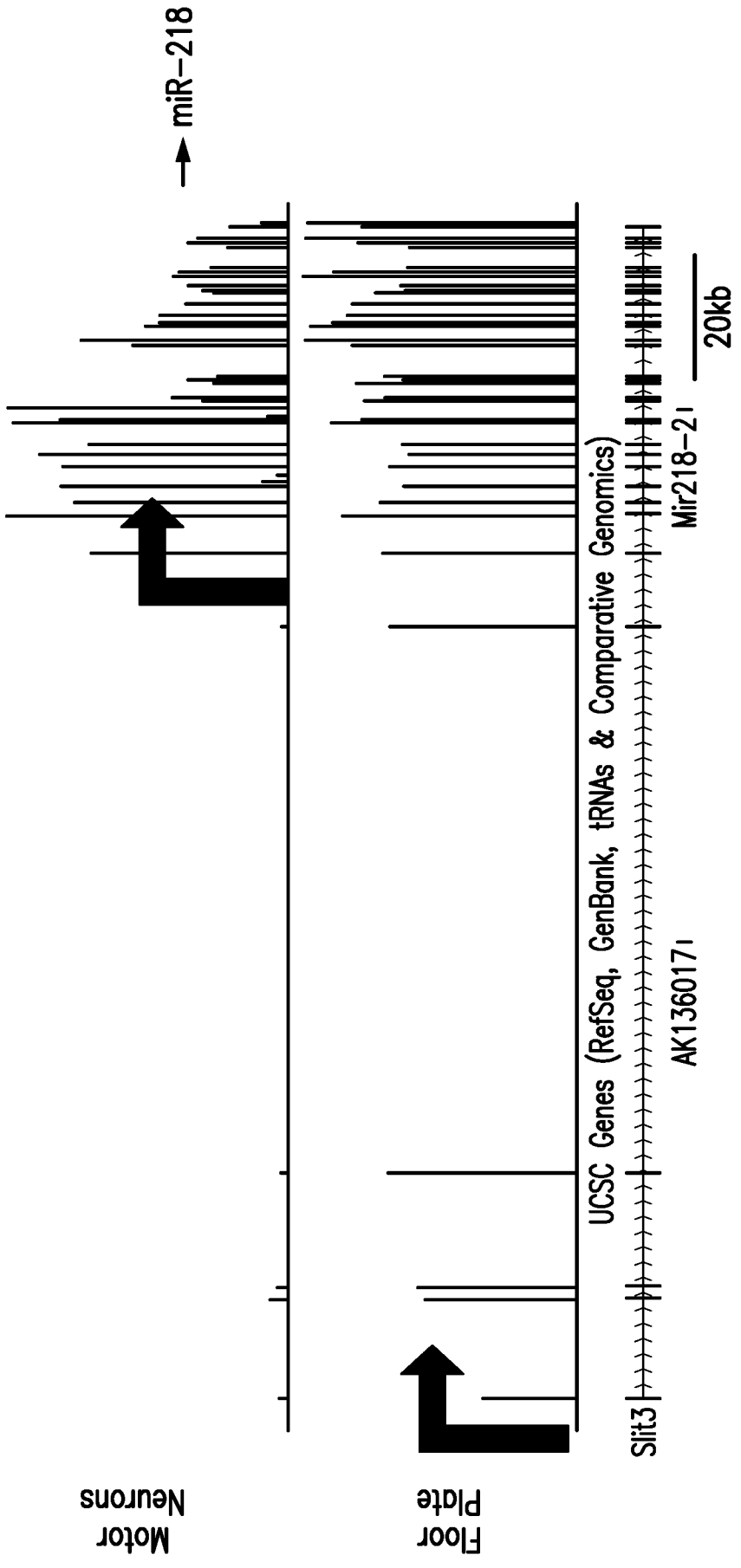

miR-218
Motor Neurons
Floor Plate
Slit3
UCSC Genes (RefSeq, GenBank, tRNAs & Comparative Genomics)
AK136017
Mir218-2
20kb

