Target Genes of Transcription Factor Sp4 in Neuronal Development

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Abstract

The nervous system is a well-coordinated network that depends on the formation of proper connections among diverse types of neurons. Dendritic arborization patterns determine the way a neuron integrates inputs. Defects in this process are associated with neurodevelopmental disorders, such as schizophrenia (SZ). N-methyl-D-aspartate (NMDA) neurotransmission plays an important role in neuronal plasticity, cognition and memory. Altered NMDA neurotransmission may underlie the pathophysiology of multiple psychiatric disorders.

Transcription factor (TF) Sp4 controls dendritic patterning during cerebellar development by limiting branch formation and promoting activity-dependent pruning. Sp4 is associated with multiple neuropsychiatric disorders, such as schizophrenia and bipolar disorders (BD). Sp4 regulates NMDA receptor 1 (NMDAR1) protein level, as has been shown by a Sp4 hypomorphic mouse model. However, little is known about the target genes of Sp4 involved in regulating neuron development and NMDA neurotransmission.

We identified Sp4-regulated genes by comparing global gene expression between wild type and Sp4 hypomorphic mouse cerebellum. Of the differentially expressed genes, several have roles in neuron development, and several have altered expression in neuropsychiatric disorders.

In particular, we identified Nervous Wreck 2 (Nwk2) as an activation target of Sp4 that regulates dendritic patterning and NMDAR protein levels. Nwk2 in mammals is a homolog of Nwk in Drosophila, which plays a vital role in synapse growth. Nwk2

expression was reduced in Sp4 hypomorphic cerebellum and cerebellar granule neurons (CGNs). Sp4 localized to the Nwk2 promoter (CGNs). Knockdown of Nwk2 in CGNs produced increased number of primary dendrites, which phenocopied the effect of Sp4 knockdown on primary dendrites. Importantly, exogenous expression of Nwk2 in Sp4 depleted CGNs rescued the morphology of neurons. Furthermore, NMDA receptor protein level was reduced in Nwk2 knockdown CGNs, which was consistent with the observation of NMDAR1 loss in Sp4 hypomorphic and knockdown CGNs. Overexpression of NMDAR in Sp4 or Nwk2 depleted neurons rescued the increase in dendrite number. Our studies identify Sp4-Nwk2-NR1 as a new pathway that contributes to normal neuronal morphogenesis and may be disrupted in neuropsychiatric diseases.

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Abbreviations

BD	Bipolar disorder
BMP	Bone morphogenetic protein
CGN	Cerebellar granule neuron
ChIP	Chromatin immunoprecipitation
CNS	Central nervous system
CNV	Copy number variation
DIV	Day in vitro
EGL	External granular layer
F-BAR	FCH-BAR, FES-CIP4 homology and Bin/Amphiphysin/Rvs
Hypo	hypomorphic
IGL	Internal granular layer
IHC	Immunohistochemistry
LDP	Long-term depression
LTP	Long-term potentiation
MDD	Major depressive disorder
ML	Molecular layer
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate Receptor
NMJ	Neuromuscular junction
NR1	N-methyl-D-aspartate Receptor 1
NT3	Neurotrophin-3
NWK	Nervous Wreck
PPI	prepulse inhibition
RT-qPCR	reverse transcription-quantitative polymerase chain reaction
SDF-1	Stromal cell-derived factor 1
SH2 and 3	SRC Homology 2 and 3
Shh	Sonic hedgehog
SNP	Single nucleotide polymorphism
Sp	Specificity protein
SVZ	Subventricular zone
SZ	Schizophrenia
TF	Transcription factor
WT	Wild type

Chapter 1. Introduction

Transcription factor Sp4 in neuronal development

Transcription regulation in neuronal development

The nervous system is a complex and well-coordinated network that depends on the formation of proper connections among diverse types of neurons. A neuron has highly specialized morphology, typically with a long axon and multiple branching dendrites, which is important for formation of neuronal connections and neuronal function. To integrate into neuronal network, newly generated neurons from neural stem cells engage in a series of stereotypical developmental processes. After exit from the cell cycle, post-mitotic neurons first determine axon and dendrites identity, and at the same time undergo extensive migration to reach their final destinations in the brain. Axons grow to their appropriate targets, dendrites arborize and prune to cover their receptive field, and synapses form and are refined to establish proper connections.

These fundamental developmental events are regulated by both extrinsic cues and cell-intrinsic cues. The extrinsic cues include secreted polypeptide growth factors, adhesion molecules, extracellular matrix components, and neuronal activity [10-15]. Extrinsic cues are thought to regulate both the overall design of neuronal shape and their fine structural elements such as axon branch points and dendritic spines. Growth factors, guidance proteins, and other extrinsic cues act via specific cell surface receptor proteins, which in turn regulate intracellular signaling proteins that directly influence cytoskeletal elements. Members of the Rho GTPase family of proteins and protein kinases have emerged as key signaling intermediaries that couple the effects of extrinsic cues to the control of actin and microtubule dynamics [16-22].

Cell-