FIG. 14

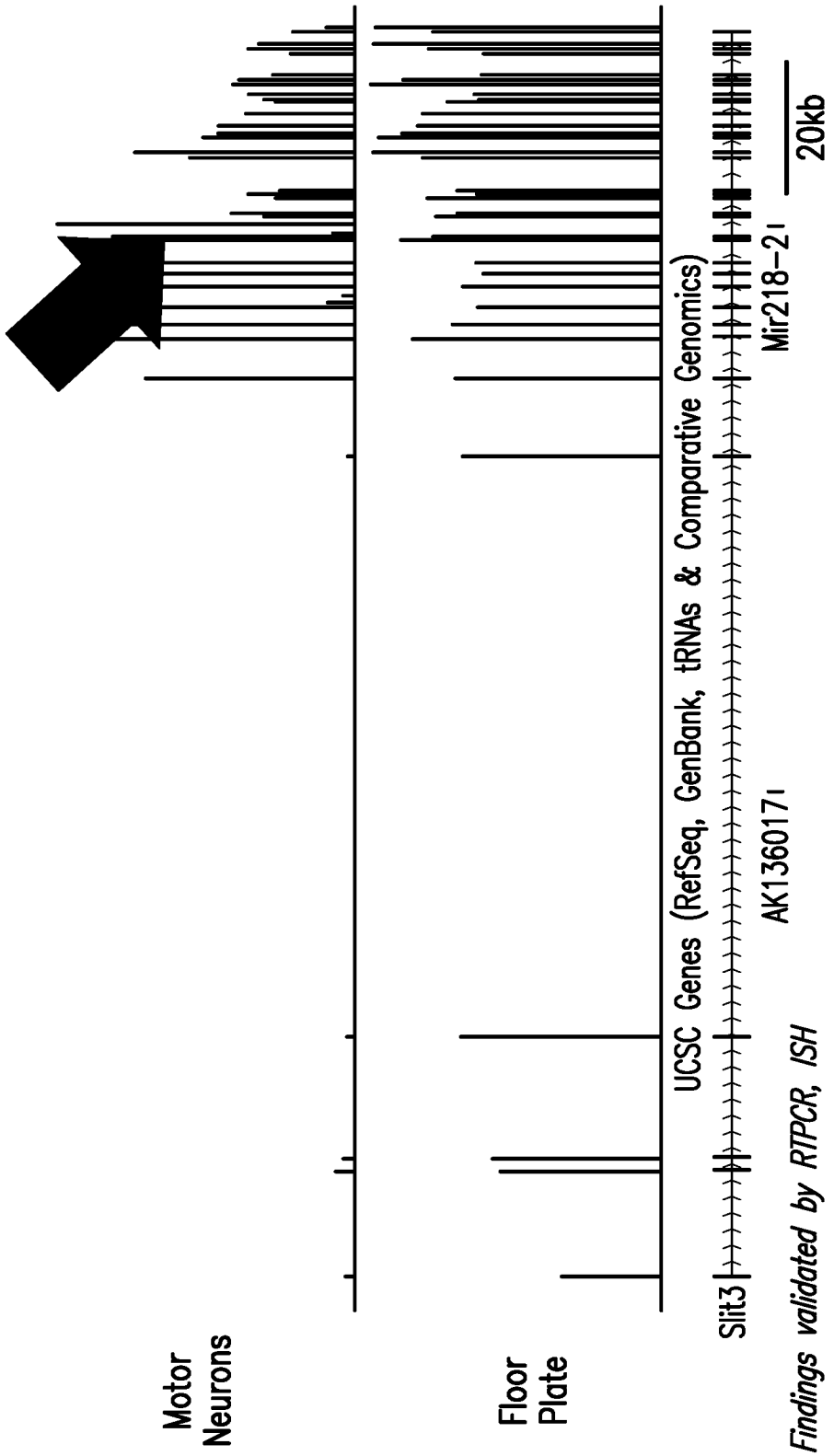


FIG. 14 Continued

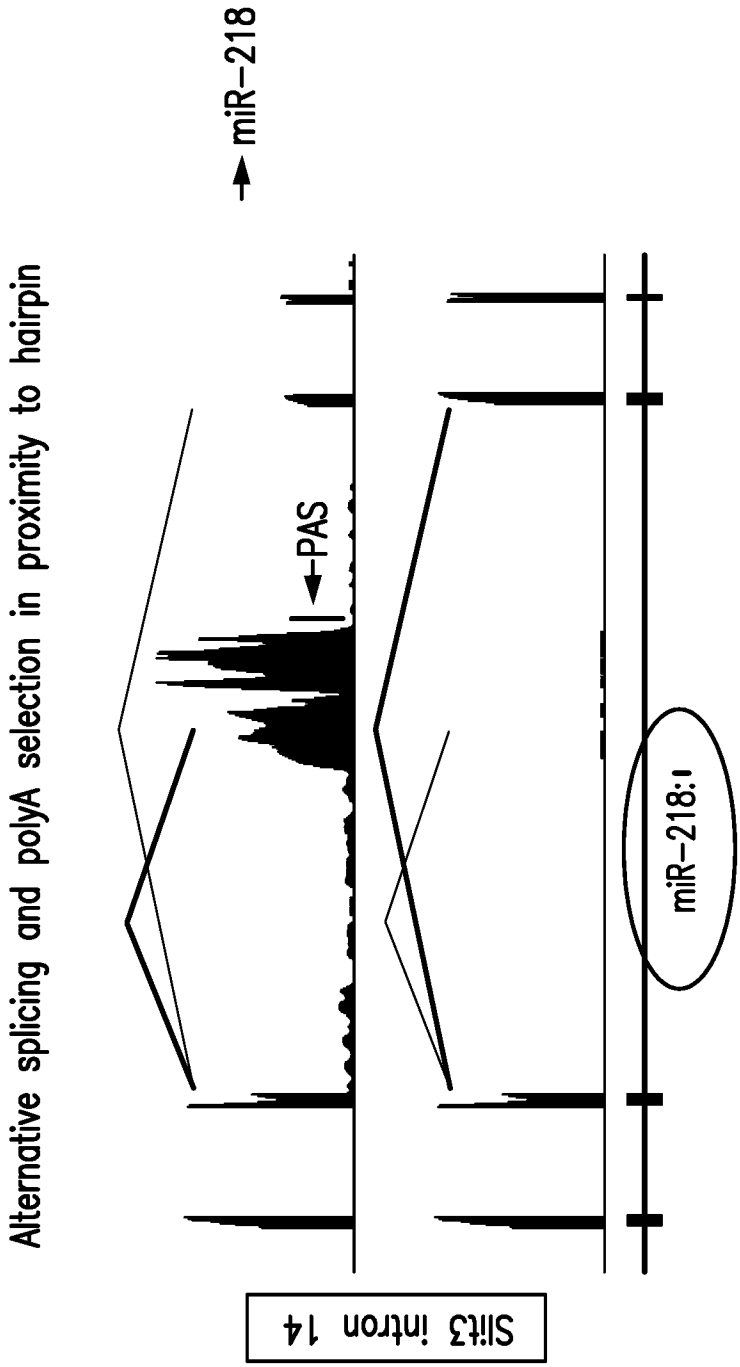


FIG. 14 Continued

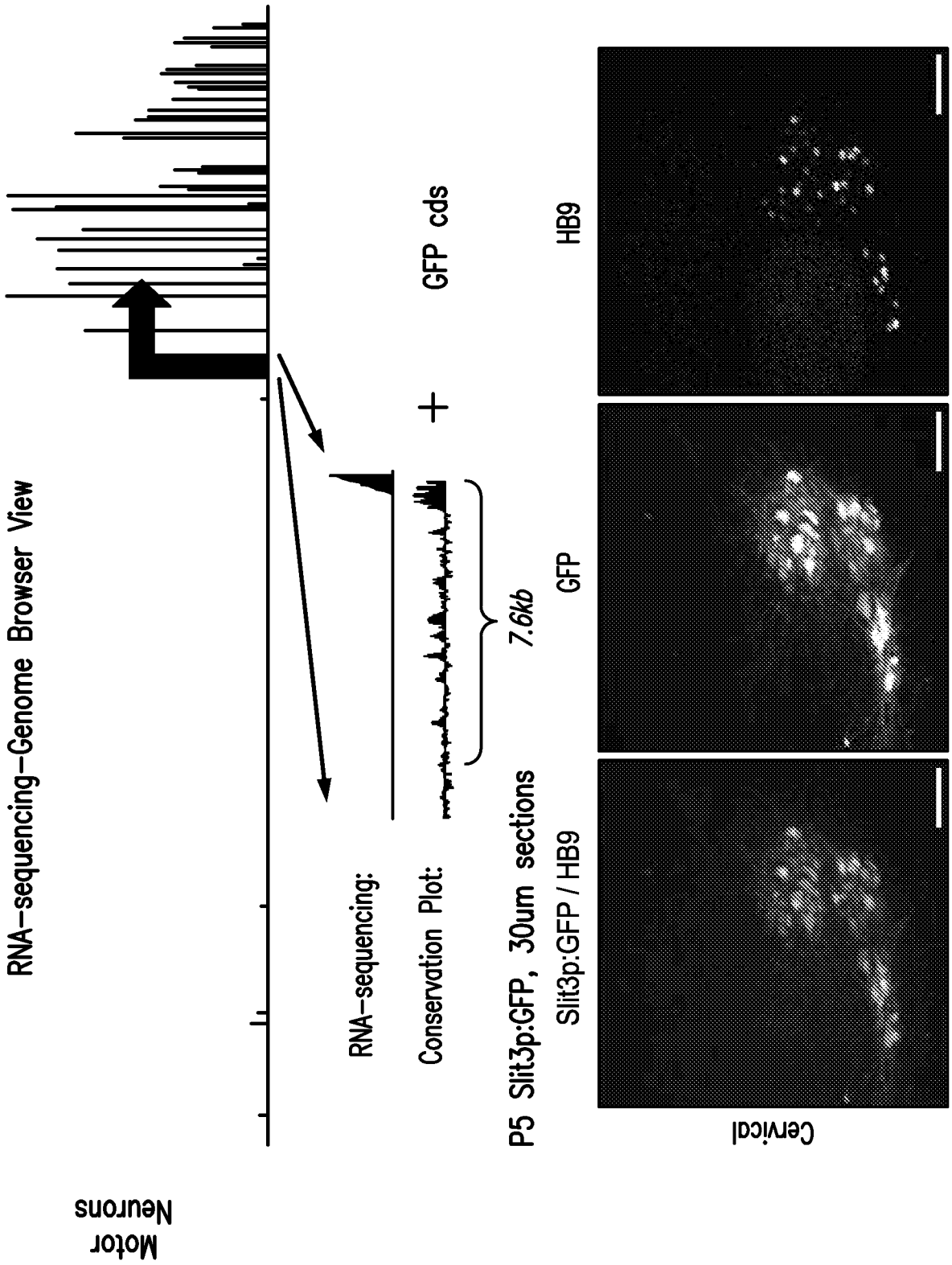


FIG. 14 Continued

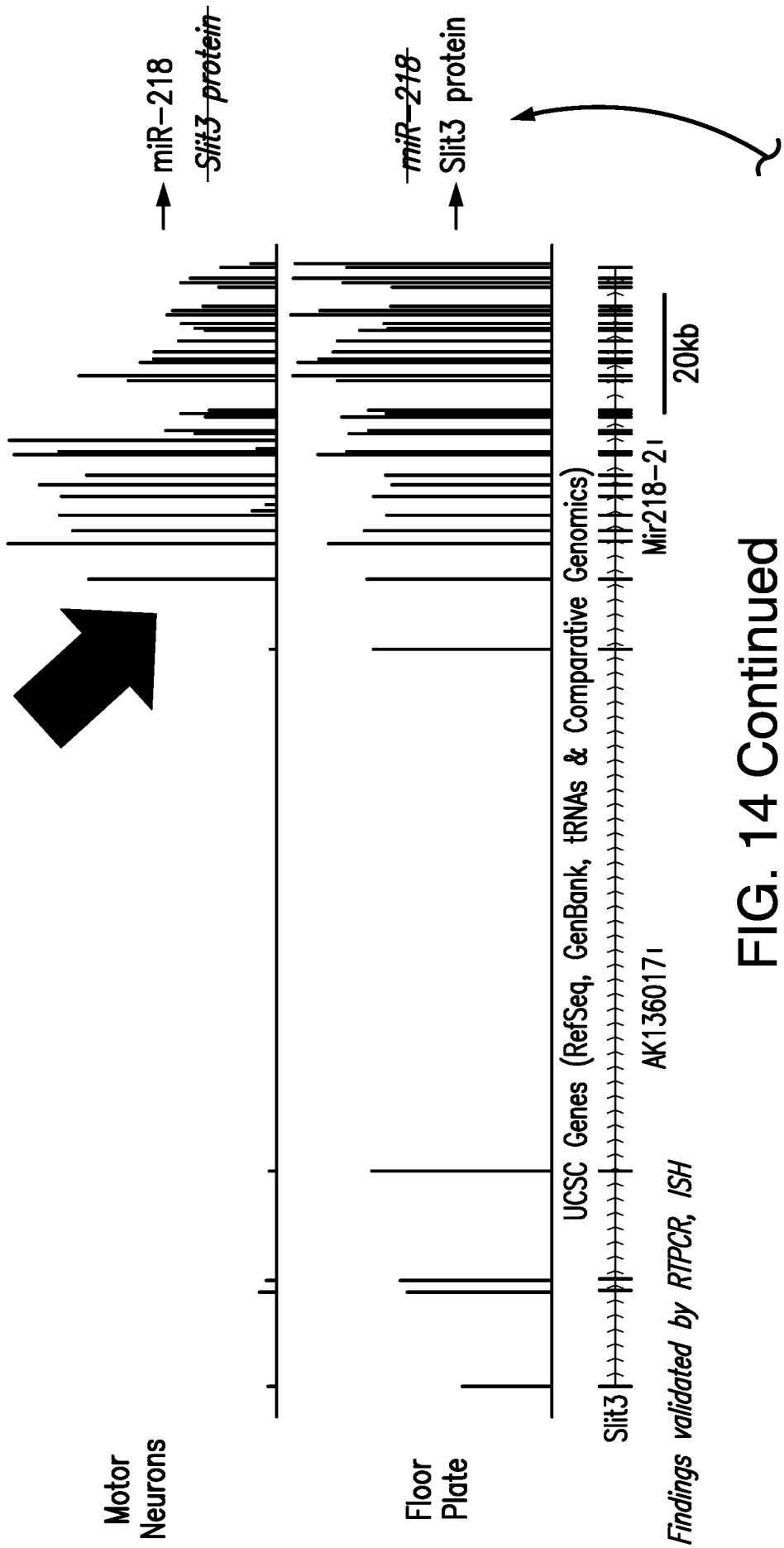


FIG. 14 Continued

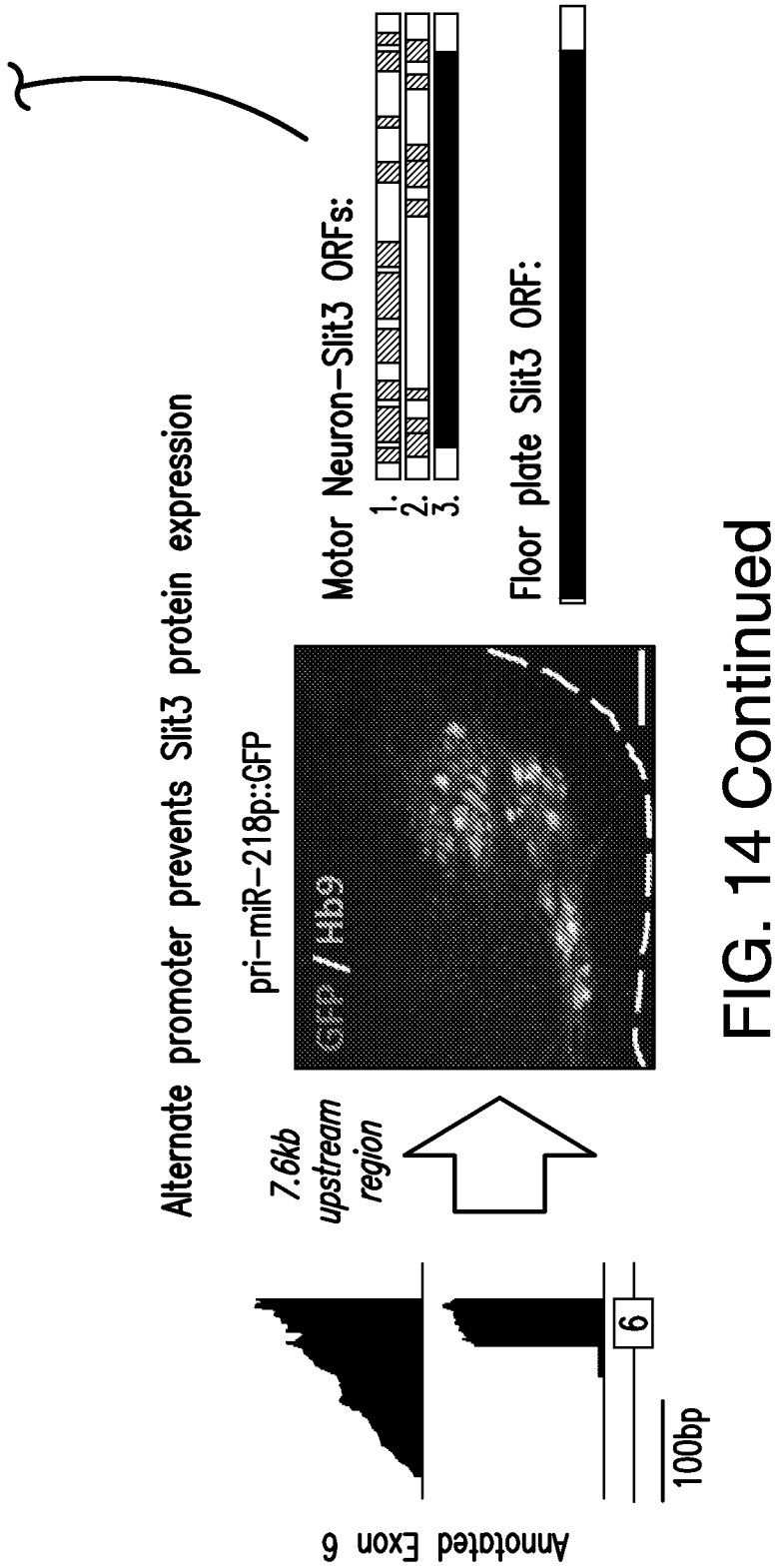


FIG. 14 Continued

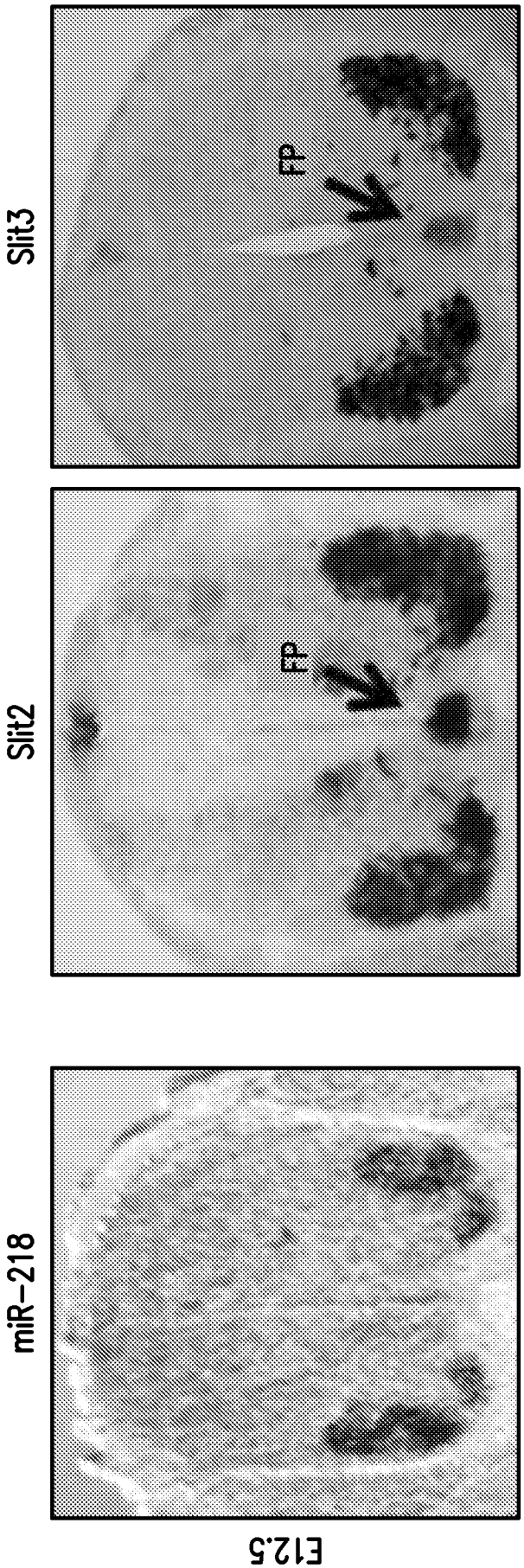


FIG. 15

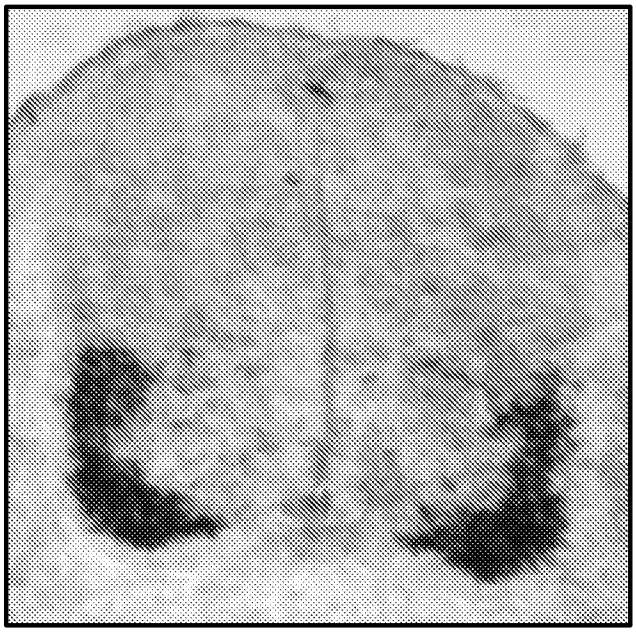


FIG. 15 Continued

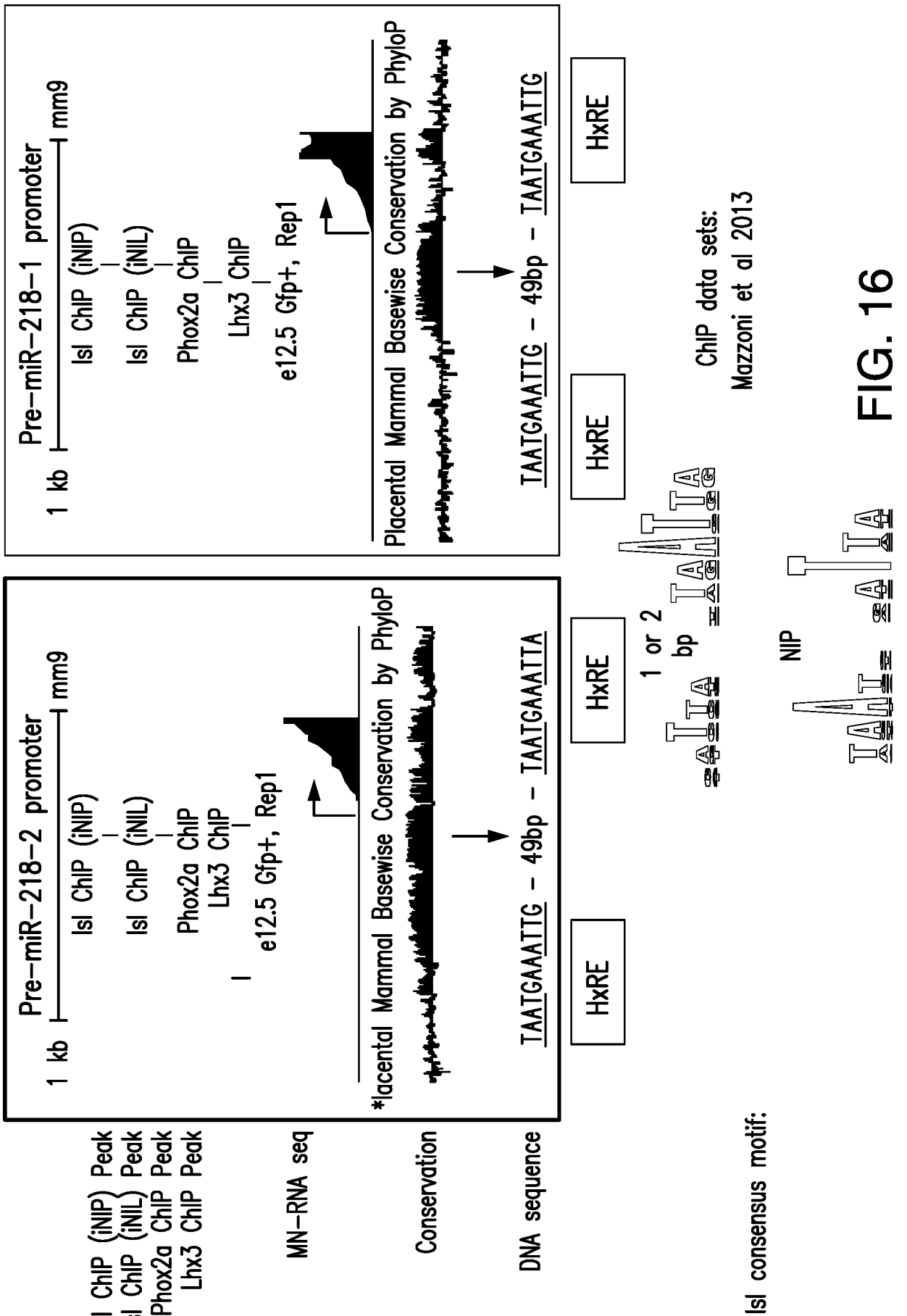

Pre-miR-218-1 promoter
1 kb
mm9
Isl ChIP (iNIP)
Isl ChIP (iNIL)
Phox2a ChIP
Lhx3 ChIP
e12.5 Gfp+, Rep1
Placental Mammal Basewise Conservation by PhyloP
TAATGAAATTG - 49bp - TAATGAAATTG
HxRE
HxRE
HxRE
Pre-miR-218-2 promoter
1 kb
mm9
Isl ChIP (iNIP)
Isl ChIP (iNIL)
Phox2a ChIP
Lhx3 ChIP
e12.5 Gfp+, Rep1
*lacental Mammal Basewise Conservation by PhyloP
TAATGAAATTG - 49bp - TAATGAAATTA
Isl ChIP (iNIP) Peak
Isl ChIP (iNIL) Peak
Phox2a ChIP Peak
Lhx3 ChIP Peak
MN-RNA seq
Conservation
DNA sequence
1 or 2 bp
ChIP data sets:
Mazzoni et al 2013
Isl consensus motif:
NIP

FIG. 16

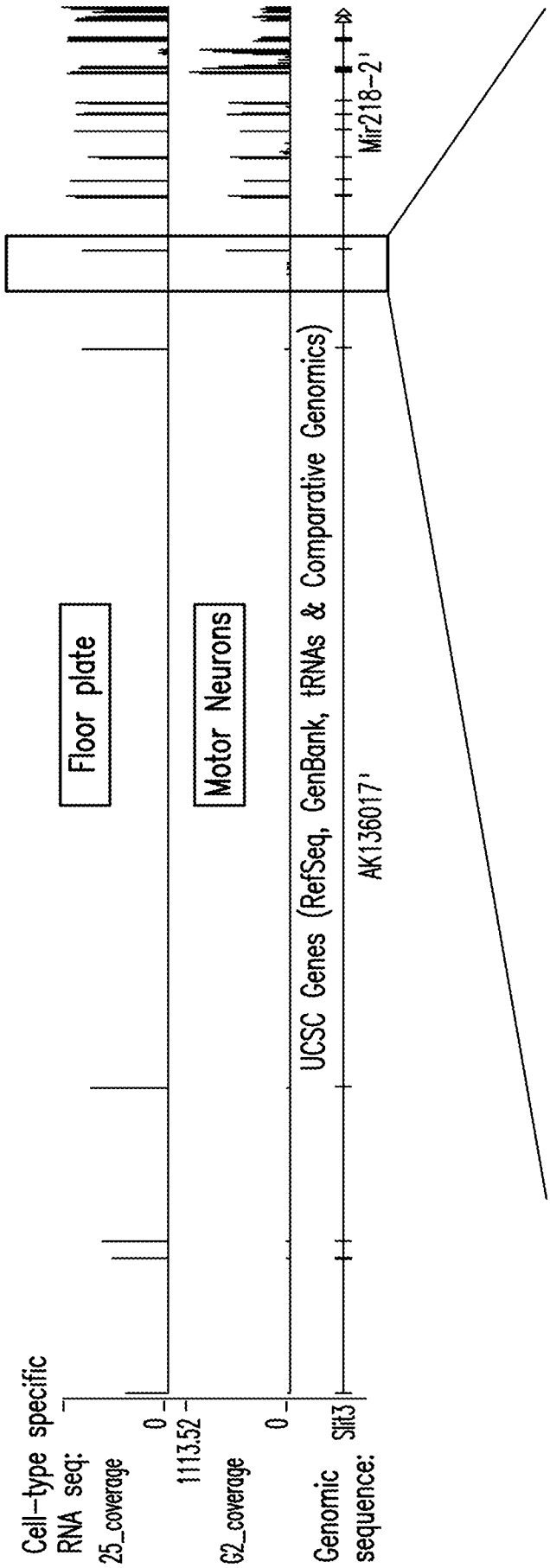

Cell-type specific RNA seq:
25_coverage
0
1113.52
G2_coverage
0
Genomic sequence:
Slit3
Floor plate
Motor Neurons
UCSC Genes (RefSeq, GenBank, tRNAs & Comparative Genomics)
AK136017
Mir218-2

FIG. 17

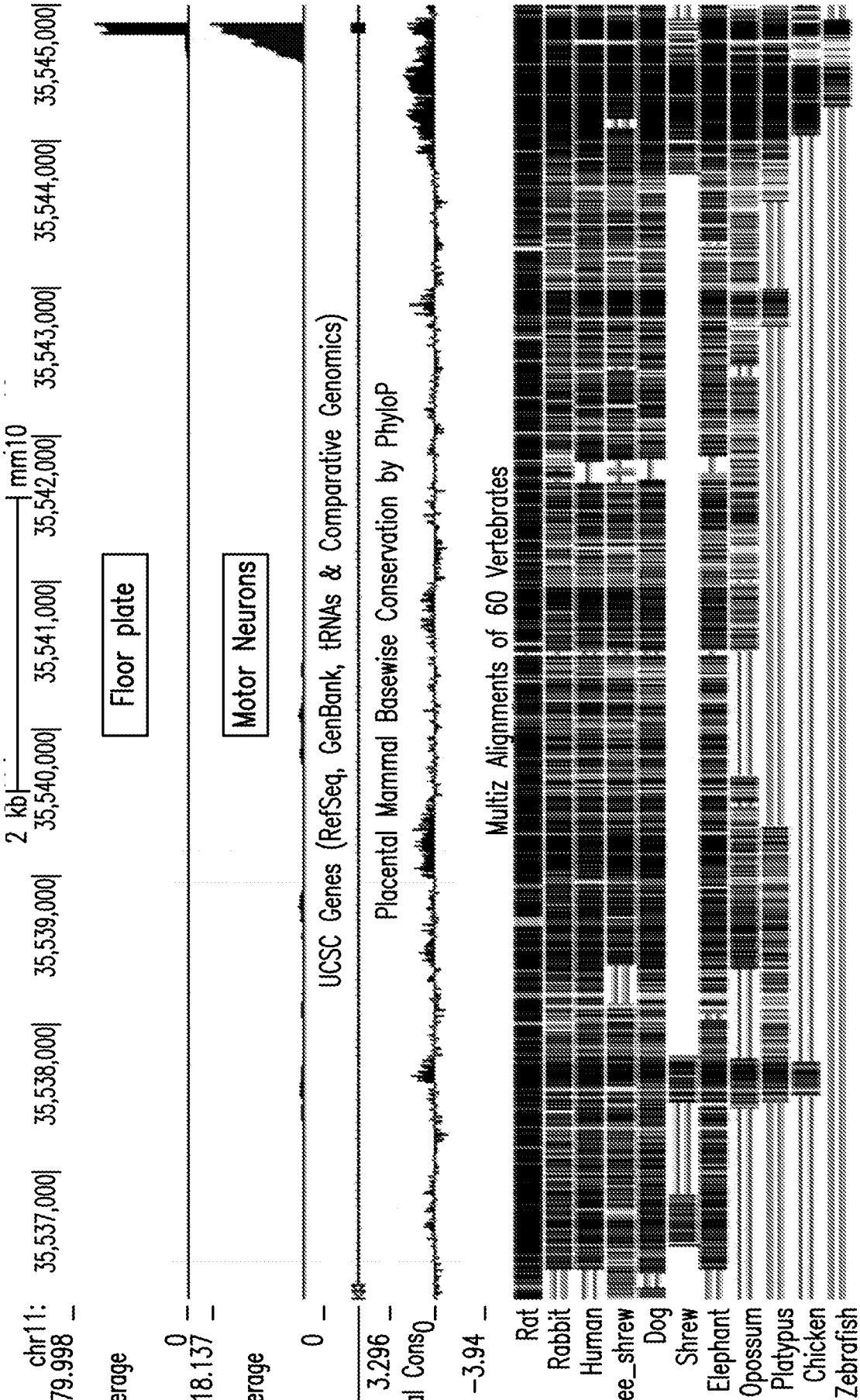


FIG. 17 Continued

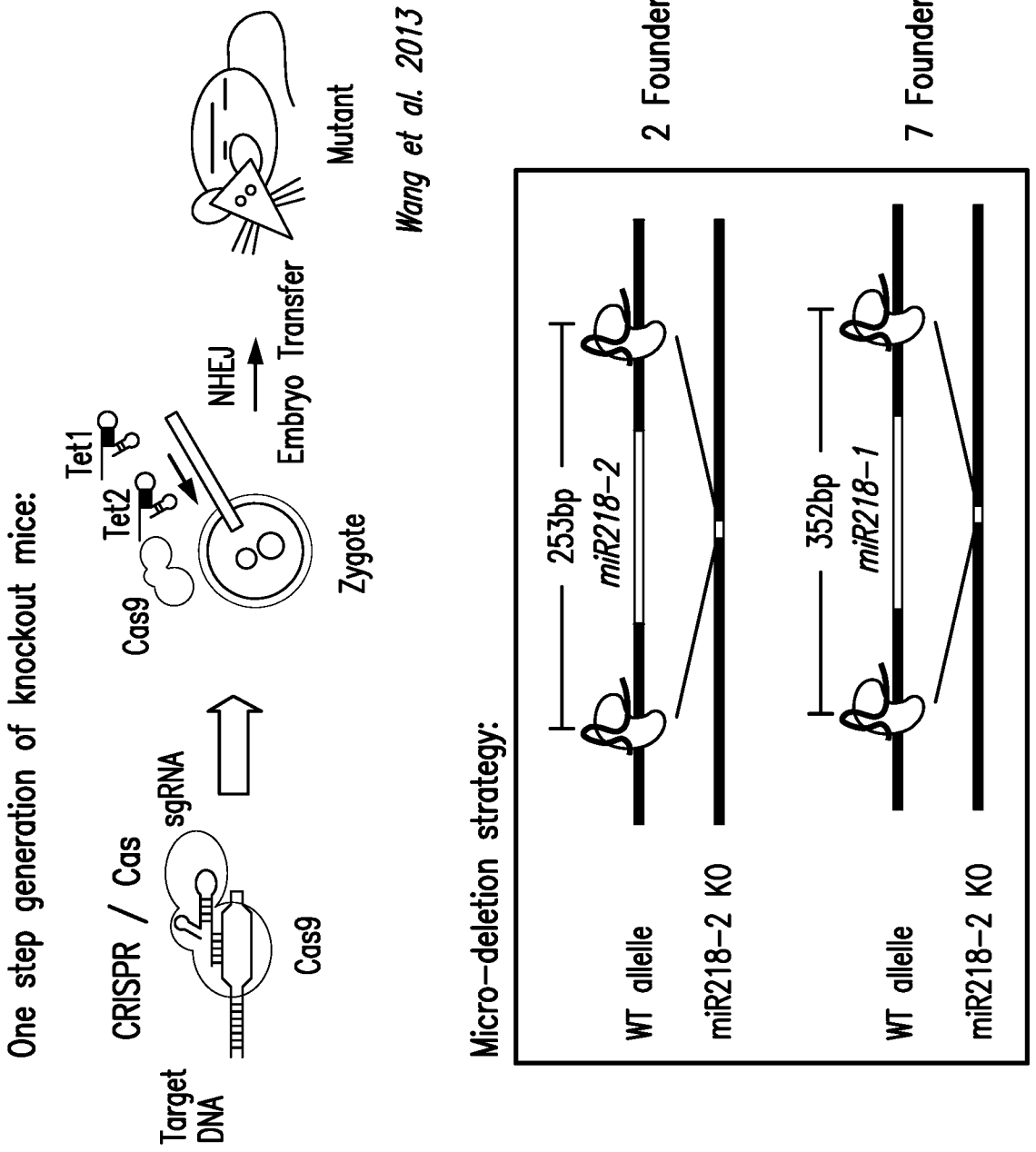

One step generation of knockout mice:
CRISPR / Cas
sgRNA
Cas9
Target DNA
Tet1
Tet2
Cas9
NHEJ
Embryo Transfer
Zygote
Mutant
Wang et al. 2013
Micro-deletion strategy:
253bp
miR218-2
WT allele
miR218-2 KO
2 Founders
352bp
miR218-1
WT allele
miR218-2 KO
7 Founders

FIG. 18

Genotype	Observed	Expected	Deaths:
WT	8	9	0 (0%)
Het	19	18	0 (0%)
218–2 KO	9	9	7 (77%)

(miR–218–1$^{-/-}$ 2$^{-/-}$ mice are pending) All deaths between P1–P3

- Morphologically normal at birth, no cyanosis
- Death possibly due to inability to suckle milk

Surviving mice (n=2) have motor weakness:

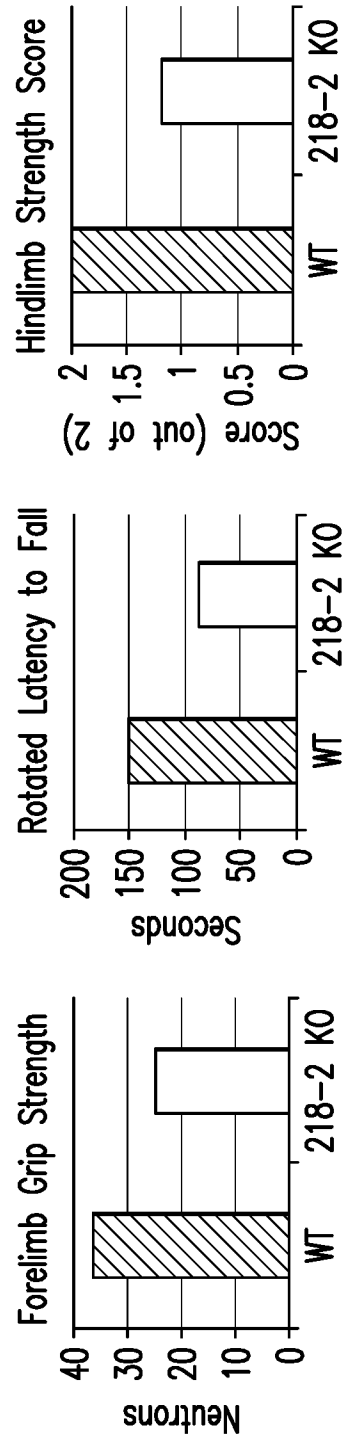
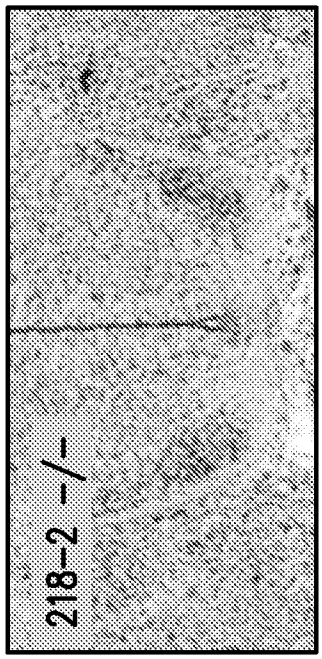


FIG. 19



Genotype	Observed (p0)	Expected	Deaths (p1-3):
WT	8	9	0 (0%)
Het	19	18	0 (0%)
KO	9	9	8 (88%)

FIG. 19 Continued

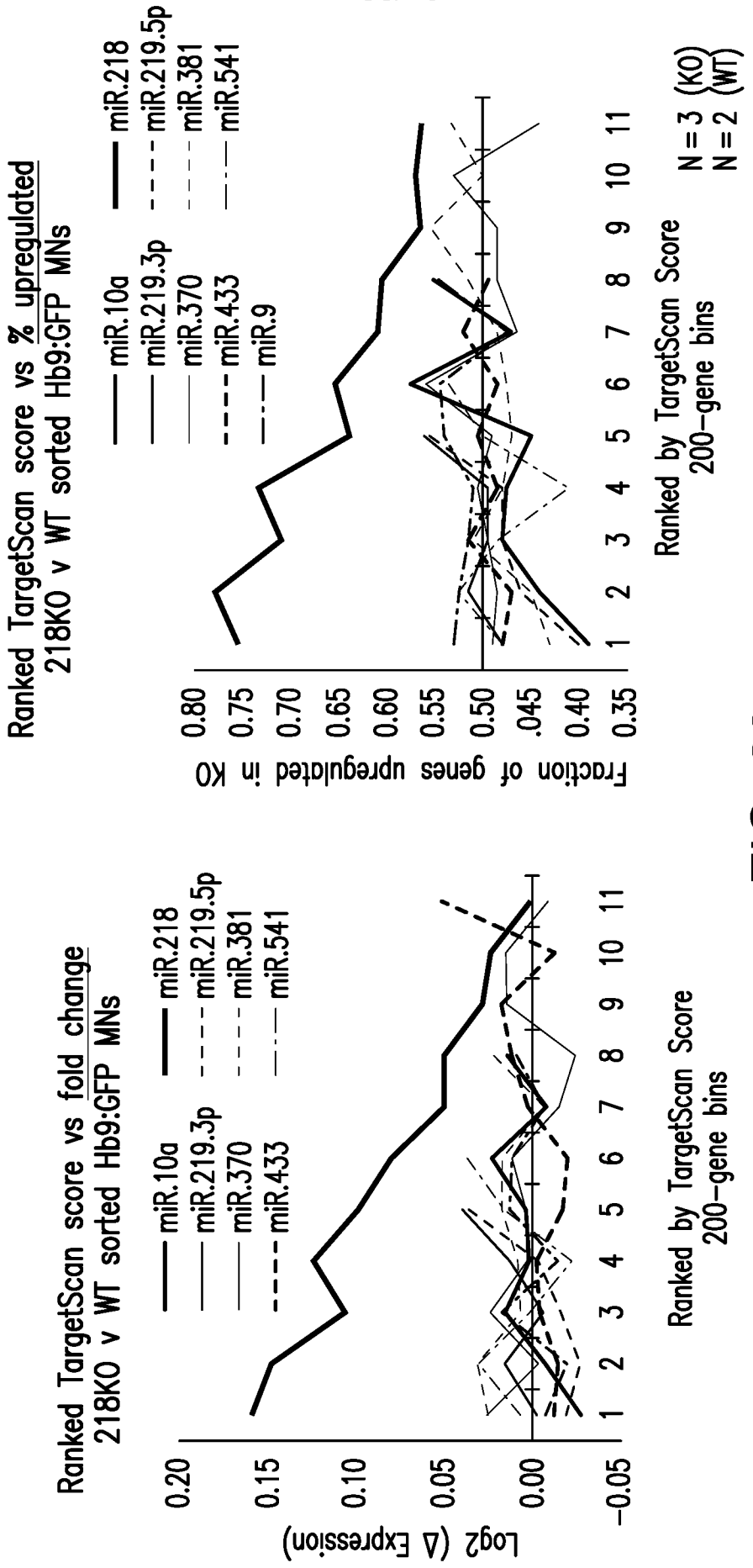

Ranked TargetScan score vs % upregulated
218KO v WT sorted Hb9:GFP MNs
miR.10a
miR.219.3p
miR.370
miR.433
miR.9
miR.218
miR.219.5p
miR.381
miR.541
Fraction of genes upregulated in KO
0.80
0.75
0.70
0.65
0.60
0.55
0.50
.045
0.40
0.35
1 2 3 4 5 6 7 8 9 10 11
Ranked by TargetScan Score
200-gene bins
N = 3 (KO)
N = 2 (WT)
Ranked TargetScan score vs fold change
218KO v WT sorted Hb9:GFP MNs
miR.10a
miR.219.3p
miR.370
miR.433
miR.218
miR.219.5p
miR.381
miR.541
Log2 (Δ Expression)
0.20
0.15
0.10
0.05
0.00
−0.05
1 2 3 4 5 6 7 8 9 10 11
Ranked by TargetScan Score
200-gene bins

FIG. 20

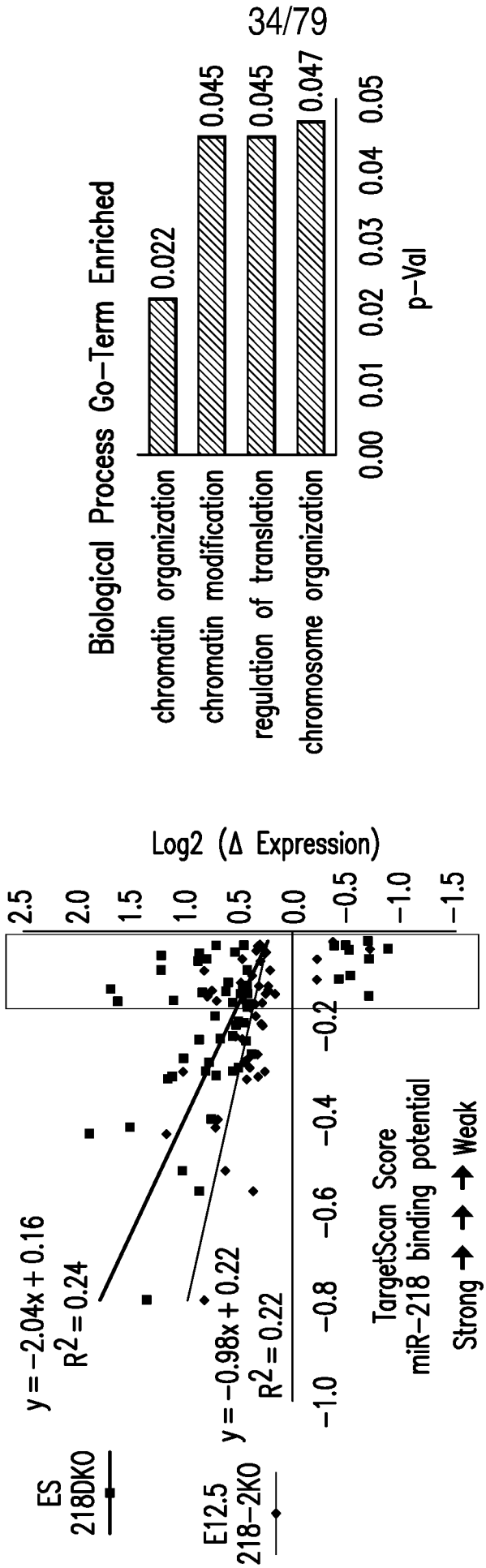

Biological Process Go-Term Enriched
chromatin organization
chromatin modification
regulation of translation
chromosome organization
0.022
0.045
0.045
0.047
0.00 0.01 0.02 0.03 0.04 0.05
p-Val
Log2 (Δ Expression)
2.5 2.0 1.5 1.0 0.5 0.0 −0.5 −1.0 −1.5
−1.0 −0.8 −0.6 −0.4 −0.2
TargetScan Score
miR-218 binding potential
Strong → → → Weak
ES
218DKO
E12.5
218-2KO
y = −2.04x + 0.16
R2 = 0.24
y = −0.98x + 0.22
R2 = 0.22

FIG. 21

Gene Name	Symbol	e12.5 %up	ESMN %up	TargetScan Score
one cut domain, family member 2	**Onecut2**	**78%**	**157%**	**−0.786**
predicted gene 9514; similar to sorting nexin 4; sorting nexin 4	Snx4	30%	83%	−0.553
glucuronyl C5−epimerase	Glce	55%	106%	−0.512
solute carrier family 1 (glutamatre reuptake)−EAAT2	Slc1a2	126%	273%	−0.431
synaptotagmin XIII	Syt13	66%	187%	−0.417
Ras association (RalGDS/AF−6) domain family member2	Rassf2	63%	70%	−0.401
YEATS domain containing 4	Yeats4	35%	125%	−0.314
Bmi1 polycomb ring finger oncogene	Bmi1	25%	119%	−0.309
expressed sequence AW146242	Vopp1	66%	65%	−0.308
one cut domain, family member 3	**Onecut3**	**104%**	**76%**	**−0.297**
splicing factor, arginine/serine−rich 6	Srsf6	20%	48%	−0.297
cytospinA	Specc1l	32%	43%	−0.291
guanine nucleotide binding protein (G protein), gamma 3	Gng3	37%	72%	−0.278
regulator of chromosome condensation (RCC1) and BTB (POZ) domain containing protein 1	Rcbtb1	33%	103%	−0.272
lysine (K)−specific demethylase 5A	Kdm5a	26%	31%	−0.261

FIG. 21 Continued

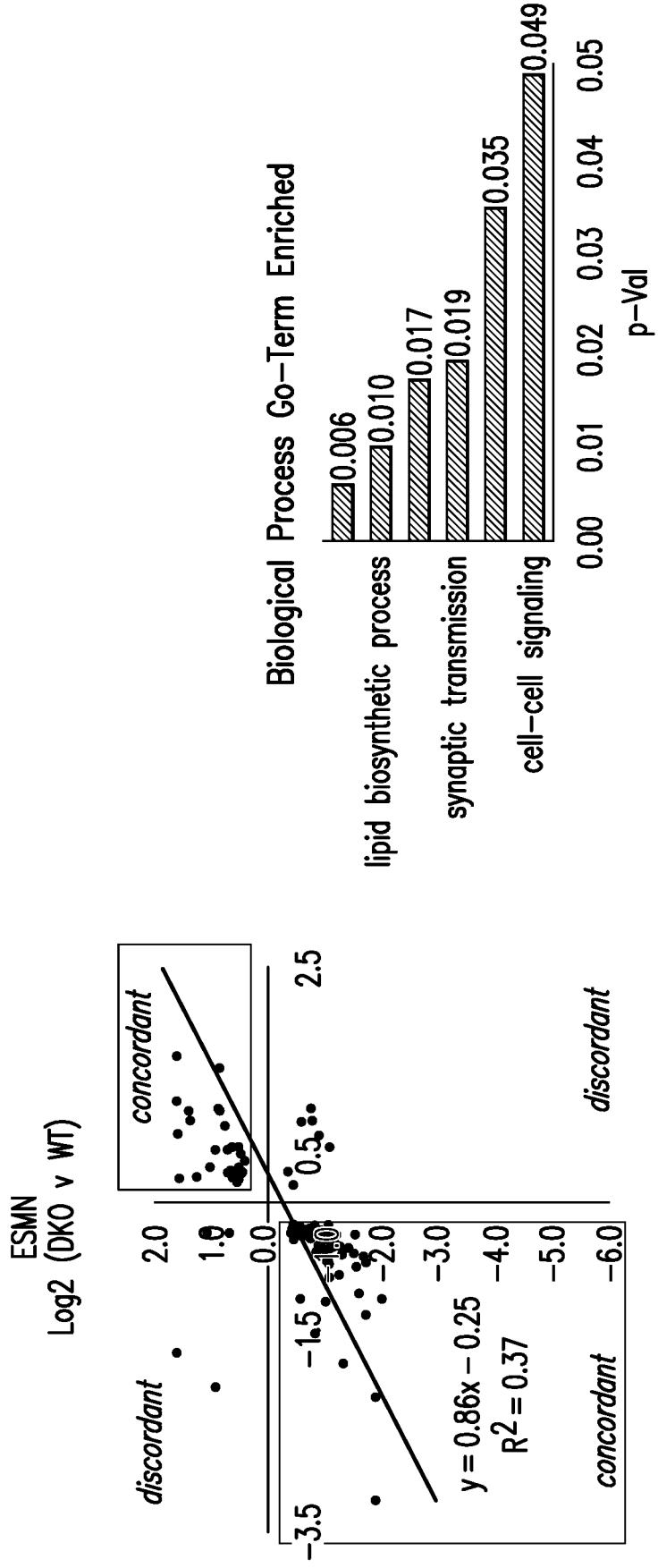
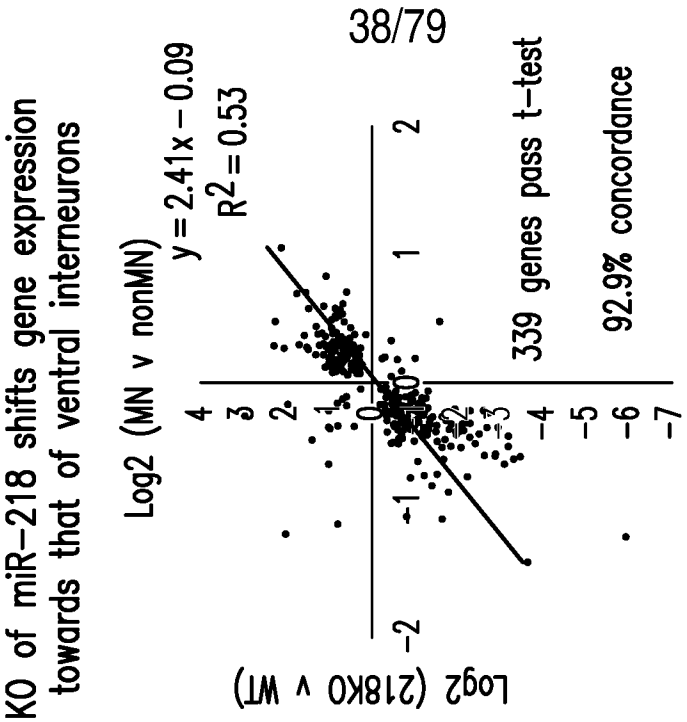

Biological Process Go-Term Enriched
lipid biosynthetic process
synaptic transmission
cell-cell signaling
0.006
0.010
0.017
0.019
0.035
0.049
0.00 0.01 0.02 0.03 0.04 0.05
p-Val
ESMN
Log2 (DKO v WT)
E12.5
Log2 (-2KO v WT)
concordant
discordant
discordant
concordant
y = 0.86x - 0.25
R2 = 0.37
2.0 1.0 0.0 -1.0 -2.0 -3.0 -4.0 -5.0 -6.0
2.5 0.5 -1.5 -3.5
90% of 'indirect' targets are dysregulated in the same direction (concordance) in vitro and in vivo by miR-218KO

FIG. 22

Synaptic-related indirect targets are downregulated in the absence of miR-218

Symbol	Gene name	Function	e12.5 %down	ESMN %down
Nrp1	neuropilin 1	Glycoprotein receptor	-10%	-23%
Pygb	brain glycogen phosphorylase	Glycogen degradation	-11%	-20%
Magee1	melanoma antigen, family E, 1	a-dystrobrevin associated	-11%	-16%
Syngr3	synaptogyrin 3	Ca++ dependent exocytosis	-12%	-23%
Cplx1	complexin 1	SNARE complex	-12%	-24%
Stx1a	syntaxin 1A (brain)	SNARE complex	-12%	-30%
Ache	**acetylcholinesterase**	**Acetylcholine turnover**	**-15%**	**-31%**
Sigmar1	sigmanon-opioid intracellular receptor 1	ALS gene-chaperone protein	-17%	-27%
Adora	adenosineA1 receptor	Target of sedatives	-20%	-36%
Trpv2	transient receptor potential cation channel V2	Mechano receptor	-20%	-34%
Sv2c	synaptic vesicle glycoprotein 2c	NMJ localized	-24%	-27%
Prph	peripherin	ALS gene-Int. Filament	-32%	-38%
Qk	similar to Quaking protein; quaking	KO results in seizures	-41%	-40%

FIG. 22 Continued


KO of miR-218 shifts gene expression towards that of ventral interneurons
Log2 (MN v nonMN)
y = 2.41x − 0.09
R2 = 0.53
339 genes pass t-test
92.9% concordance
Log2 (218KO v WT)

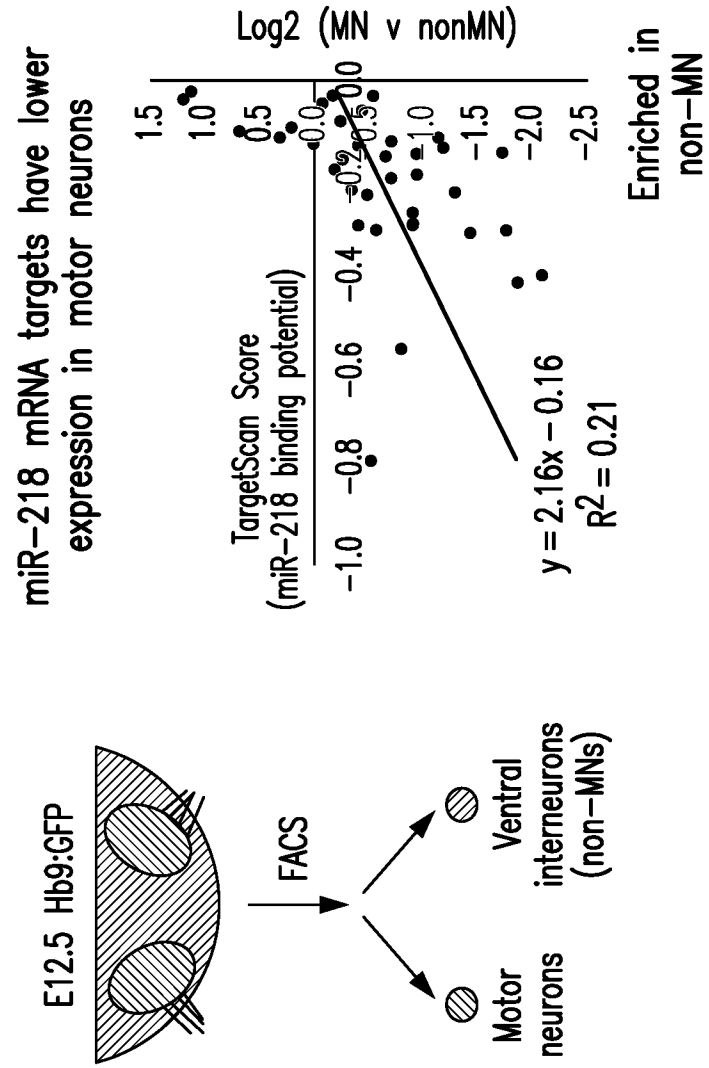

miR-218 mRNA targets have lower expression in motor neurons
Log2 (MN v nonMN)
TargetScan Score
(miR-218 binding potential)
y = 2.16x − 0.16
R2 = 0.21
Enriched in non-MN
E12.5 Hb9:GFP
FACS
Motor neurons
Ventral interneurons (non-MNs)

FIG. 23

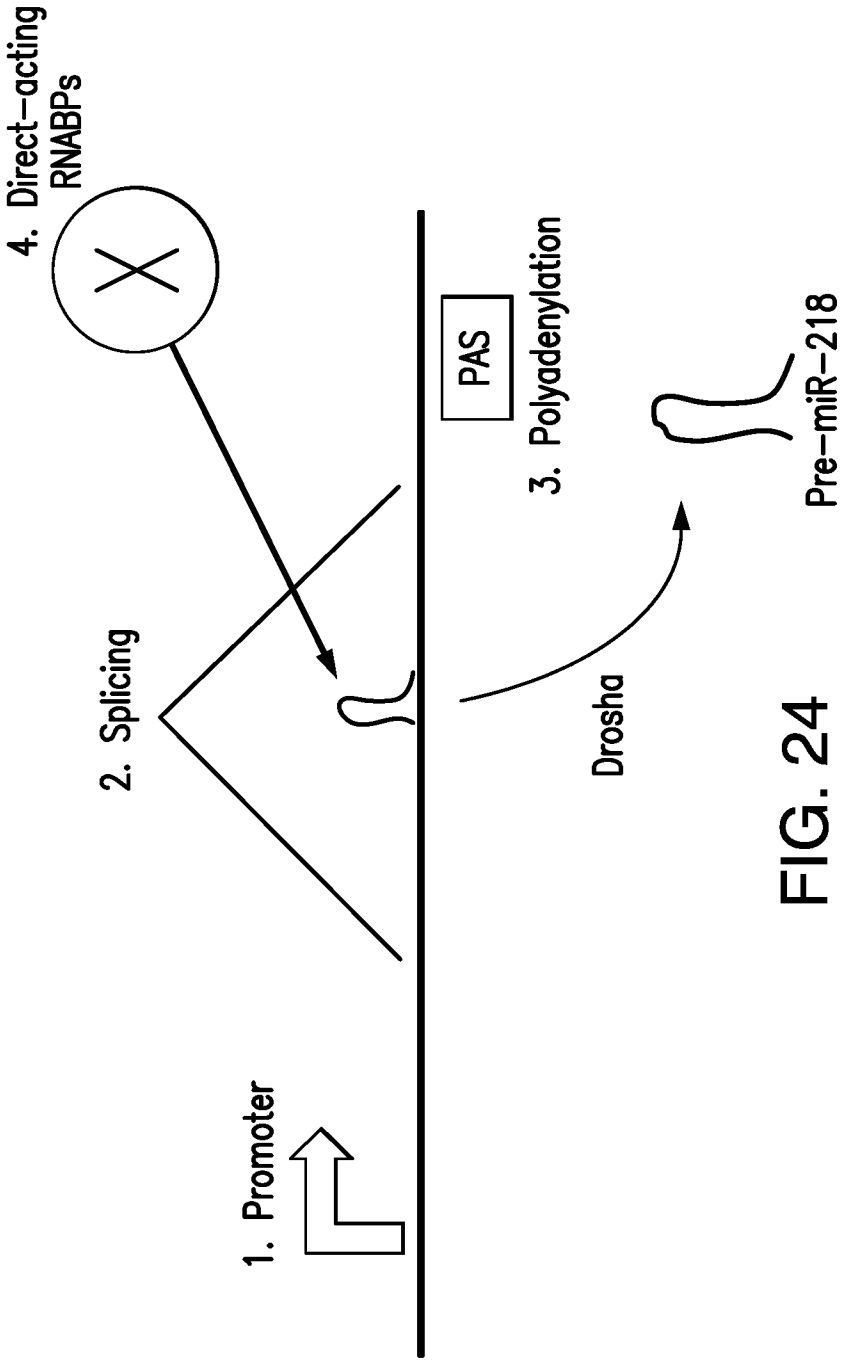

4. Direct-acting RNABPs
2. Splicing
1. Promoter
PAS
3. Polyadenylation
Drosha
Pre-miR-218

FIG. 24

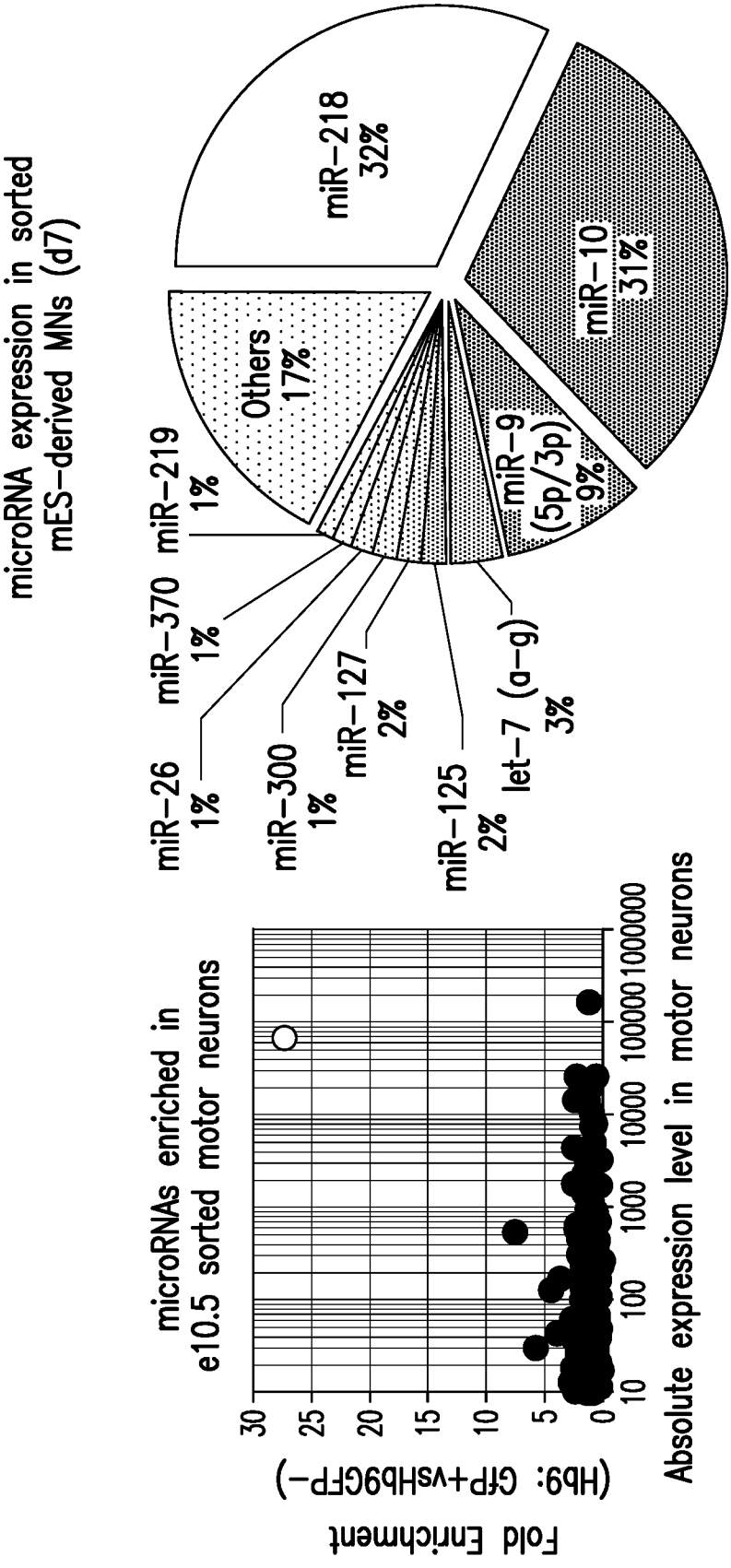

microRNA expression in sorted
mES-derived MNs (d7)
miR-218
32%
miR-10
31%
miR-9
(5p/3p)
9%
let-7 (a-g)
3%
miR-125
2%
miR-127
2%
miR-300
1%
miR-26
1%
miR-370
1%
miR-219
1%
Others
17%
microRNAs enriched in
e10.5 sorted motor neurons
Fold Enrichment
(Hb9: GFP+vsHb9GFP-)
30
25
20
15
10
5
0
10
100
1000
10000
100000
1000000
Absolute expression level in motor neurons

FIG. 25

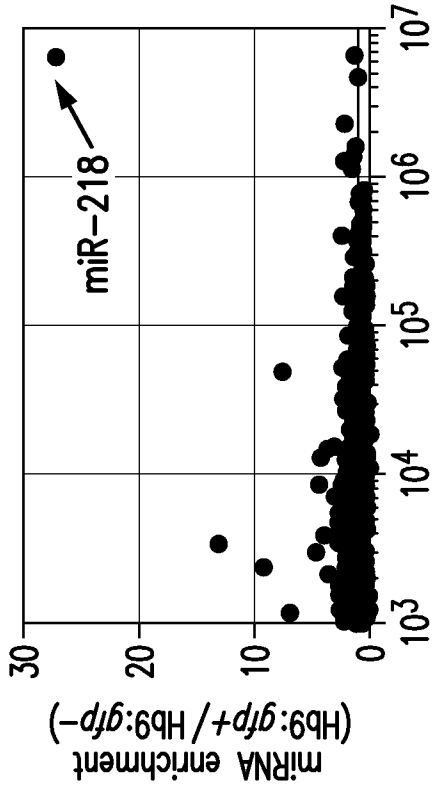


FIG. 26A

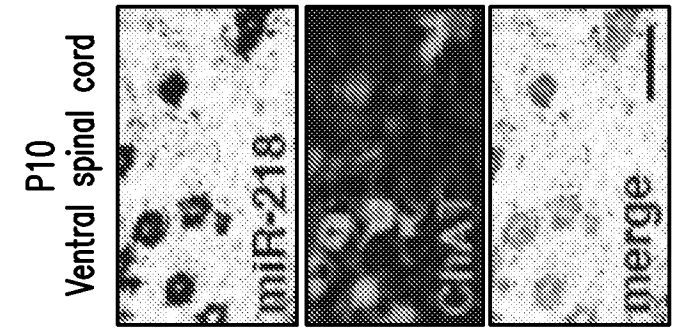


FIG. 26C

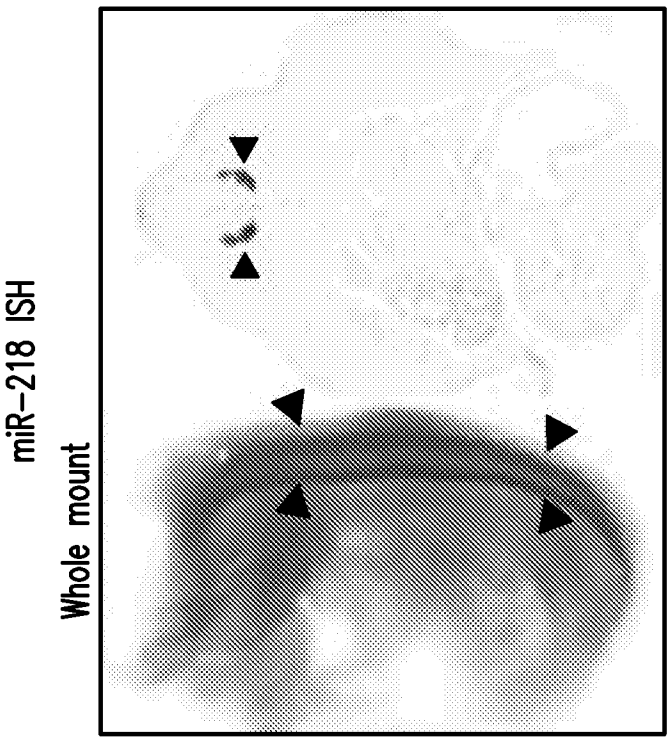


FIG. 26B

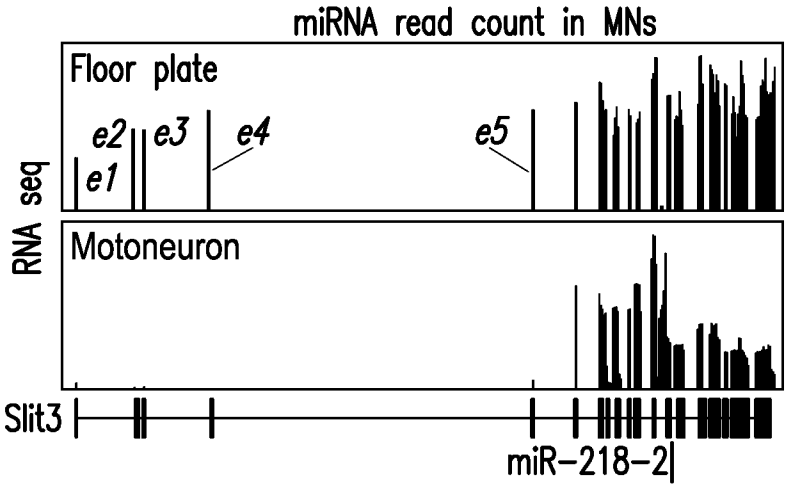


FIG. 26D

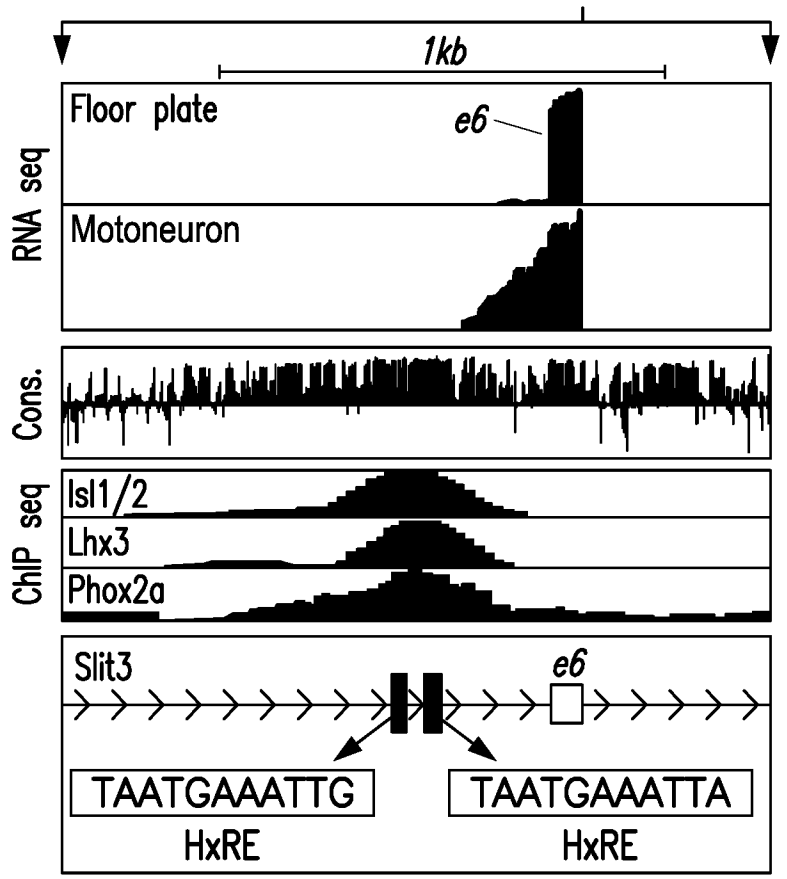


FIG. 26E

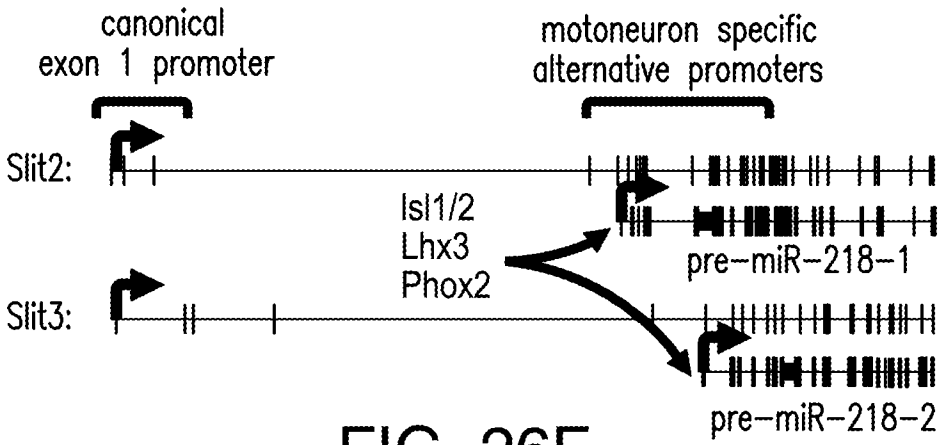


FIG. 26F

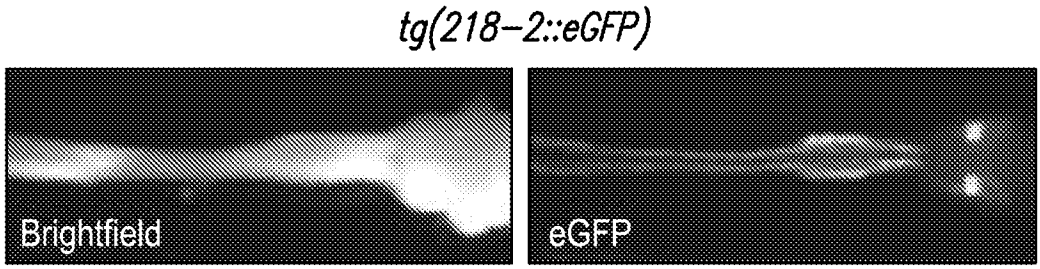


FIG. 26G

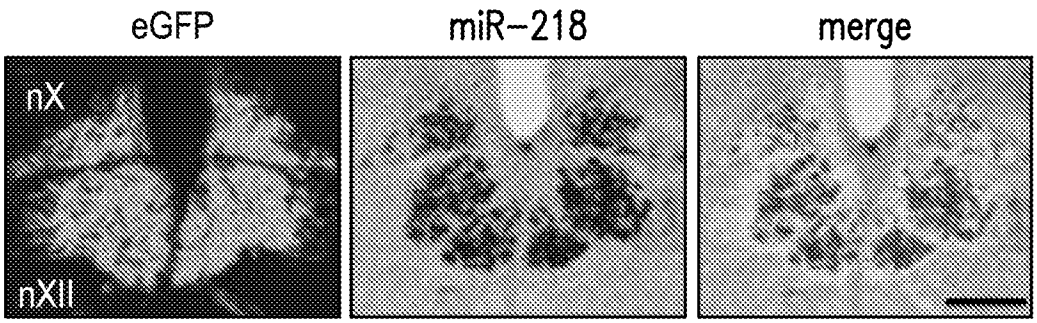


FIG. 26H

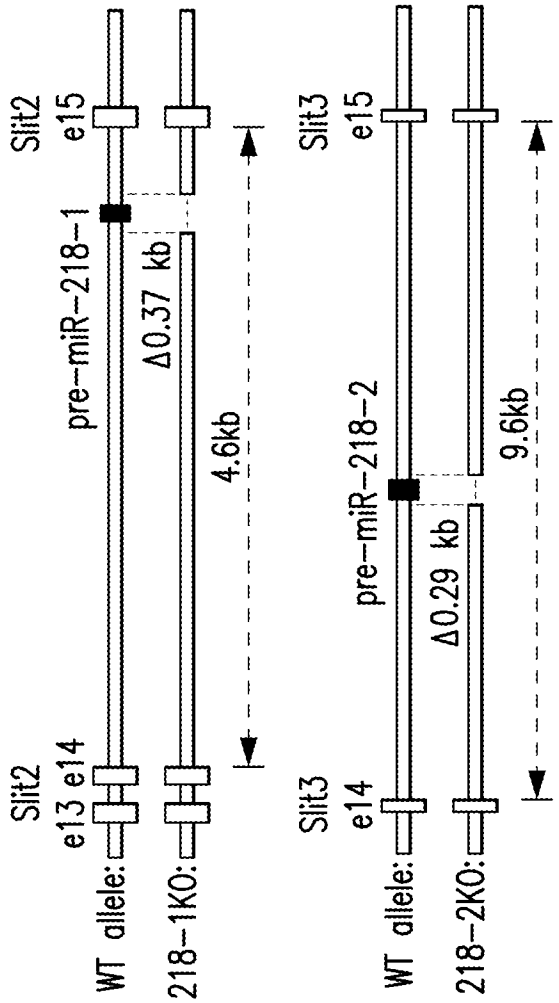


FIG. 27A

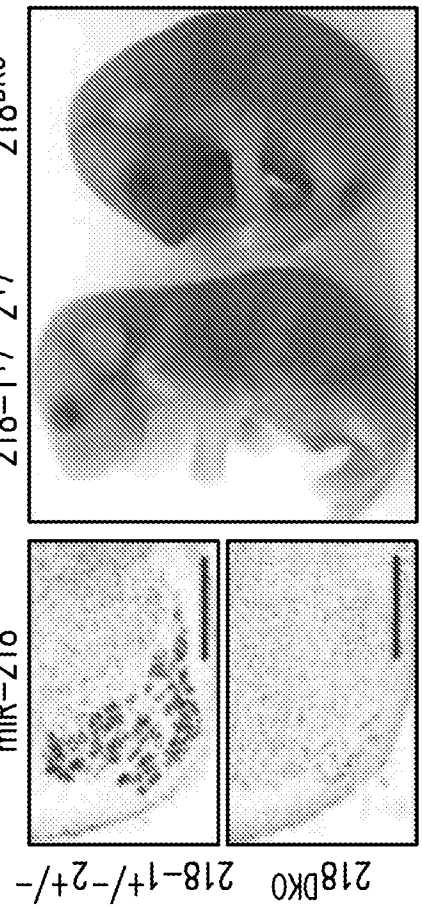


FIG. 27B

FIG. 27C

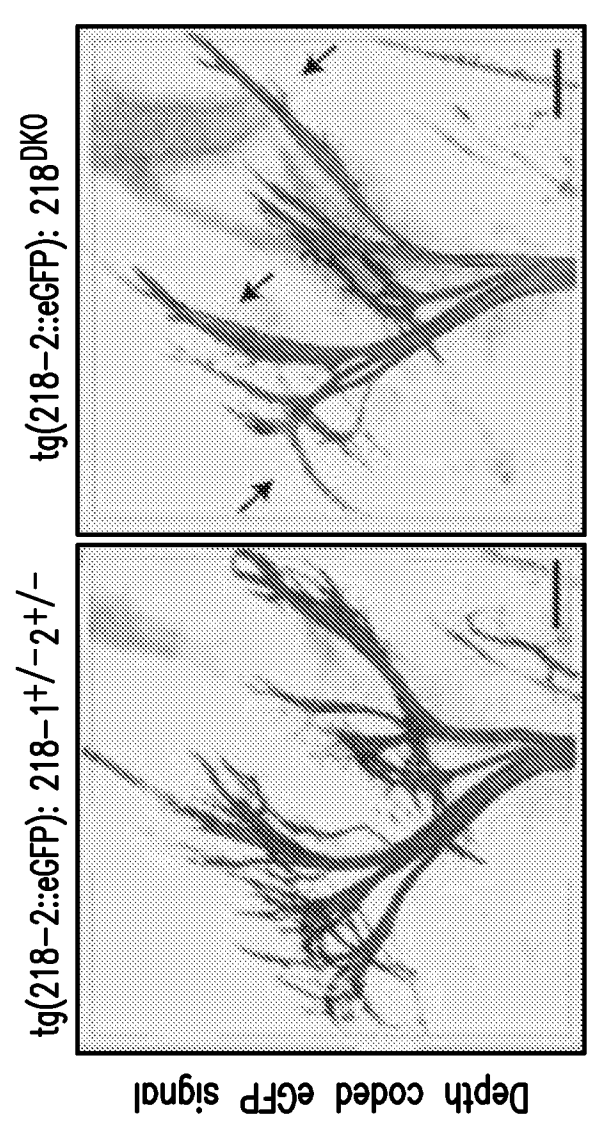


FIG. 27D

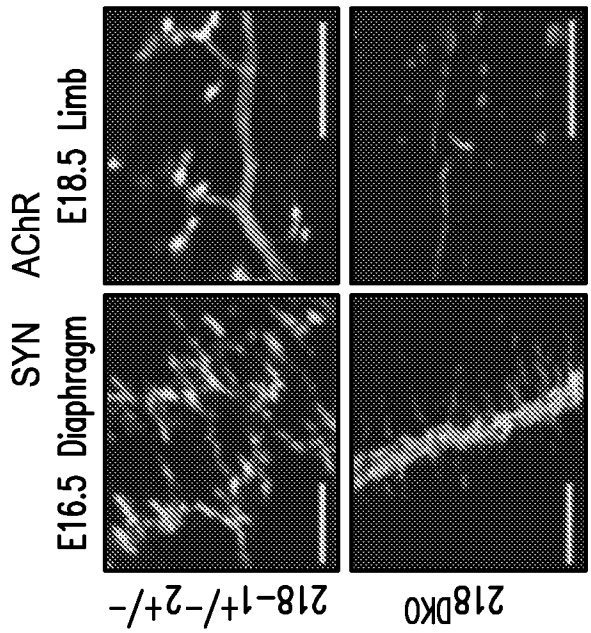


FIG. 27E

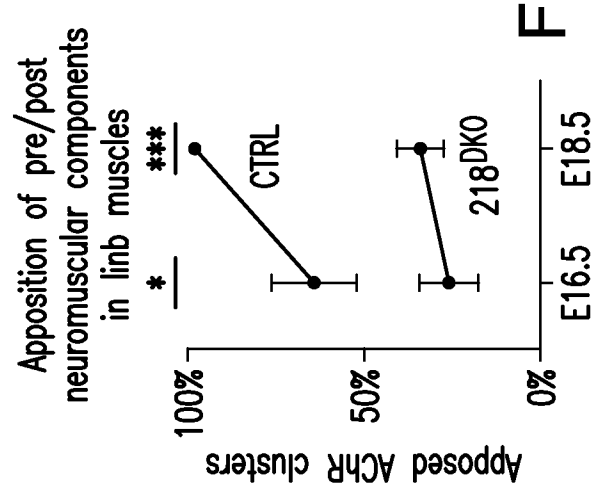


FIG. 27F

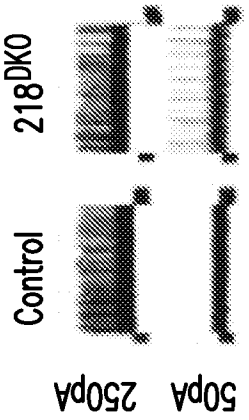


FIG. 27I

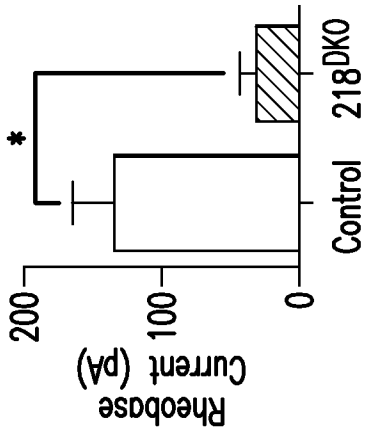


FIG. 27J

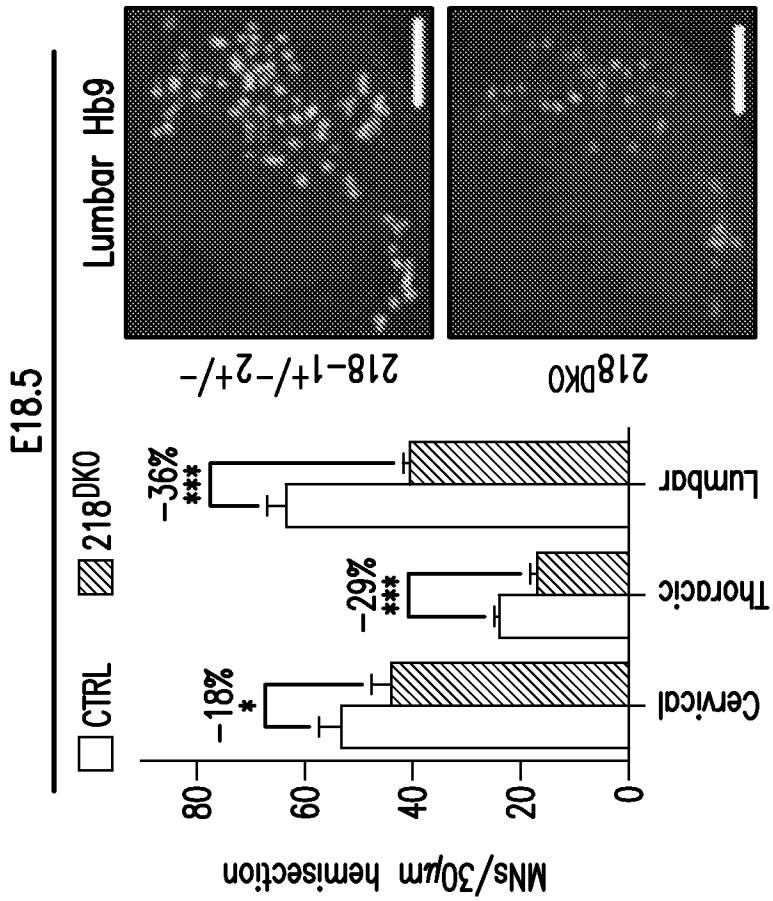


FIG. 27H

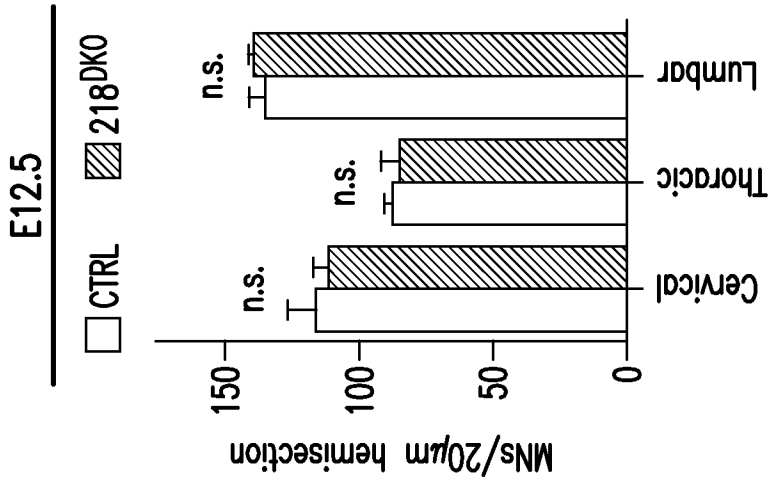


FIG. 27G

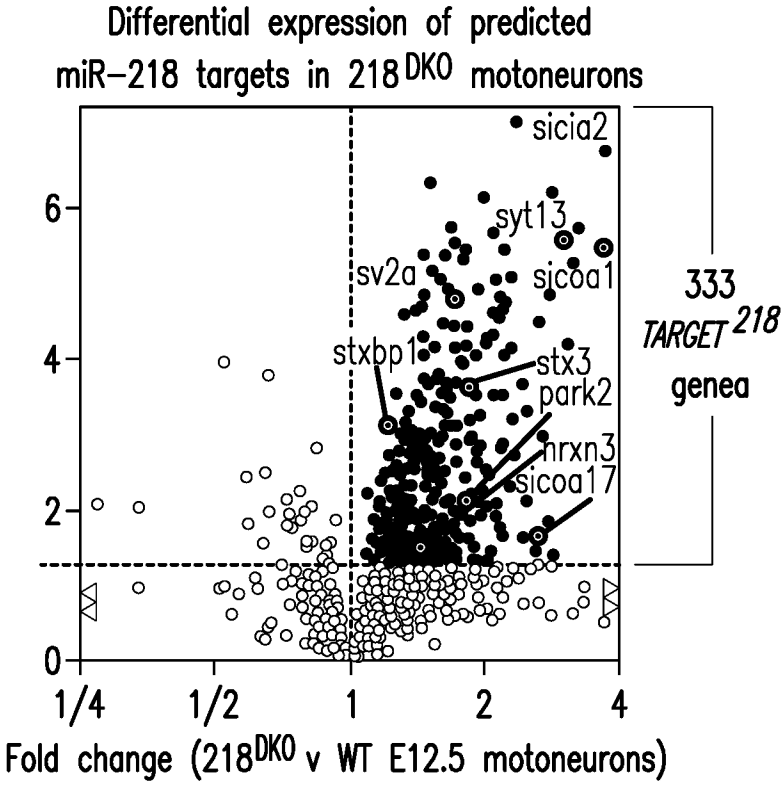


FIG. 28A

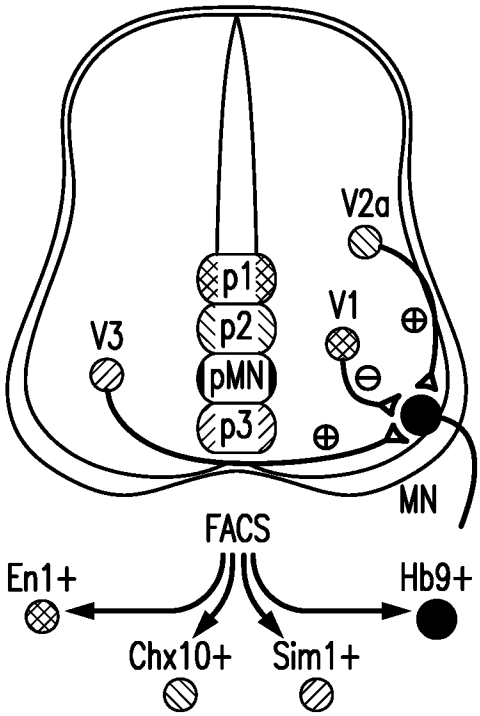


FIG. 28B

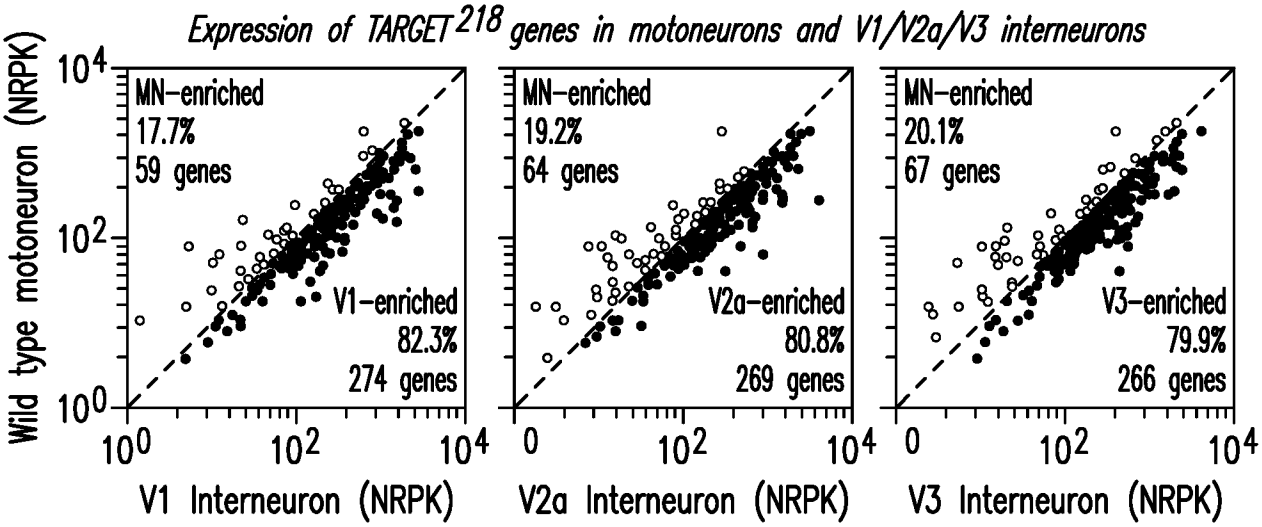


FIG. 28C

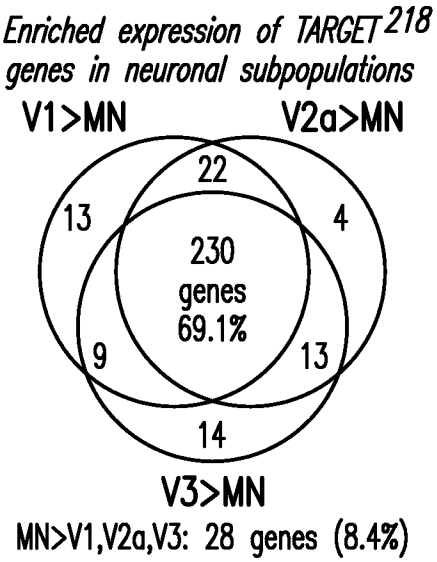


FIG. 28D

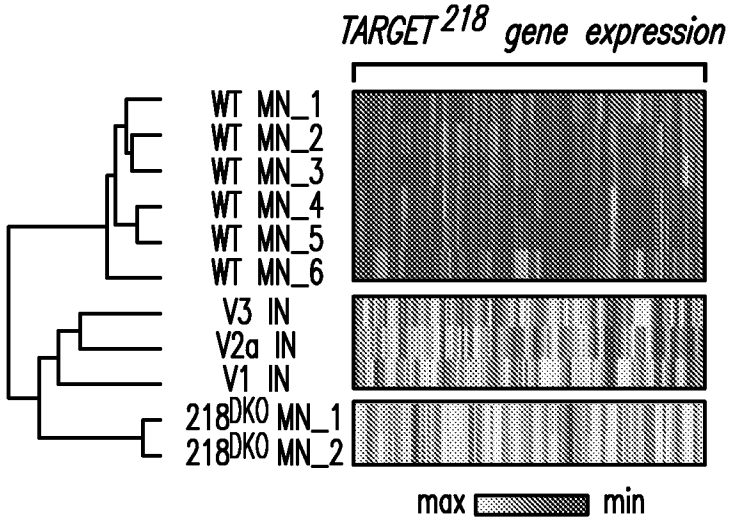


FIG. 28E

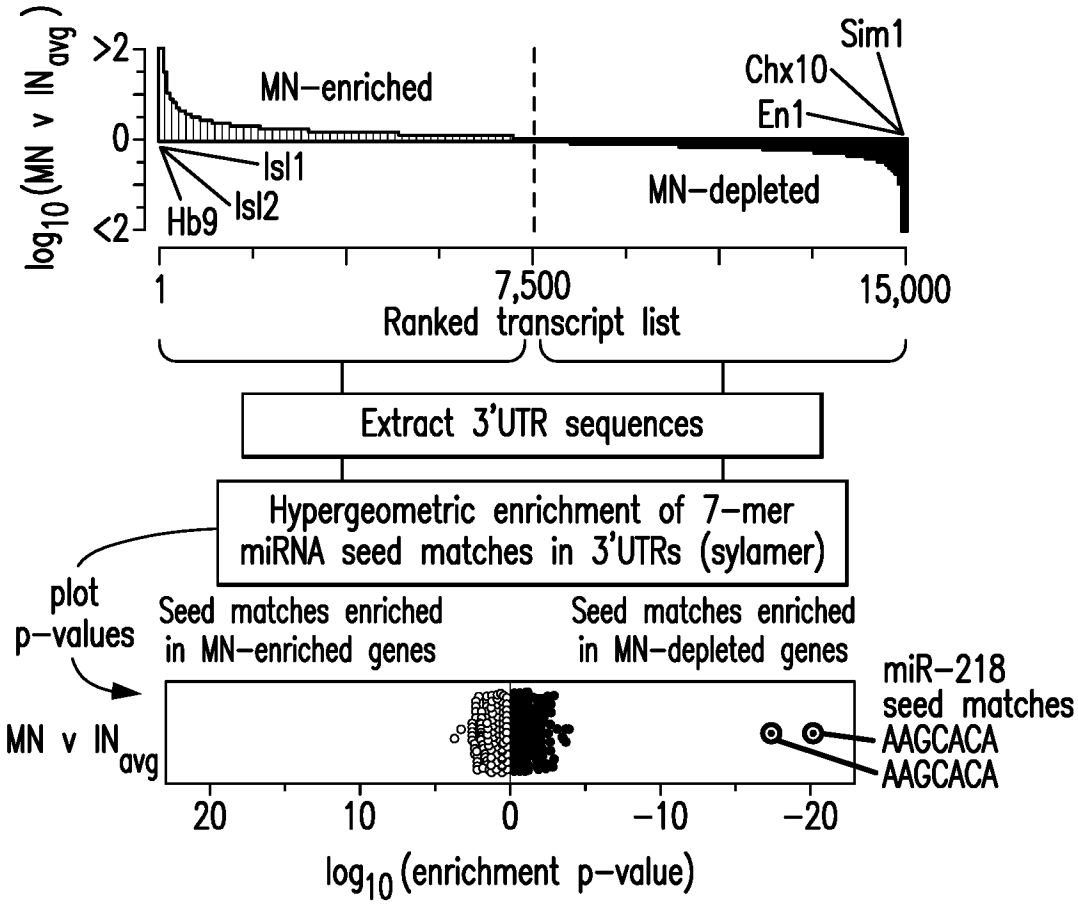

log10(MN v INavg)
>2
0
<2
MN−enriched
Isl1
Hb9
Isl2
Sim1
Chx10
En1
MN−depleted
1
7,500
15,000
Ranked transcript list
Extract 3'UTR sequences
Hypergeometric enrichment of 7−mer miRNA seed matches in 3'UTRs (sylamer)
plot p−values
Seed matches enriched in MN−enriched genes
Seed matches enriched in MN−depleted genes
miR−218 seed matches
AAGCACA
AAGCACA
MN v INavg
20
10
0
−10
−20
log10 (enrichment p−value)

FIG. 29A

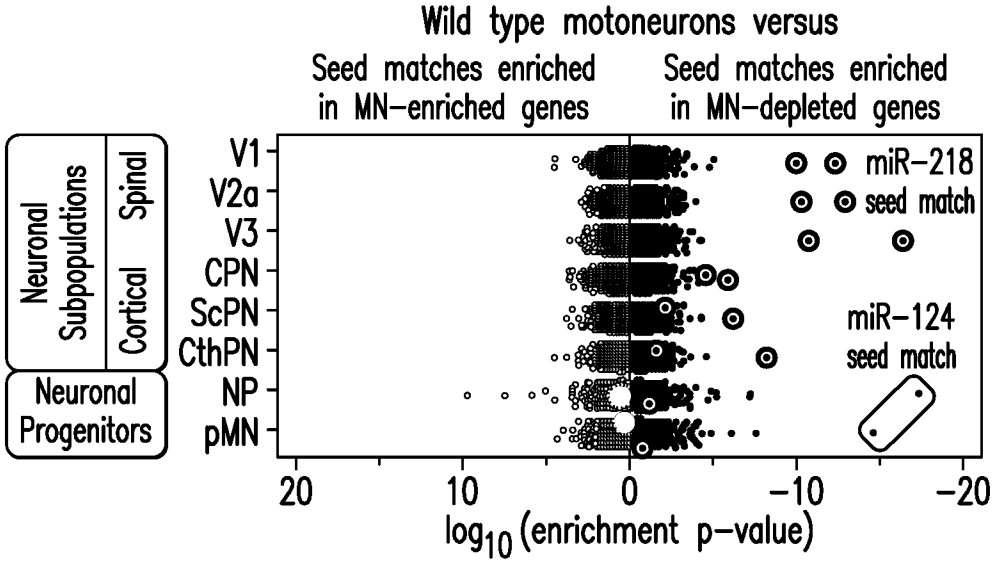


FIG. 29B

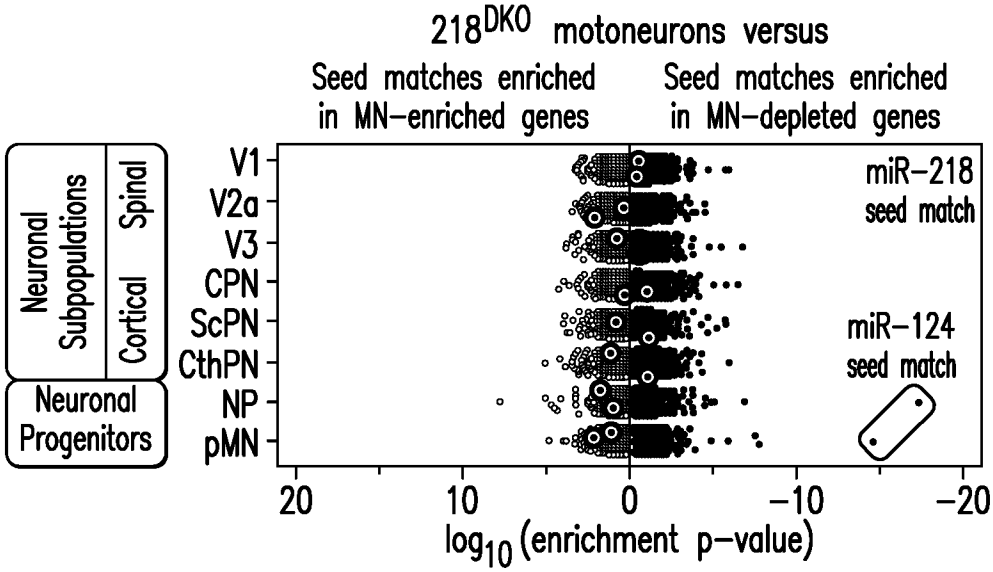


FIG. 29C

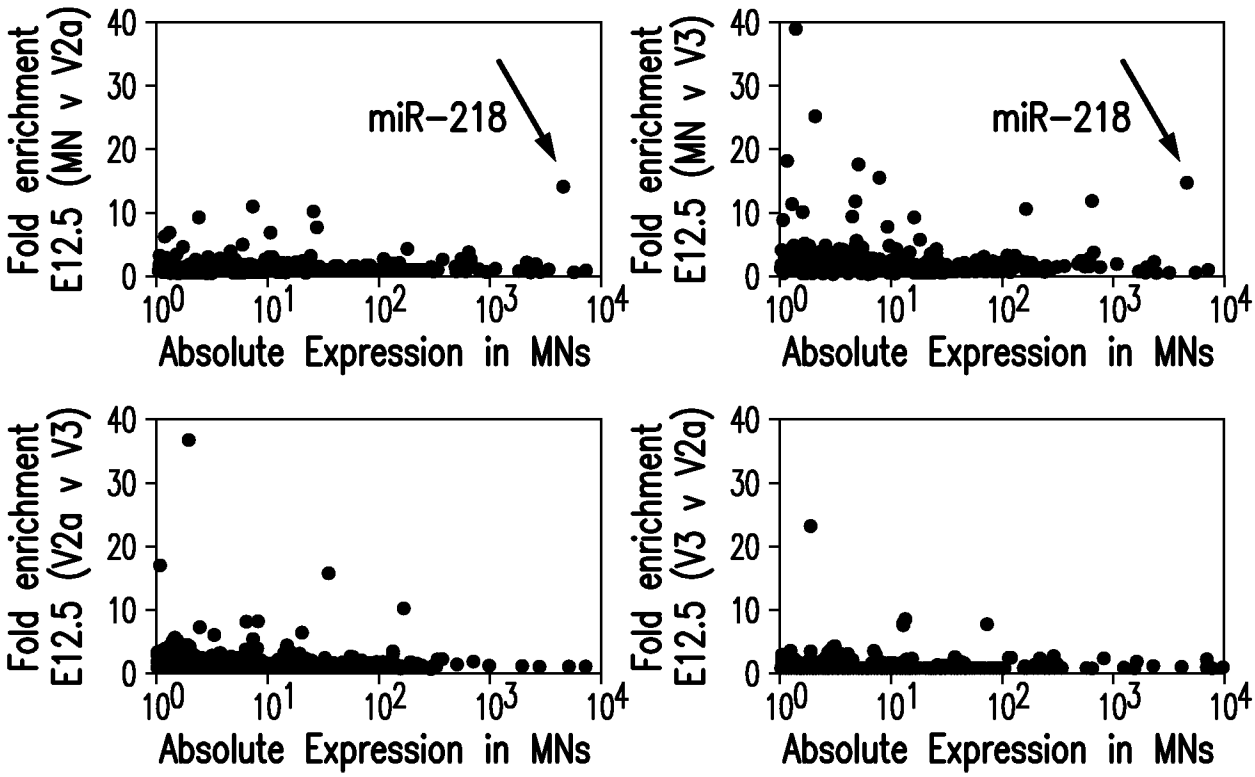


FIG. 30A

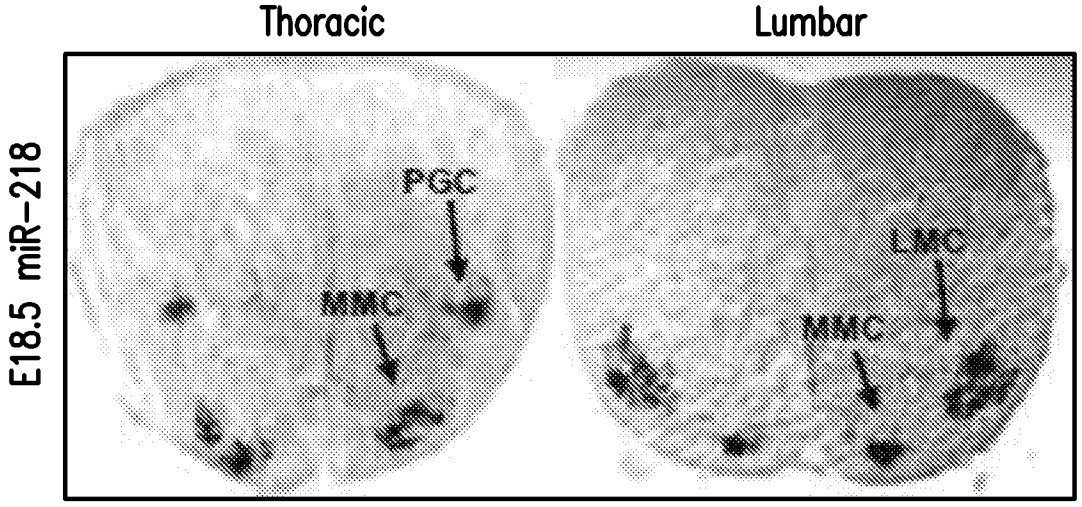


FIG. 30B

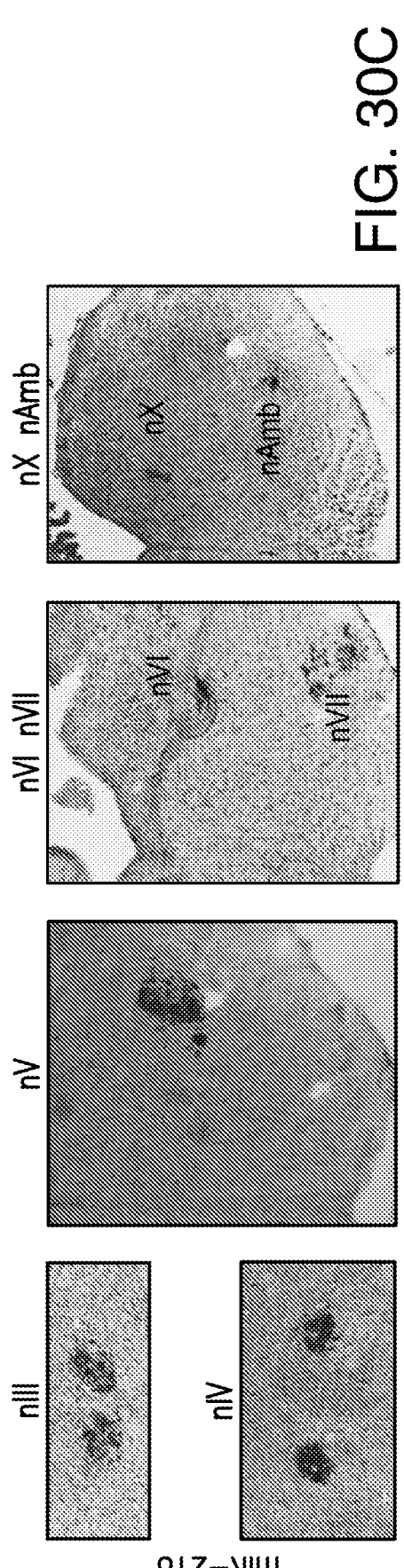


FIG. 30C

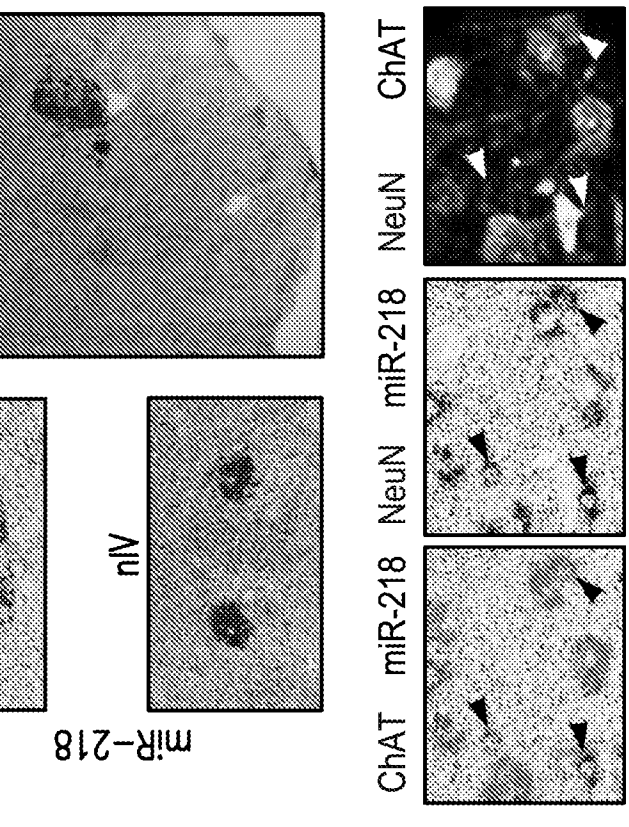


FIG. 30D

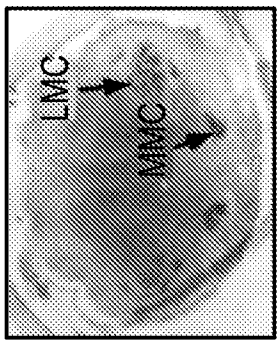


FIG. 30F

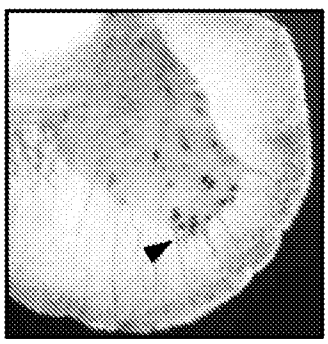


FIG. 30G

FIG. 30E

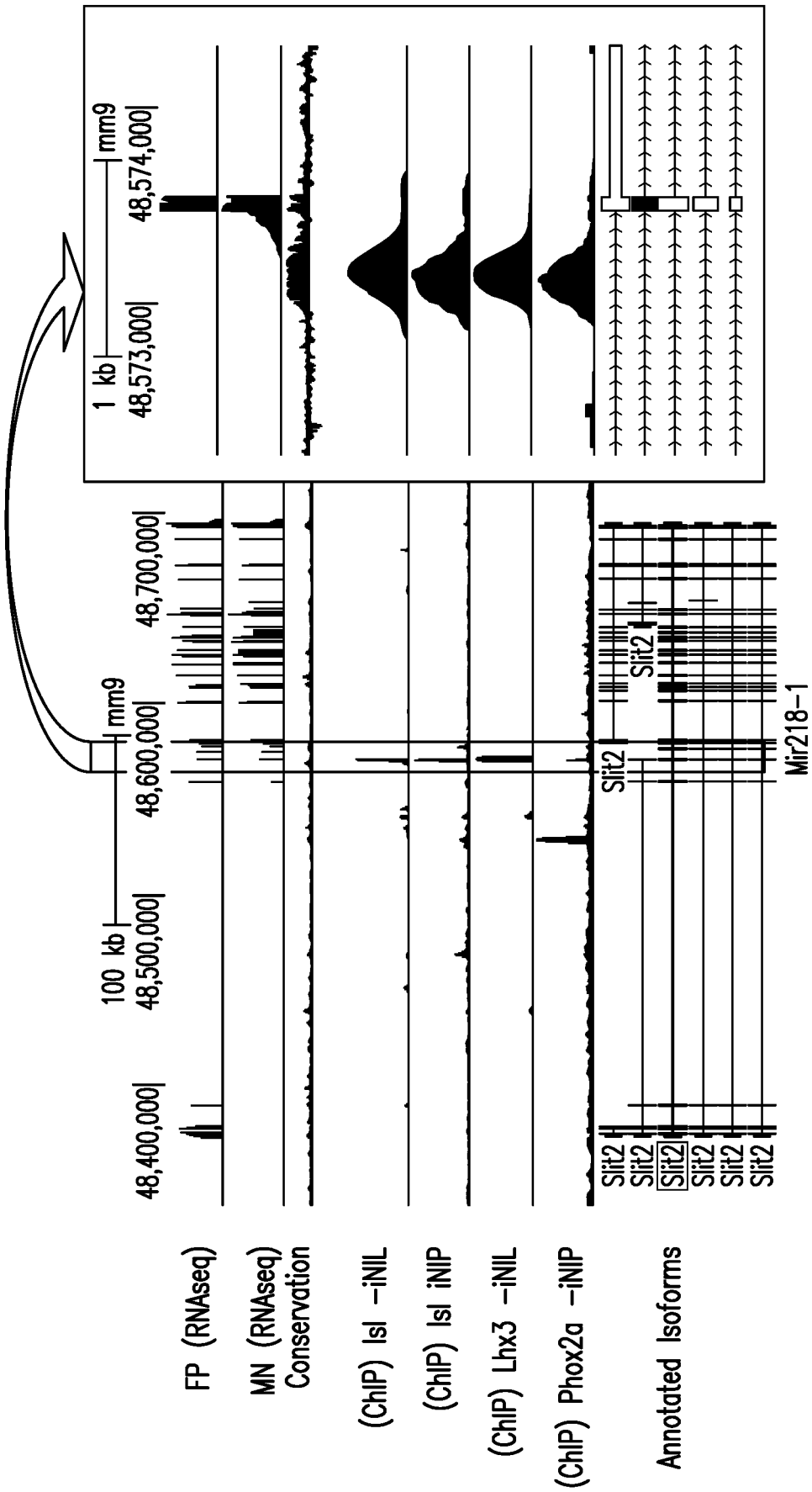

1 kb
48,573,000
mm9
48,574,000
100 kb
48,500,000
48,600,000
48,700,000
48,400,000
FP (RNAseq)
MN (RNAseq)
Conservation
(ChIP) Isl -iNIL
(ChIP) Isl iNIP
(ChIP) Lhx3 -iNIL
(ChIP) Phox2a -iNIP
Annotated Isoforms
Slit2
Mir218-1

FIG. 31A

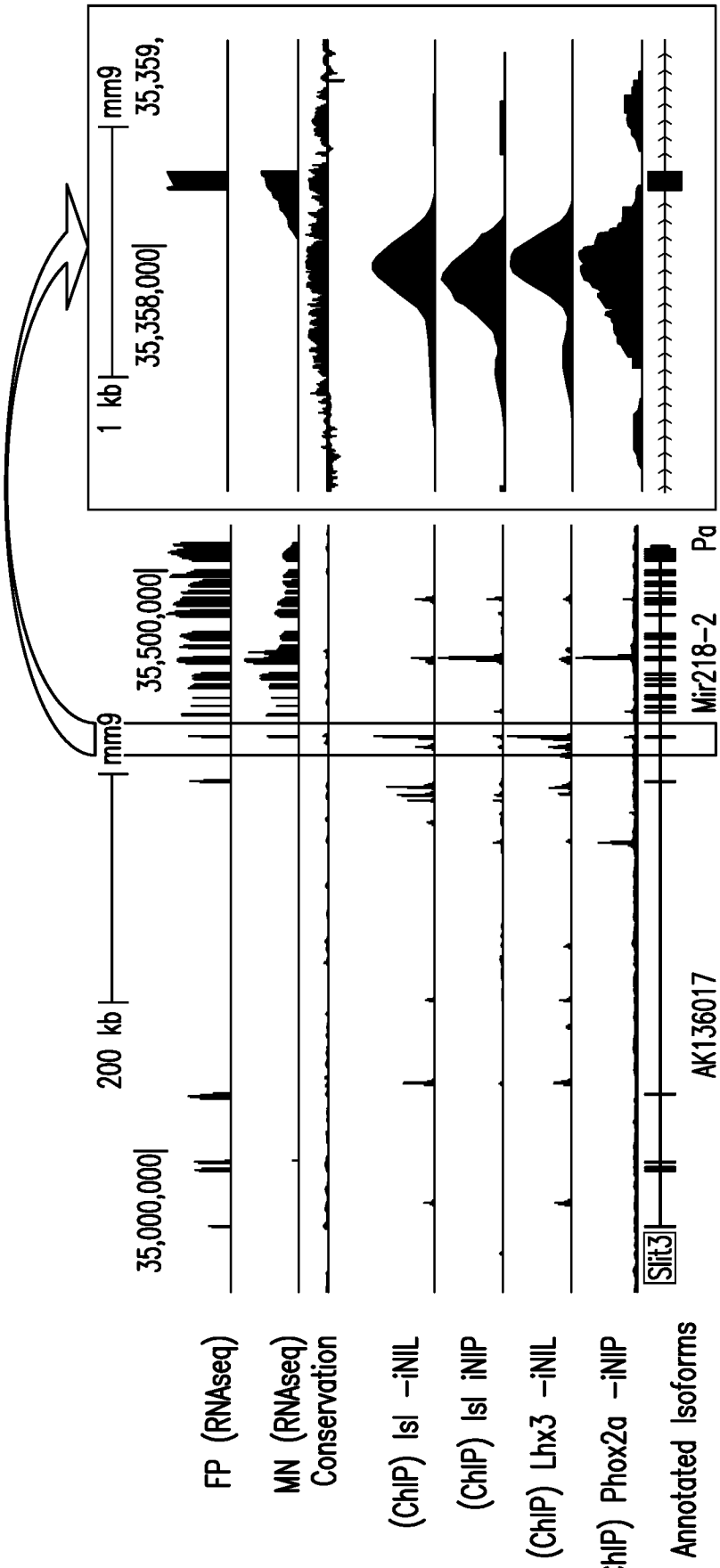

1 kb
35,358,000
mm9
35,359,
200 kb
35,000,000
mm9
35,500,000
Mir218-2
Pa
AK136017
Slit3
FP (RNAseq)
MN (RNAseq)
Conservation
(ChIP) Isl -iNIL
(ChIP) Isl iNIP
(ChIP) Lhx3 -iNIL
(ChIP) Phox2a -iNIP
Annotated Isoforms

FIG. 31B

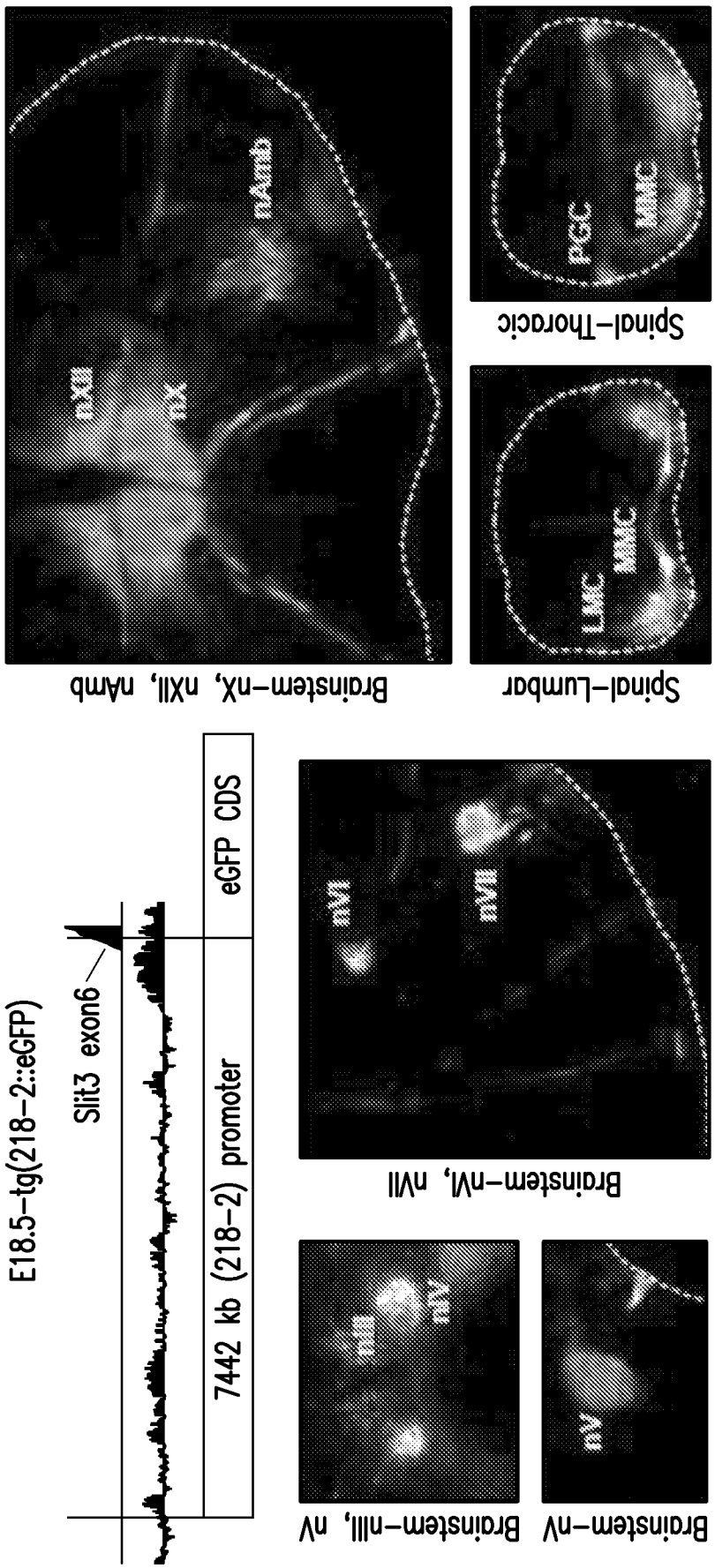

E18.5–tg(218–2::eGFP)
Slit3 exon6
7442 kb (218–2) promoter
eGFP CDS
Brainstem–nV
nV
Brainstem–nIII, nV
nIII
nIV
Brainstem–nVI, nVII
nVI
nVII
Brainstem–nX, nXII, nAmb
nXII
nX
nAmb
Spinal–Lumbar
LMC
MMC
Spinal–Thoracic
PGC
MMC

FIG. 31C

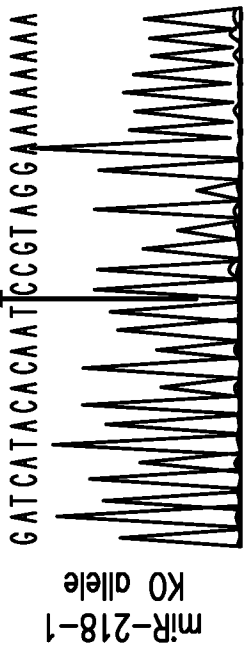


FIG. 32B

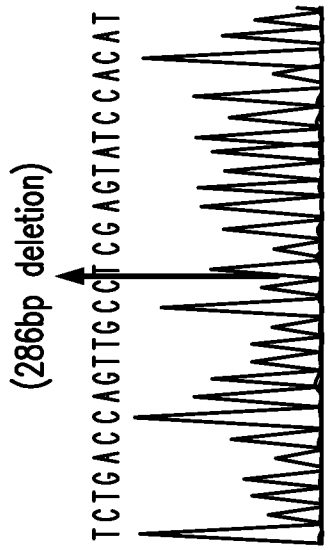


FIG. 32D

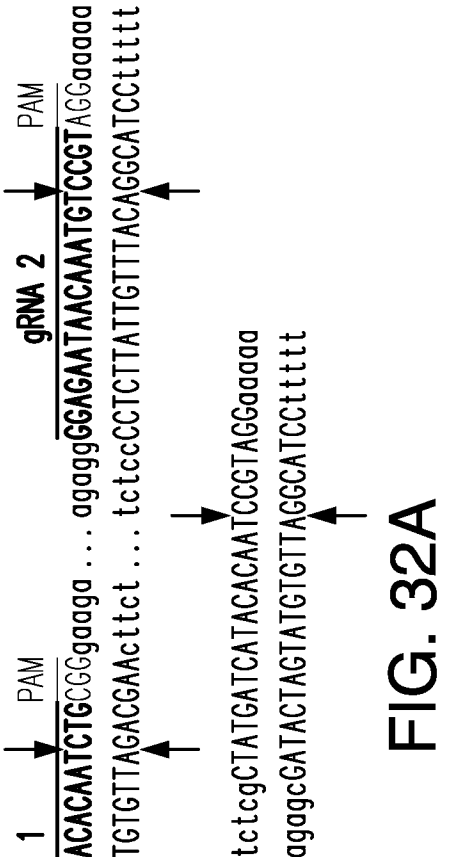


FIG. 32A

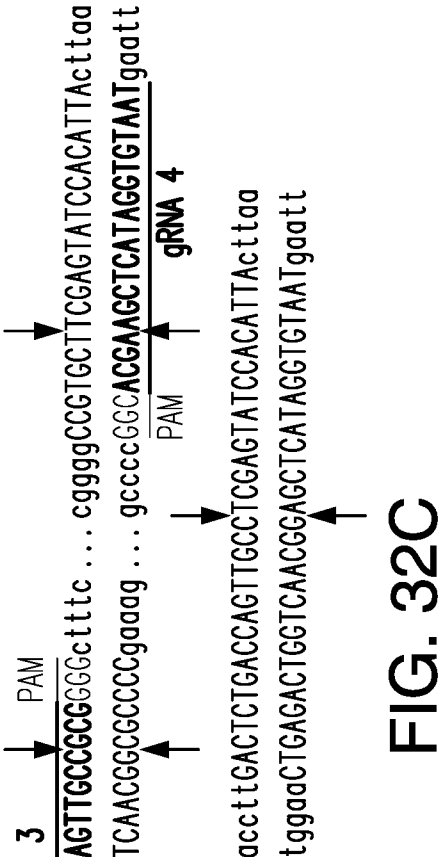


FIG. 32C

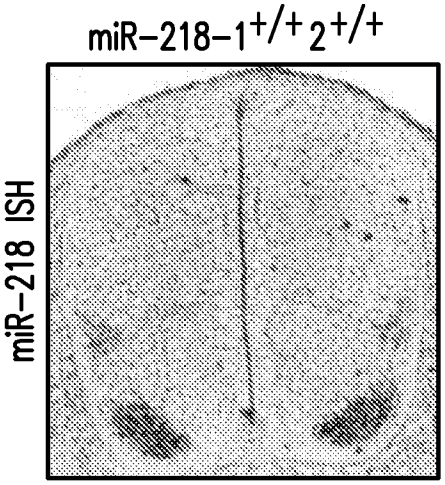


FIG. 32E

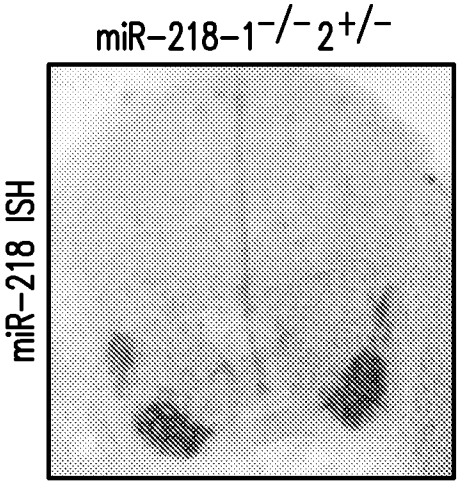


FIG. 32F

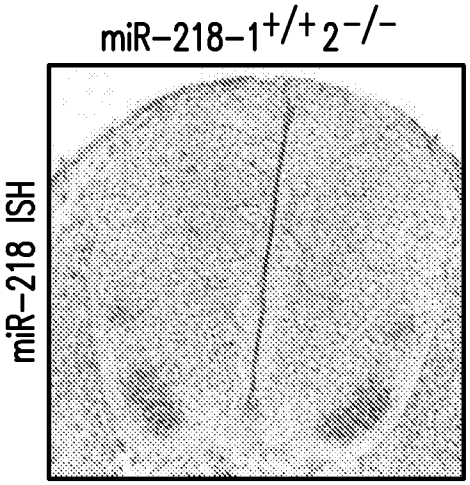


FIG. 32G

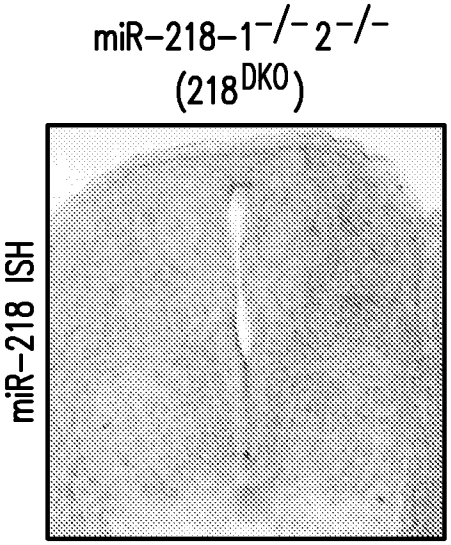


FIG. 32H

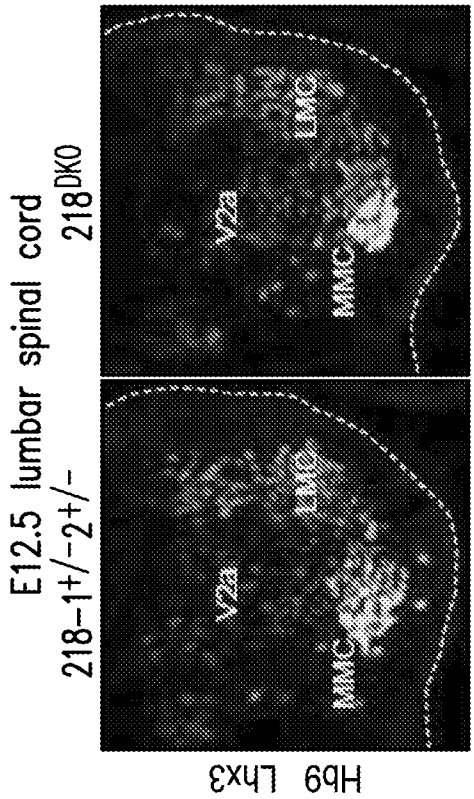


FIG. 33A

FIG. 33B

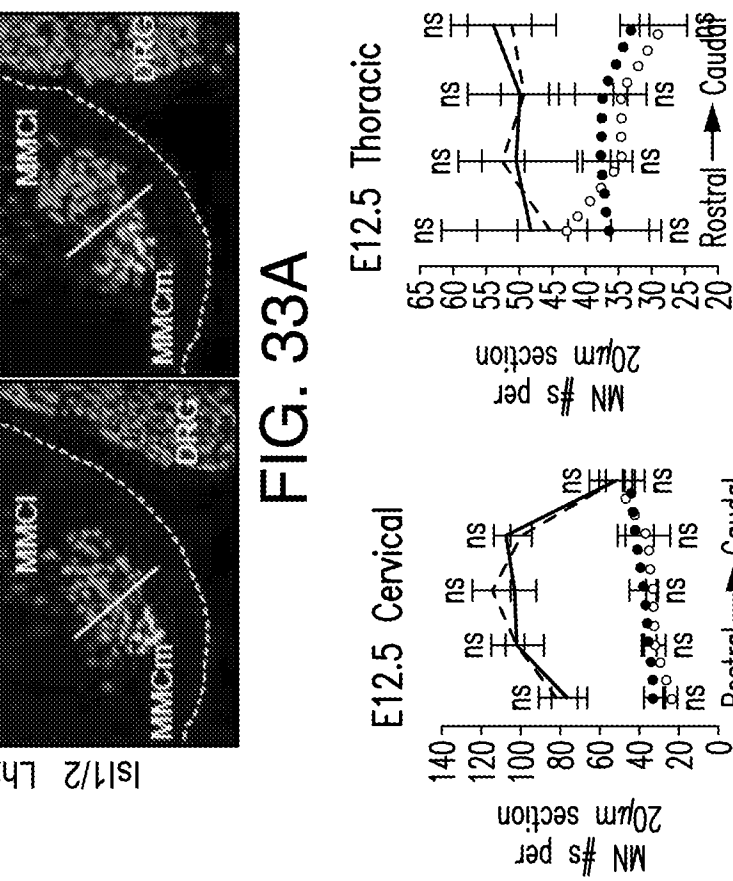


FIG. 33C

FIG. 33D

FIG. 33E

FIG. 33F

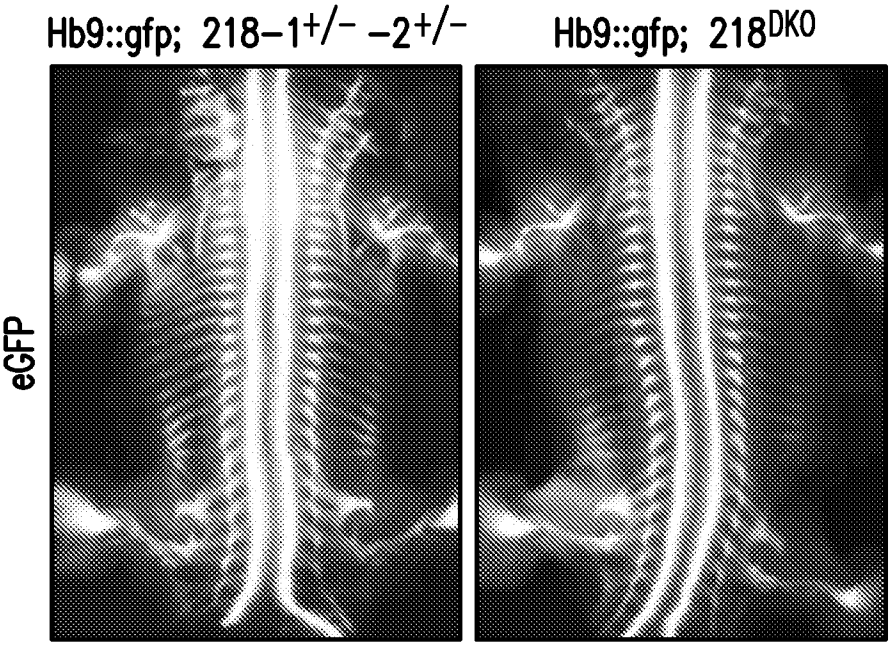


FIG. 33G

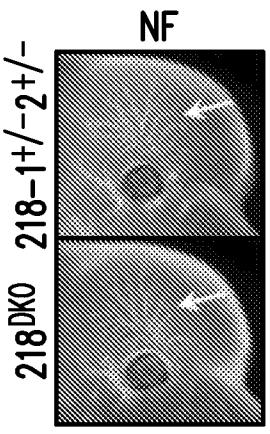


FIG. 33H

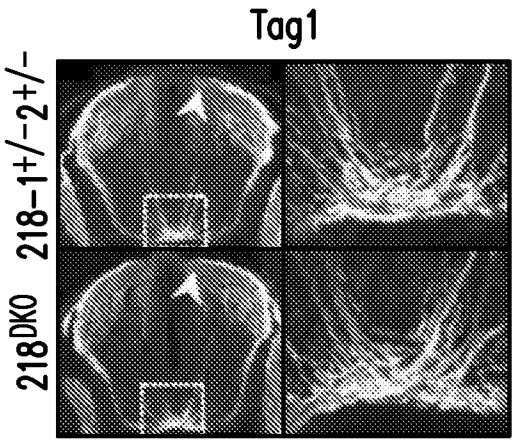


FIG. 33I

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		

FIG. 34A

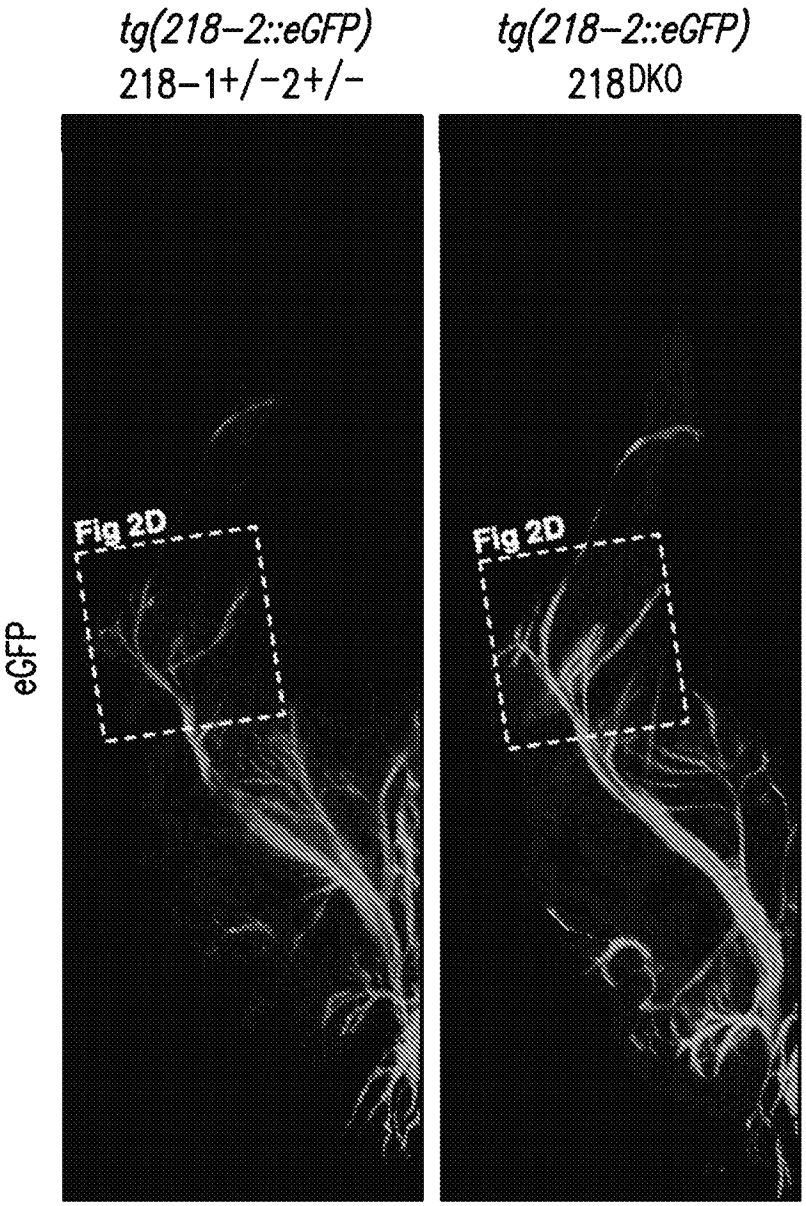


FIG. 34B

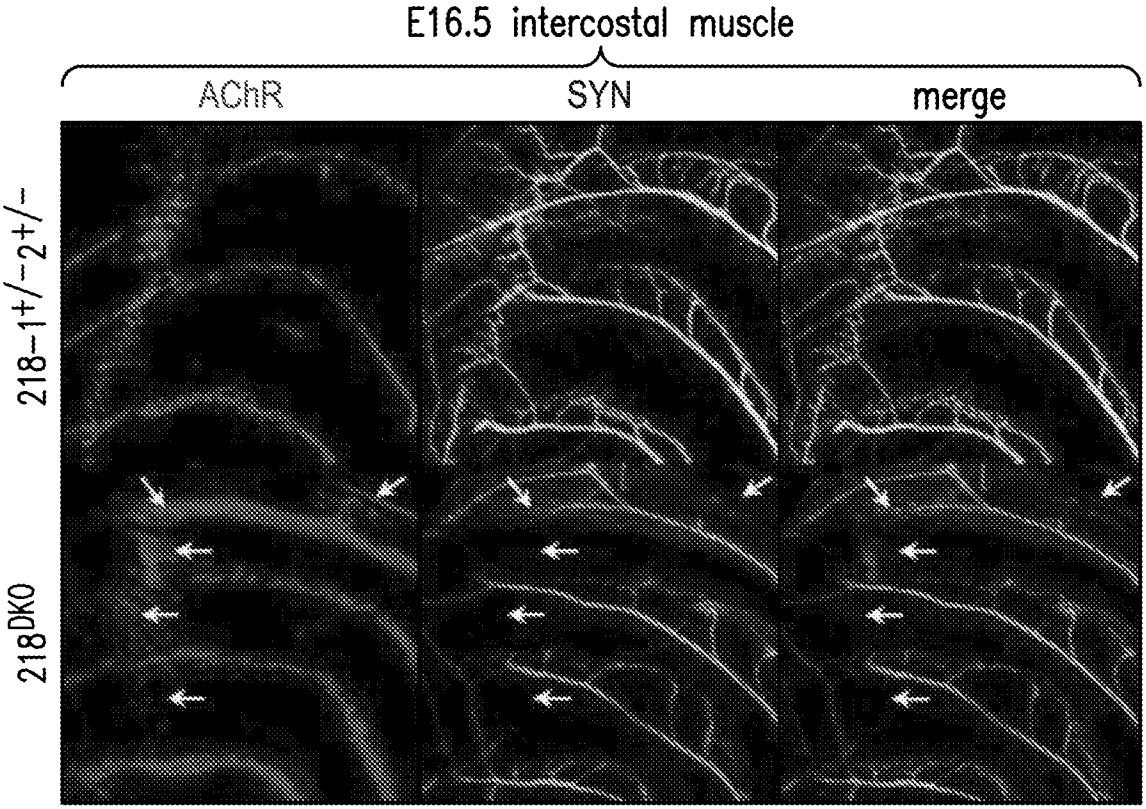


FIG. 35A

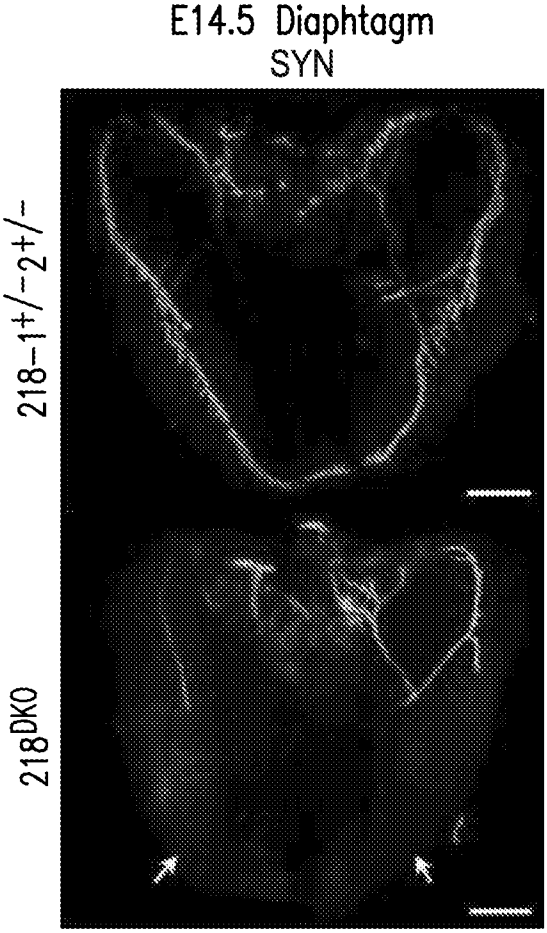


FIG. 35B

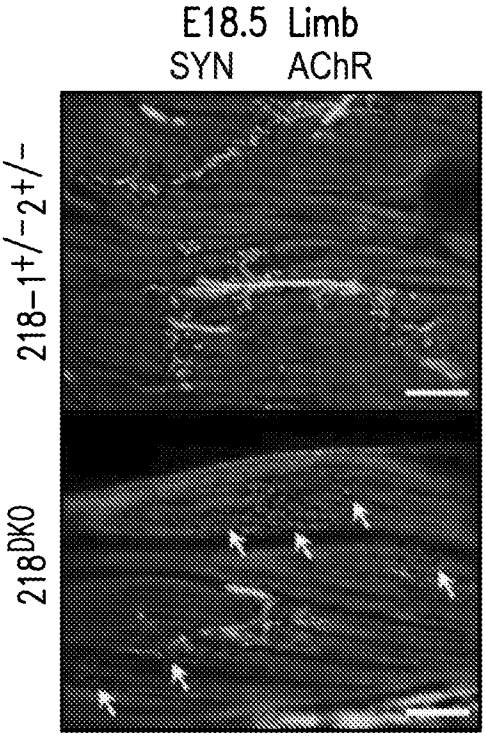


FIG. 35C

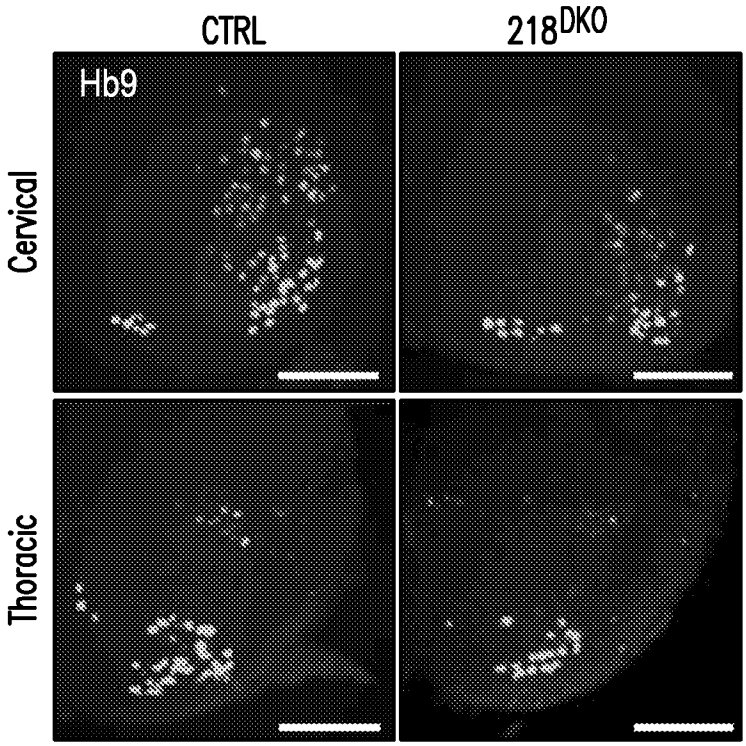


FIG. 36A

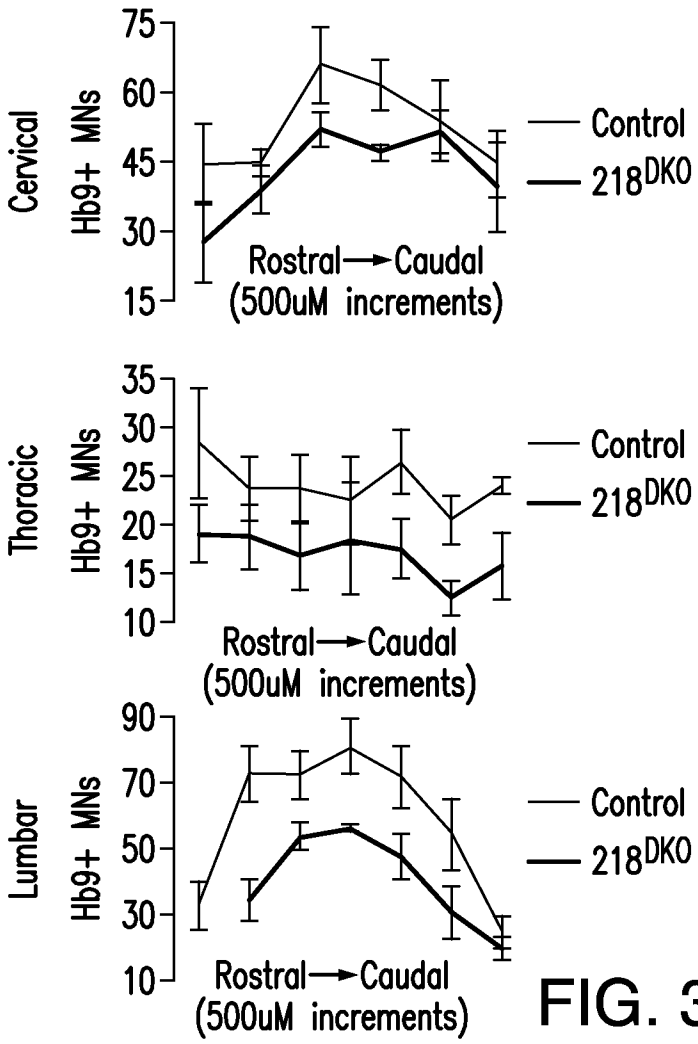


FIG. 36B

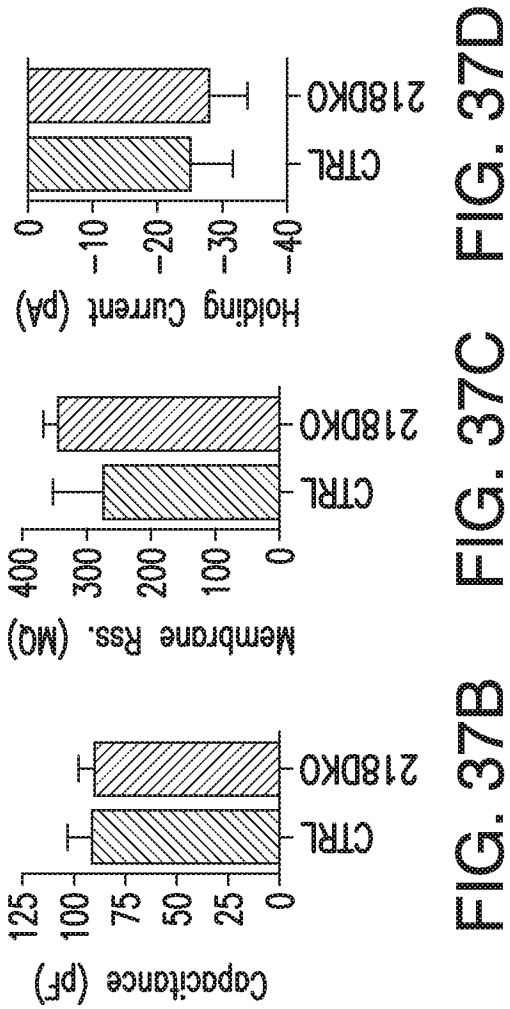


FIG. 37B

FIG. 37C

FIG. 37D

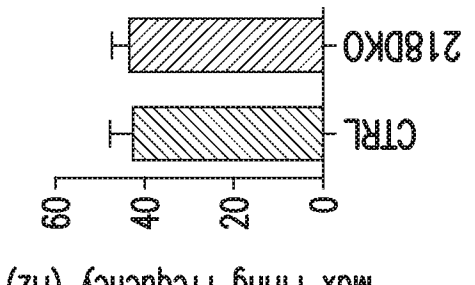


FIG. 37E

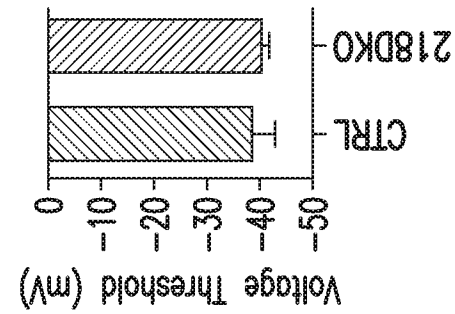


FIG. 37F

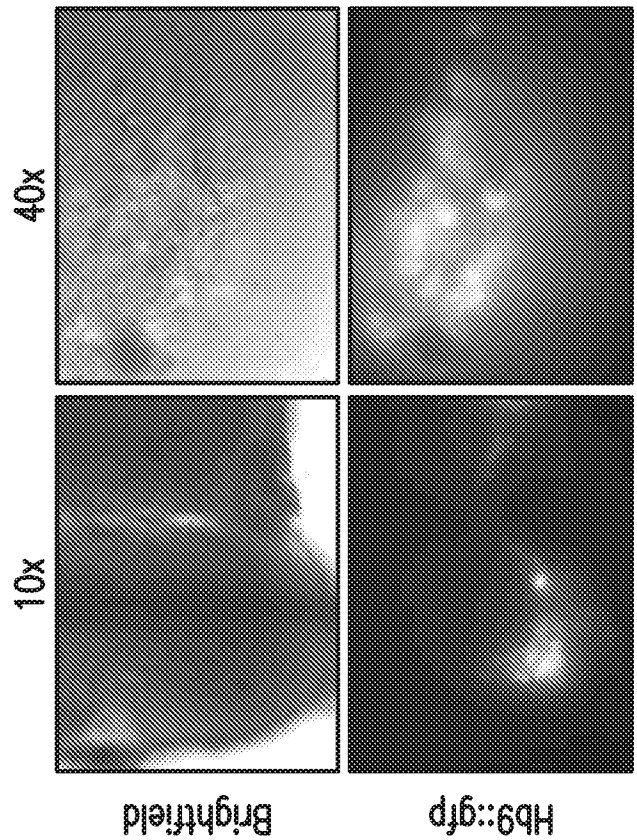


FIG. 37A

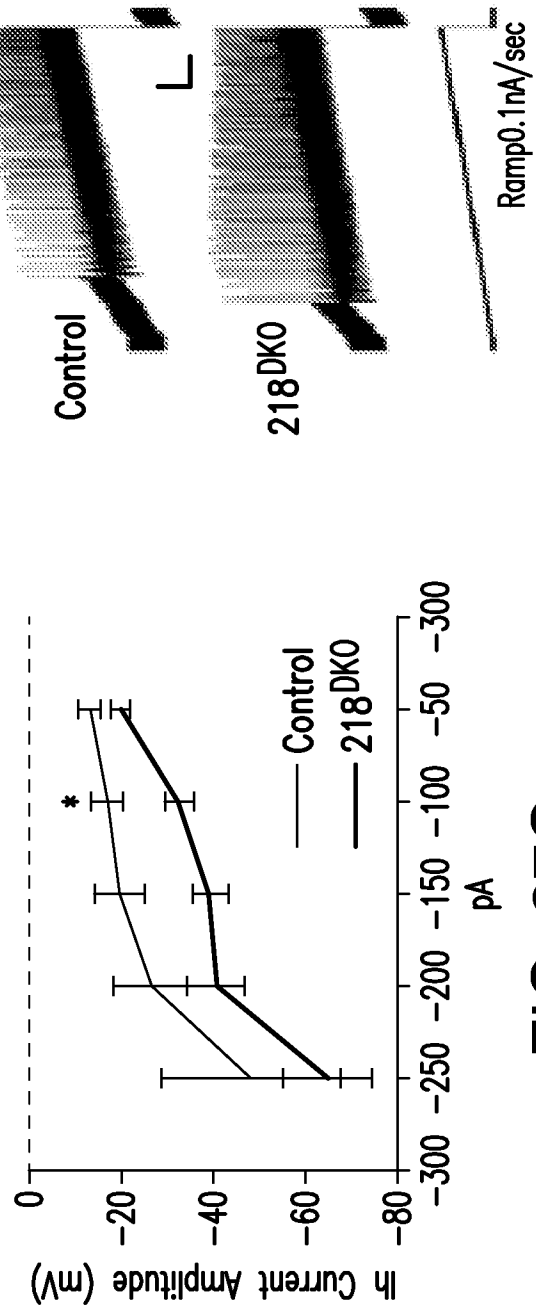


FIG. 37G

FIG. 37H

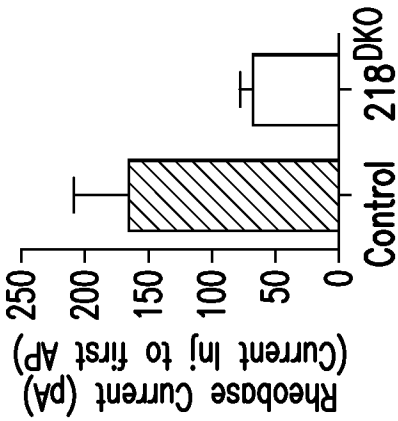


FIG. 37I

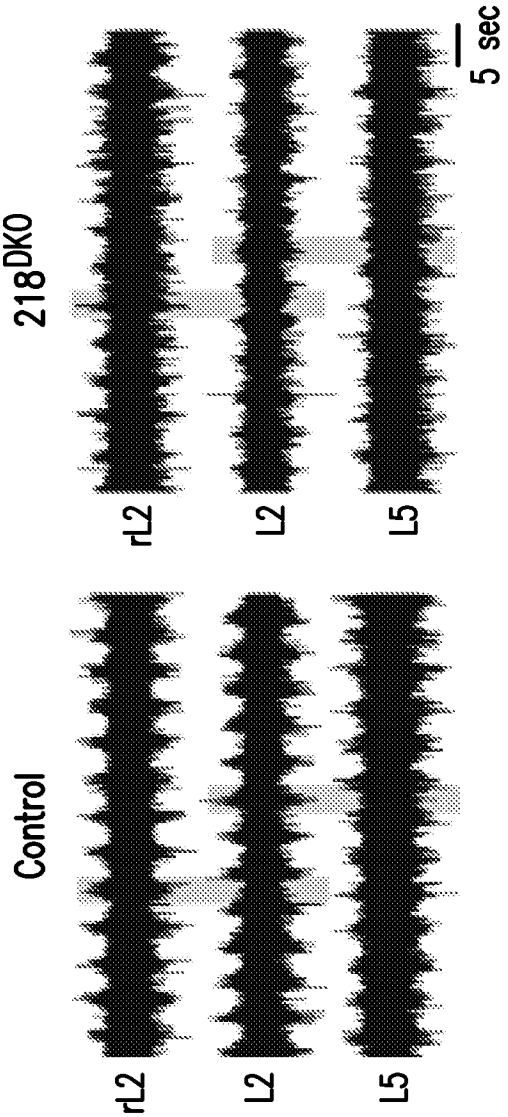


FIG. 37J

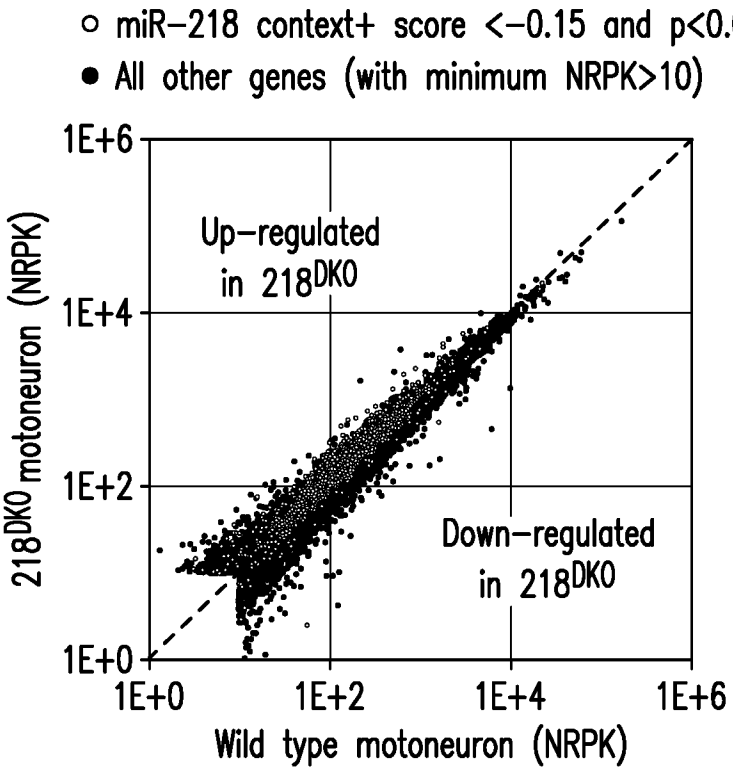


FIG. 38A

```
miR-218: 5' UUGUGCUUGAUCUAACCAUGU (G) 3'
miR-218: 3' (G) UGUACCAAUCUAGUUCGUGUU 5'
                                ||||||||
3' UTR:                         ||||||||
8mer      match:                AAGCACAA
7mer      match:                AAGCACA
7mer-1A   match:                 AGCACAA
6mer      match:                 AGCACA
6mer-1A   match:                  GCACAA
6mer-6A   match:                AAGCAC
```

FIG. 38B

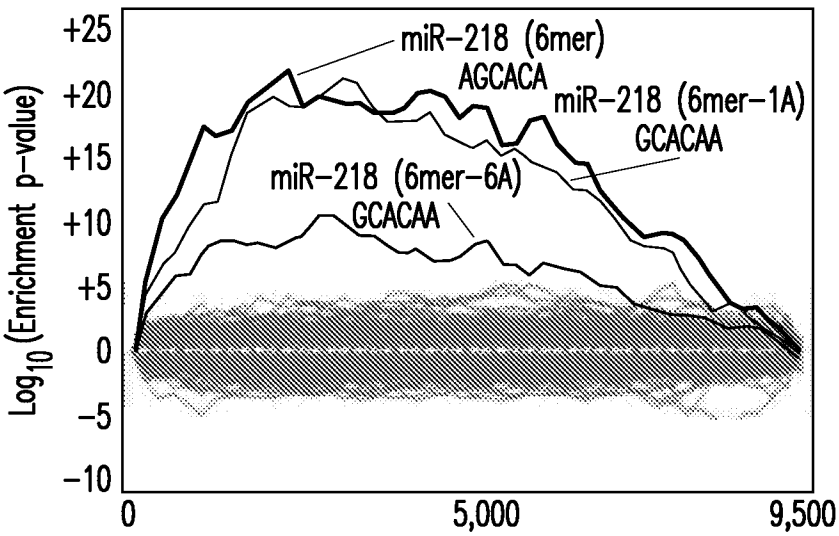


FIG. 38C

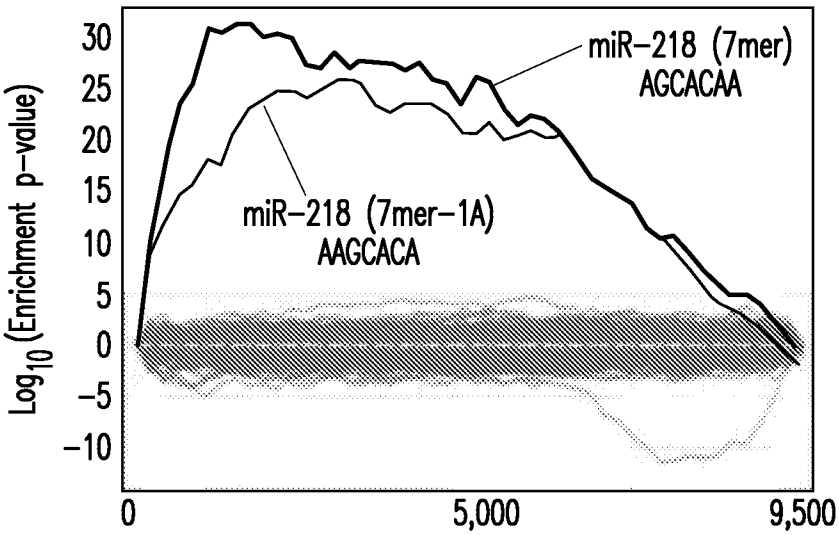


FIG. 38D

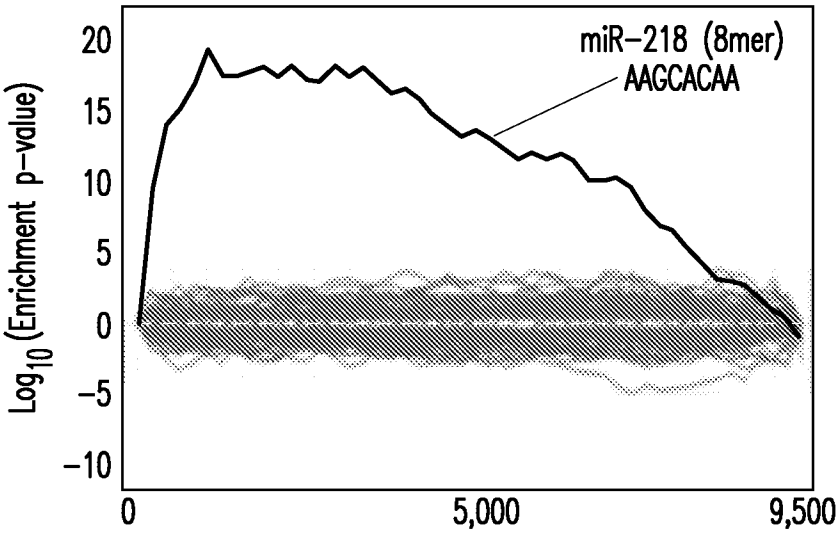


FIG. 38E

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

FIG. 38F

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

FIG. 38G

Neuronal population	Genetic reporter mouse line
Motor neurons	Hb9:gfp
V1 interneurons	En1:Cre, LSL:tdtomato
V2a inerneurons	Chx10:Cre; LSL:tdtomato
V3 interneurons	Sim1:Cre; LSL:tdtomato

Progenitors	Genetic reporter embryonic stem cell line
NP	(unsorted) neurospheres on d4 of differentiation
pMN	Olig2:Cre x LSL:tdtomato sorted on d4 of differentiation

FIG. 39A

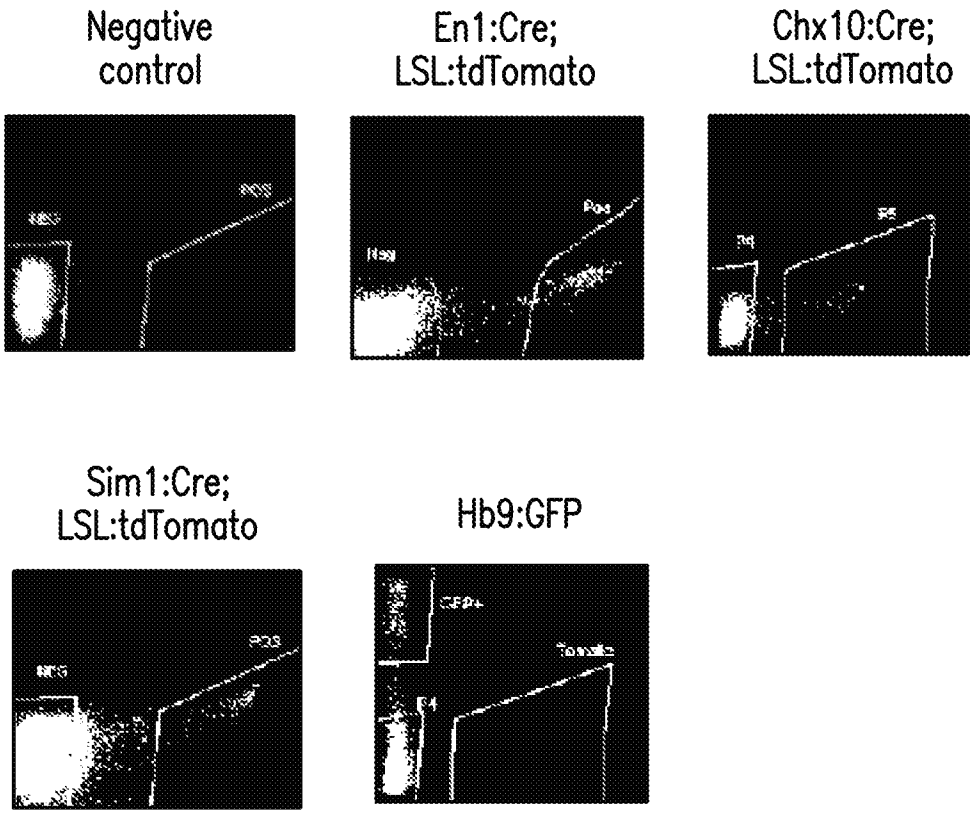


FIG. 39B

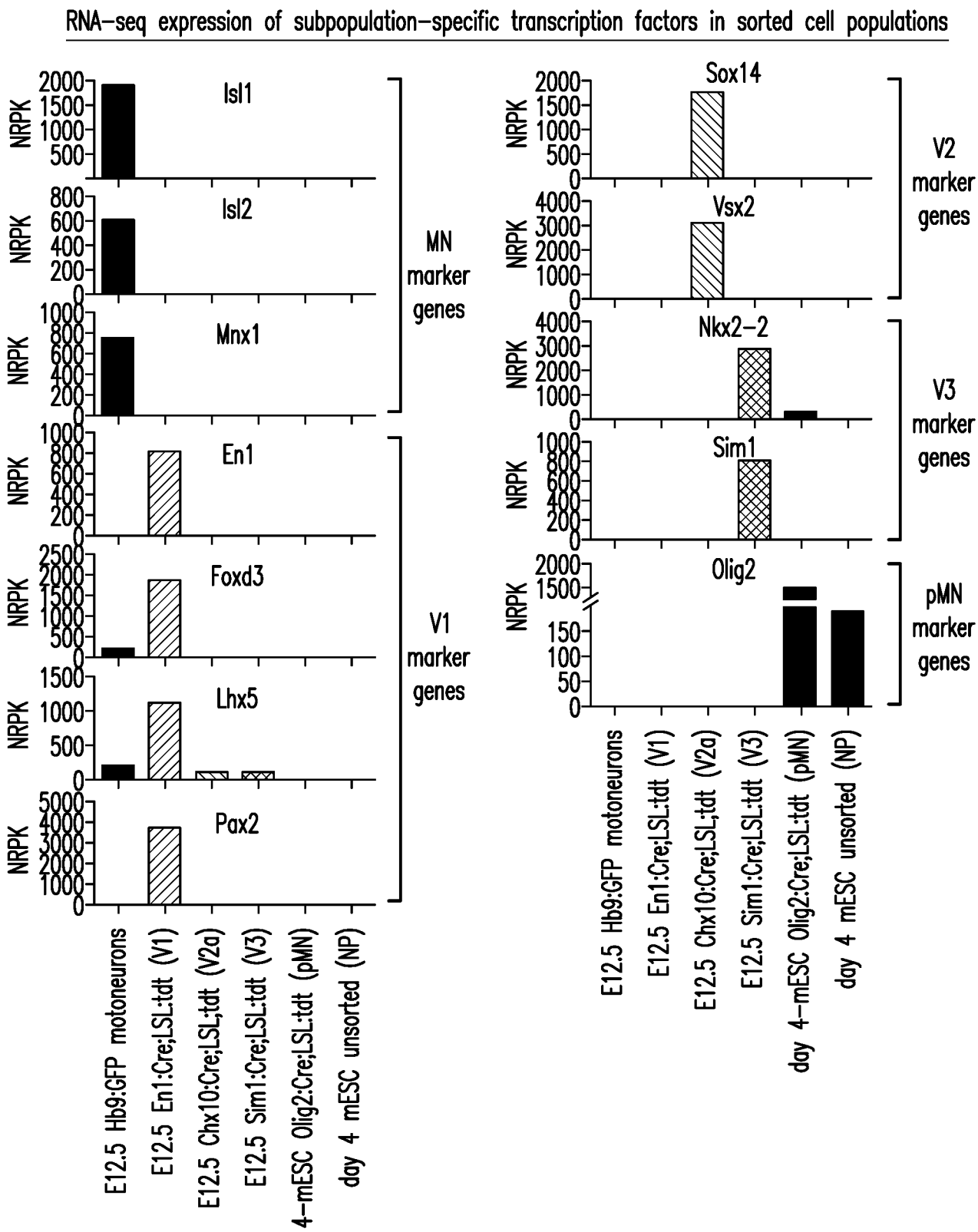

RNA-seq expression of subpopulation-specific transcription factors in sorted cell populations
Isl1
Isl2
Mnx1
En1
Foxd3
Lhx5
Pax2
Sox14
Vsx2
Nkx2-2
Sim1
Olig2
NRPK
MN marker genes
V1 marker genes
V2 marker genes
V3 marker genes
pMN marker genes
E12.5 Hb9:GFP motoneurons
E12.5 En1:Cre;LSL:tdt (V1)
E12.5 Chx10:Cre;LSL;tdt (V2a)
E12.5 Sim1:Cre;LSL:tdt (V3)
day 4-mESC Olig2:Cre;LSL:tdt (pMN)
day 4 mESC unsorted (NP)

FIG. 39C

TARGET218 gene list Gene Symbol	Context+ score	NRPK WT MNs	NRPK 218DKO MNs	Fold increase
Slc1a2	-0.43	136.2	499.0	266%
Slc6a1	-0.35	161.0	585.2	264%
Itm2c	-0.23	198.8	634.9	219%
2510009E07 Rik	-0.75	102.3	318.9	212%
Kif21b	-0.28	661.8	2004.6	203%
Syt13	-0.42	69.9	207.3	197%
Chl1	-0.22	266.4	751.0	182%
Cetn2	-0.15	140.9	394.0	180%
Plgrkt	-0.37	144.0	397.0	176%
Mdfic	-0.28	3.9	10.8	174%
Yeats4	-0.31	509.8	1357.5	166%
Scrt1	-0.39	326.0	849.1	160%
Slc6a17	-0.15	95.2	247.7	160%
6330403A02 Rik	-0.16	18.5	47.5	157%
Lrrc39	-0.22	18.1	44.6	146%
Rassf2	-0.40	86.5	212.5	146%
2010107G23 Rik	-0.29	100.4	242.0	141%
Sertad4	-0.23	40.7	97.9	141%
Psap	-0.15	1840 .8	4279.0	132%
Prrg3	-0.15	94.1	217.5	131%
Prkx	-0.21	142.1	325.3	129%
Zmat4	-0.16	71.7	162.4	127%
Necab1	-0.18	9.1	20.6	126%
Syne1	-0.16	6.9	15.5	125%
Gpr153	-0.29	117.6	259.2	120%
Cdkn2aip	-0.35	140.2	307.2	119 %
Snx4	-0.55	301.8	660.2	119%
Pex2	-0.30	102.4	223.0	118%
Scai	-0.20	106.2	230.8	117%
AU041133	-0.41	37.6	81.7	117%
Slit2	-0.18	1800.9	3911.8	117%
Mcf2	-0.21	15.8	34.1	116%
Ankrd44	-0.16	61.7	132.5	115%
Vav3	-0.17	81.9	175.4	114%
Tub	-0.23	332.9	710.5	113%
Sec61a1	-0.23	441.0	931.6	111%
Pold3	-0.37	81.9	171.8	110%
Serinc5	-0.22	111.4	233.4	110%
Lmo3	-0.34	225.1	467.3	108%
Zbtb11	-0.30	93.2	193.3	107%
Bmi1	-0.31	283.3	586.0	107%
N28178	-0.32	204.8	423.2	107%
Fam65b	-0.30	30.6	62.4	104%
Lypd6b	-0.35	59.8	121.2	102%
Fam63b	-0.29	82.8	166.6	101%
Epha7	-0.41	164.8	331.3	101%
Sh3rf1	-0.32	210.8	423.4	101%

FIG. 40

Gucy1a2	-0.29	60.1	119.9	100%
Tmem25	-0.33	67.6	134.7	99%
Paip2b	-0.19	365.0	719.1	97%
Clcc1	-0.25	56.8	111.3	96%
Brdt	-0.22	27.5	53.6	95%
Lin28b	-0.32	47.8	92.8	94%
Pik3c2a	-0.25	61.9	119.8	94%
Snapc2	-0.32	171.7	329.4	92%
Nav3	-0.32	105.1	201.4	92%
Insig1	-0.17	655.4	1254.0	91%
Plxnc1	-0.32	116.5	222.8	91%
Ptprk	-0.38	66.5	126.8	91%
Herpud1	-0.32	313.1	596.2	90%
Reln	-0.36	279.6	531.0	90%
Cacnb4	-0.27	77.2	146.2	89%
Fam118a	-0.45	108.8	203.5	87%
Clec2g	-0.39	5.9	10.9	86%
Park2	-0.31	17.9	33.3	86%
Dach2	-0.20	40.4	75.0	85%
Pclo	-0.17	150.7	278.9	85%
B3gat1	-0.28	817.5	1512.7	85%
Fcho2	-0.29	98.6	182.2	85%
Rabgap1l	-0.74	250.5	460.9	84%
Rabep1	-0.25	427.9	786.4	84%
Jmy	-0.32	80.3	147.6	84%
Kit	-0.19	49.0	89.9	84%
Stx3	-0.15	55.9	102.5	83%
Them6	-0.47	34.5	62.7	82%
Pdzd4	-0.24	591.9	1070.8	81%
Zcchc9	-0.18	97.9	177.1	81%
Prune2	-0.17	24.9	44.8	80%
Gng3	-0.28	1660.8	2984.2	80%
Rnf165	-0.22	388.0	696.7	80%
Arhgef12	-0.20	364.0	653.4	79%
Samd8	-0.23	140.3	249.8	78%
Adrbk2	-0.18	75.7	134.6	78%
Phc3	-0.21	87.1	154.4	77%
Pkd2l2	-0.18	56.1	99.0	77%
Nox4	-0.18	10.7	18.9	76%
Gabra5	-0.17	135.7	236.3	74%
B630005N14 Rik	-0.29	124.3	216.1	74%
G2e3	-0.15	78.8	136.4	73%
Specc1l	-0.29	254.0	438.4	73%
Sfmbt1	-0.29	53.6	92.4	72%
Mob1b	-0.29	85.9	147.8	72%
Gadd45a	-0.27	109.5	188.5	72%
Fam178 a	-0.15	208.3	358.2	72%
Mtmr1	-0.30	109.9	188.8	72%
Vat1	-0.34	1125.0	1930.9	72%
St18	-0.20	145.1	248.8	71%
Satb2	-0.32	81.1	138.8	71%
Sgsm1	-0.19	111.0	190.0	71%
Dnal1	-0.20	60.7	103.8	71%

FIG. 40 Continued

Glce	-0.51	82.4	140.5	71%
Sv2a	-0.20	959.5	1632.9	70%
Rnf103	-0.43	155.9	265.1	70%
Kirrel3	-0.36	95.9	162.7	70%
Csmd3	-0.20	64.3	109.0	69%
Serpini1	-0.22	835.7	1413.6	69%
Mxd1	-0.16	154.6	259.4	68%
Adarb1	-0.22	65.5	109.8	68%
Celf4	-0.26	924.5	1544.5	67%
Irak1bp1	-0.16	124.5	207.3	67%
Ugt8a	-0.15	25.4	42.2	66%
Chuk	-0.15	206.6	342.6	66%
Enoph1	-0.15	156.8	259.0	65%
Prkar2b	-0.32	892.0	1469.2	65%
Sh3d19	-0.19	43.6	71.5	64%
Caskin1	-0.21	303.0	496.3	64%
Kdm5a	-0.26	107.9	176.7	64%
Lrrfip1	-0.17	191.7	313.9	64%
Eif2ak3	-0.40	137.2	224.6	64%
Dcbld2	-0.25	271.6	444.0	64%
Stam2	-0.37	229.2	373.0	63%
Ccp110	-0.19	295.8	480.6	62%
Ankrd45	-0.29	82.6	133.9	62%
Hectd2	-0.42	47.1	76.3	62%
Cntnap2	-0.22	270.7	438.3	62%
Elmo1	-0.18	178.2	287.5	61%
Rnasel	-0.17	237.5	383.1	61%
Sh3rf3	-0.21	20.4	32.7	61%
Myt1l	-0.27	647.8	1040.7	61%
Rcbtb1	-0.27	303.3	487.1	61%
Prox2	-0.28	8.0	12.9	60%
Pum2	-0.28	555.1	890.0	60%
Klhl13	-0.17	524.1	838.1	60%
B3gat2	-0.27	102.3	163.6	60%
Tmpo	-0.17	543.8	868.8	60%
Kctd9	-0.68	56.3	89.9	60%
Hdac7	-0.24	243.7	387.0	59%
Mpv17	-0.21	181.1	286.7	58%
Rmdn2	-0.19	26.3	41.5	58%
Mdga2	-0.32	86.3	135.5	57%
Eogt	-0.15	38.2	59.9	57%
Rab43	-0.21	62.5	98.0	57%
Tspan5	-0.23	587.1	920.5	57%
Trhde	-0.24	49.3	77.3	57%
L3hypdh	-0.24	39.2	61.3	56%
Kcnh1	-0.41	15.8	24.5	56%
Ipp	-0.34	48.2	75.0	56%
Slc24a3	-0.16	301.3	466.5	55%
Nrxn3	-0.16	199.5	308.7	55%
Skil	-0.17	232.6	359.7	55%
Ptp4a1	-0.27	371.1	573.5	55%
1600012H06 Rik	-0.42	87.3	134.5	54%
Asxl2	-0.16	57.8	89.0	54%

FIG. 40 Continued

Zeb2	-0.37	164.6	253.5	54%
Synm	-0.18	83.0	127.7	54%
Sde2	-0.17	194.6	299.2	54%
Snai2	-0.19	10.6	16.2	54%
Robo2	-0.31	700.5	1074.9	53%
Edem1	-0.34	51.5	78.9	53%
Zfp810	-0.26	146.2	223.8	53%
Rock1	-0.19	181.3	277.2	53%
Ap4e1	-0.15	105.4	161.0	53%
Onecut2	-0.79	129.7	196.5	52%
Golt1b	-0.17	234.2	354.9	52%
Tmem132b	-0.17	97.8	148.2	51%
Snx13	-0.17	95.4	144.6	51%
Zmiz2	-0.24	906.9	1372.9	51%
Snx8	-0.17	184.7	279.0	51%
Adipor2	-0.26	277.3	418.5	51%
Galnt13	-0.28	59.8	90.3	51%
Camkk2	-0.28	295.9	445.3	50%
Abca3	-0.18	447.5	672.5	50%
Aacs	-0.18	314.6	471.6	50%
Jtb	-0.18	205.9	307.3	49%
Tenm3	-0.29	212.2	316.4	49%
Sash1	-0.19	74.1	110.3	49%
Tspan3	-0.15	1659.4	2467.3	49%
Rhobtb1	-0.30	51.0	75.8	49%
Clk3	-0.15	391.1	580.2	48%
Lmnb1	-0.17	790.3	1170.7	48%
Ing3	-0.18	64.7	95.8	48%
Sdccag3	-0.15	546.3	803.8	47%
Ppp2r5a	-0.21	28.4	41.8	47%
Fam161b	-0.18	107.7	158.3	47%
Actn1	-0.27	54.9	80.7	47%
Piezo2	-0.70	11.0	16.2	47%
Heca	-0.28	151.1	220.6	46%
Ubr3	-0.34	364.3	530.9	46%
Fam196a	-0.26	63.9	93.1	46%
Crtc3	-0.23	51.3	74.7	46%
Ick	-0.16	327.0	475.9	46%
Itga9	-0.15	65.4	95.1	45%
Ranbp10	-0.23	176.7	256.8	45%
Rpl7l1	-0.21	226.8	329.3	45%
Ubqln2	-0.25	1313.9	1905.2	45%
Rnf114	-0.20	518.1	751.0	45%
Gemin6	-0.15	47.0	68.1	45%
Shisa6	-0.37	50.2	72.5	45%
Nmral1	-0.31	209.3	302.1	44%
Med4	-0.17	267.6	385.9	44%
Ermp1	-0.47	89.2	128.6	44%
Socs6	-0.23	140.8	202.6	44%
Zbtb10	-0.27	136.7	196.2	44%
Pbx2	-0.25	435.4	622.4	43%
Hoxd10	-0.25	785.2	1121.7	43%
Morc4	-0.16	23.4	33.5	43%

FIG. 40 Continued

Dph6	-0.17	61.6	87.6	42%
Slc36a4	-0.20	229.7	325.8	42%
Camkmt	-0.37	67.9	96.0	41%
Vamp7	-0.24	171.8	242.1	41%
Tyw3	-0.23	28.1	39.6	41%
2010315B03 Rik	-0.29	57.0	79.9	40%
Aff4	-0.25	415.1	581.6	40%
Necap2	-0.21	123.4	172.5	40%
Uhrf1bp1l	-0.22	145.4	202.9	40%
Pkn2	-0.25	86.8	121.0	39%
Agpat3	-0.15	234.6	325.7	39%
Prosc	-0.21	231.4	320.2	38%
Net1	-0.30	33.3	46.0	38%
Sema6b	-0.16	205.9	284.0	38%
D17H6S53E	-0.16	44.3	60.9	38%
Gabpa	-0.16	96.7	132.6	37%
Sh3gl1	-0.20	320.7	440.0	37%
Cstf2t	-0.22	339.3	463.9	37%
Epha5	-0.20	234.7	320.0	36%
Fam86	-0.24	21.5	29.3	36%
Pou3f3	-0.15	332.0	452.6	36%
Pcgf2	-0.30	502.4	684.1	36%
Nufip2	-0.19	101.5	138.2	36%
Gnai2	-0.20	932.1	1267.9	36%
Lgr4	-0.55	68.5	92.6	35%
Mast4	-0.18	240.1	324.0	35%
Srxn1	-0.24	82.3	110.9	35%
Zfp521	-0.19	293.1	395.0	35%
Wnt2b	-0.29	9.6	12.9	35%
Ebf3	-0.17	369.4	497.4	35%
Cab39l	-0.25	57.8	77.7	35%
Rap1gap	-0.23	371.3	499.2	34%
Tmem206	-0.28	106.2	142.4	34%
Fam45a	-0.39	144.7	194.1	34%
9830147E19 Rik	-0.31	43.2	57.9	34%
Mier3	-0.44	98.7	132.3	34%
Smchd1	-0.24	163.2	218.5	34%
Hecw1	-0.20	274.2	366.2	34%
Gca	-0.24	12.1	16.1	33%
Jrkl	-0.35	62.3	82.6	33%
Kdm1b	-0.18	173.9	230.1	32%
Rrp1b	-0.25	97.3	128.6	32%
Col4a3bp	-0.29	192.2	253.8	32%
Rab36	-0.17	64.0	84.4	32%
Mtx3	-0.15	138.8	183.1	32%
Emc4	-0.15	663.5	874.5	32%
Pcnp	-0.19	993.7	1309.1	32%
Epc1	-0.16	605.2	795.3	31%
Ahctf1	-0.18	180.1	236.5	31%
Gpr155	-0.24	89.8	117.8	31%
Grik2	-0.26	112.3	147.2	31%
Zbtb26	-0.17	64.3	84.0	31%

FIG. 40 Continued

Pld5	-0.27	105.0	137.0	30%
Mtmr12	-0.39	54.0	70.4	30%
Uvssa	-0.26	44.9	58.5	30%
Kalrn	-0.27	300.4	390.2	30%
Nol7	-0.25	330.7	427.8	29%
Pde7a	-0.24	290.8	375.6	29%
Arf6	-0.24	235.3	303.8	29%
Mmp24	-0.16	560.4	723.5	29%
Dnajc1	-0.25	58.1	74.8	29%
Tgfbrap1	-0.30	140.5	180.9	29%
Iba57	-0.20	52.8	67.9	29%
Creb1	-0.18	217.9	280.2	29%
Wipi2	-0.18	273.9	352.0	28%
Zfp719	-0.23	47.8	61.4	28%
Tmem150c	-0.25	178.7	229.0	28%
Srsf6	-0.30	1194.9	1531.3	28%
Rcc1	-0.21	133.7	171.0	28%
Cacna1g	-0.24	440.9	563.6	28%
Uck1	-0.15	310.6	396.8	28%
Onecut3	-0.30	79.7	101.4	27%
Zfp445	-0.23	836.0	1062.0	27%
Opn3	-0.23	40.4	51.3	27%
Zfp202	-0.28	72.2	91.4	27%
Insr	-0.25	288.6	365.4	27%
Sqstm1	-0.20	1748.2	2212.5	27%
Pex5l	-0.28	98.4	124.4	26%
Lasp1	-0.16	954.5	1204.0	26%
Smim19	-0.15	165.4	208.1	26%
Shprh	-0.23	173.2	216.4	25%
Efcab2	-0.29	38.4	47.8	24%
Agap1	-0.20	418.1	518.5	24%
Rint1	-0.36	114.1	141.5	24%
Lmo7	-0.31	12.7	15.8	24%
Twsg1	-0.17	211.0	261.4	24%
Gpr45	-0.31	51.5	63.7	24%
Prr14l	-0.18	85.5	105.5	23%
Rassf5	-0.23	30.3	37.3	23%
Zscan29	-0.19	54.1	66.7	23%
Shank2	-0.22	205.1	252.7	23%
Rnf24	-0.17	283.8	348.8	23%
Birc6	-0.27	236.4	289.7	23%
Gfpt1	-0.26	370.7	453.1	22%
Golga7	-0.19	1070.3	1306.1	22%
Prmt6	-0.16	202.1	246.6	22%
Hars2	-0.23	170.8	208.0	22%
Tomm5	-0.23	395.8	480.3	21%
Gnb1	-0.18	5447.6	6607.9	21%
Araf	-0.17	1419.0	1718.2	21%
Stxbp1	-0.17	2311.1	2795.0	21%
Cbx5	-0.21	835.5	1010.3	21%
Sertad2	-0.28	110.8	133.4	20%
Ctnnd2	-0.28	403.7	484.4	20%
Rnf219	-0.22	165.5	198.4	20%

FIG. 40 Continued

Psmd14	-0.18	778.4	933.0	20%
4930402H24 Rik	-0.18	946.3	1129.1	19%
Rnf19b	-0.17	596.7	711.0	19%
Ncan	-0.30	692.4	823.6	19%
D230025D16 Rik	-0.28	148.3	175.8	19%
Sec62	-0.27	563.8	666.2	18%
Rnf41	-0.32	299.7	352.9	18%
C77080	-0.17	99.4	116.4	17%
Acox1	-0.21	534.8	626.1	17%
Pum1	-0.16	552.1	644.2	17%
Micall1	-0.22	376.8	438.6	16%
Tmem129	-0.24	78.6	90.7	15%
Tmem110	-0.17	89.2	101.8	14%
Fert2	-0.15	193.2	218.8	13%
Acsl1	-0.30	126.9	143.4	13%
Ppp2r4	-0.21	783.8	884.2	13%
Papola	-0.21	596.0	669.2	12%
L3mbtl3	-0.34	172.1	193.2	12%
Tmub2	-0.23	453.7	502.1	11%
Gmeb1	-0.40	141.6	154.9	9%
Cdc42se2	-0.20	575.6	626.2	9%
Gpatch2l	-0.27	206.2	222.9	8%

FIG. 40 Continued

Primer and sequence information.

Promoter Cloning	Forward primer	Reverse primer	Promoter size
pri-mir218-2 7.4kb promoter	agatcgggtaccgagaaatacctccgctctg	agatcgaccggtgaaggctccatcttcaatgc	7442bp

CRISPR gRNA sequences		gRNA target sequence	PAM
mir-218-1	Left guide (5')	tatgatcatacacaatctgc	ggg
mir-218-1	Right guide (3')	ggagaataacaaatgtccgt	agg
mir-218-2	Left guide (5')	gactctgaccagttgccgcg	ggg
mir-218-2	Right guide (3')	taatgtggatactcgaagca	cgg
pri-miR-218-2 promoter	Left guide (5')	tgtctccatgcattgttacg	tgg
pri-miR-218-2 promoter	Right guide (3')	caggcgagcgagcgcaccaa	agg

oligos for px330 vector		T7-sgRNa PCR	
Forward	Reverse	T7 Primer	Reverse T7 Primer
cacc g tatgatcatacacaatctgc	aaac gcagattgtgtatgatcata c	taatacgactcactatagg tatgatcatacacaatctgc	aaaagcaccgactcggtgcc
cacc ggagaataacaaatgtccgt	aaac acggacatttgttattctcc	taatacgactcactata ggagaataacaaatgtccgt	aaaagcaccgactcggtgcc
cacc gactctgaccagttgccgcg	aaac cgcggcaactggtcagagtc	taatacgactcactatag gactctgaccagttgccgcg	aaaagcaccgactcggtgcc
cacc g taatgtggatactcgaagca	aaac tgcttcgagtatccacatta c	taatacgactcactatagg taatgtggatactcgaagca	aaaagcaccgactcggtgcc
cacc g tgtctccatgcattgttacg	aaac cgtaacaatgcatggagaca	taatacgactcactatagg tgtctccatgcattgttacg	aaaagcaccgactcggtgcc
cacc g caggcgagcgagcgcaccaa	aaac ttggtgcgctcgctcgcctg	taatacgactcactatagg caggcgagcgagcgcaccaa	aaaagcaccgactcggtgcc

Genotyping	Forward primer	Reverse primer	Band Sizes	Deletion size
miR-218-2 genotyping	ggcagtcctgtgtcacaaag	tggatatcggggttcatcgg	WT: 998bp/KO: 712bp	286bp deletion
miR-218-1 genotyping	accctcttttcaatccctga	ctttctgctgatccgattctg	WT: 841bp/KO: 482bp	359bp deletion

FIG. 41

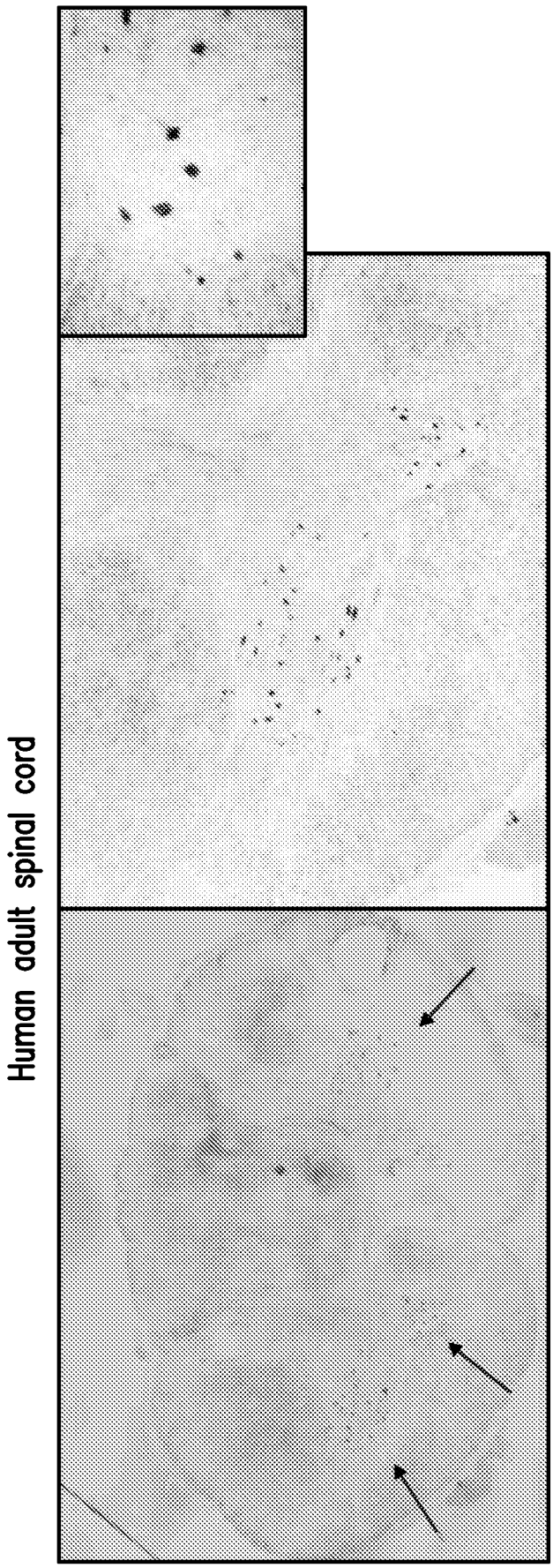


FIG. 42

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/033914

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)

   ☐ on paper

   ☐ in electronic form

   b. (time)

   ☐ in the international application as filed

   ☐ together with the international application in electronic form

   ☐ subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

   No sequence listing was provided in the application. A search was conducted on the basis of the sequence in Figure 1.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/033914

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
the subject matter listed in Rule 39 on which, under Article 17(2)(a)(i), an international search is not required to be carried out, including

2. ☒ Claims Nos.: **15-17 (in full), 13, 18-20 (in part)**
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
See Supplemental Box

3. ☐ Claims Nos:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See Supplemental Box for Details

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/033914

A. CLASSIFICATION OF SUBJECT MATTER

C12N 15/85 (2006.01) A61K 31/7088 (2006.01) A61P 25/28 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Espacenet: Salk, Amin

EPODOC, WPI, CAPLUS, MEDLINE, BIOSIS, EMBASE: mir-218, promoter, reporter, regulatory, control, sequence, motor neuron, als and like terms

GENOMEQUEST: SEQ ID NO. 1 at 90% identity

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C [ ] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 September 2016	13 September 2016
Name and mailing address of the ISA/AU	**Authorised officer**
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustralia.gov.au	Felix White AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. +61 2 62832565

INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. **PCT/US2016/033914**
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Punnamoottil, B. et al, "Motor Neuron-Expressed MicroRNAs 218 and Their Enhancers Are Nested Within Introns of *Slit2/3* Genes", genesis, 2015, vol. 53, pps 321-328. Published online 10 April 2015 Abstract, p. 324 1st paragraph	1-14, 18-21
A	Little, M. et al, "Conserved modularity and potential for alternate splicing in mouse and human *Slit* genes" International Journal of Developmental Biology, 2002, vol. 46, pps 385-391 Abstract, p. 390 rh column	1-14, 18-21
A	Eom, S. et al, "Transglutaminase II/MicroRNA-218/-181a Loop Regulates Positive Feedback Relationship between Allergic Inflammation and Tumor Metastasis" Journal of Biological Chemistry, 2014, vol. 289, pps 29483-29505 Abstract, p. 29485 4th paragraph	1-14, 18-21
A	Li, C.H. et al "Enhancer of Zeste Homolog 2 Silences MicroRNA-218 in Human Pancreatic Ductal Adenocarcinoma Cells by Inducing Formation of Heterochromatin", Gastroenterology, 2013, vol. 144, pps 1086-1097 Abstract, Fig 5D and p. 1097.e1 rh column 2nd paragraph	1-14, 18-21
A	Marson, A. et al "Connecting microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells", Cell, 2008, vol. 134, pps 521-533 Tables S6 and S7	1-14, 18-21
P,X	Amin, N.D. et al "Loss of motoneuron-specific microRNA-218 causes systemic neuromuscular failure", Science, 2015, vol. 350, pps 1525-1529, published 18 December 2015 Whole document	1-14, 18-21

INTERNATIONAL SEARCH REPORT	International application No. PCT/US2016/033914

Supplemental Box

Continuation of **Box II**
NB - this text repeats the contents of BOX VIII of the ISO.

Claims 12-20 do not satisfy the requirements of PCT Articles 5 and 6. The specification does not sufficiently disclose these inventions to enable the skilled worker to carry them out without need for further experimentation, and these claims are not supported by the specification over their full scope.

These claims are directed to methods of treating a human subject diagnosed with motor neurone disease, based on modulation of various aspects of the mir-218 pathway.

As a threshold issue, the specification demonstrates that mice engineered to lack mir-218 develop phenotypic similarities similar to motor neurone diseases. P. 47 l. 20-21 speculates that the link between mir-218 and motor neuron diseases "likely extends beyond phenotypic similarities". However the specification has not provided any demonstration that mir-218 is dysregulated in motor neuron diseases, and more importantly, has not provided any evidence that provision of mir-218 can successfully treat motor neuron disease symptoms.

Therefore, the claimed treatment methods must be considered to be merely speculative and unsupported (PCT Art 6). Additional experiments would be needed to establish the relationship of mir-218 and motor neuron disease, and there is no expectation that such experiments would be successful (PCT Art 5).

In addition to this threshold issue, various individual claims have additional support and disclosure issues as follows:

Claim 12 involves administering a construct of claims 1-10 – i.e. any nucleic acid under the control of the murine mir-218 promoter. The specification has no evidence that the murine mir-218 promoter will be effective in human cells. Further experimentaiton would be required to confirm this (PCT Art 5) Furthermore the full scope of claims 1-10 includes e.g. reporter genes under the control of the murine mir-218 promoter. These would clearly not be effective to treat disease and therefore are unsupported (PCT Art 6).

Claim 15 and also claim 13 (in part) involves administering a transcription factor that can increase mir-218 expression. These transcription factors are not specific to mir-218 nor are they all necessarily specific to motor neurons. The specification clearly does not provide sufficient disclosure to support the ability of such transcription factors to treat motor neuron disease.

Claims 16-17 involve modulating the expression or activity of a target of mir-218. The specification discloses (Figure 40) a very large number of genes whose expression is somewhat regulated by mir-218. Even assuming for the sake of argument that mir-218 dysregulation causes motor neuron disease through de-repression of these genes, there is no indication provided which of these genes are likely to be significant in mediating motor neuron disease. Therefore the specification clearly does not provide sufficient disclosure to support the ability of modulating any single one gene in Fig 40 to treat motor neuron disease.

Please note that in view of this lack of support, the search and examination of claims 12-20 has been limited insofar as they relate to mir-218 specifically, i.e. claims 12 and 14 (in full) and claims 13 and 18-20 (in part with respect to mir-218 itself).

Continuation of **Box III**
The application does not comply with the requirement for unity of invention.

This requires that all claims share a common "special technical feature" that makes a contribution over the prior art.

Invention 1: Claims 1-12 and 21 are characterised by a recombinant nucleic acid comprising the murine mir-218 promoter of SEQ ID NO: 1

Invention 2: Claims 13-20 are characterised by methods of treating motor neuron disease in a human by modulating the expression or activity of mir-218 or molecules upstream or downstream of mir-218.

The only feature shared by these groups of claims is mir-218, which is known in the art as can be seen from the documents cited in the ISR, and therefore cannot be a unifying "special technical feature".

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Supplemental Box

Another potential unifying concept is the fact that mir-218 is expressed in motor neurons; invention 1 relates to constructs for specific expression in motor neurons and invention 2 relates to treatment of motor neurons. However the relationship of mir-218 with motor neurons has already been disclosed in D1, and so this concept cannot unify the two groups of claims either and they lack unity *a posteriori*

I note that because invention 2 encompasses a large number of different target molecules for treating motor neuron disease and therefore may be considered to lack unity internally. However the international search has been restricted to mir-218 alone (see also Boxes III and VIII)

This report has been drawn up on both invention 1 (fully) and invention 2 (in part with respect to mir-218) as it did not require significant additional search effort to do so.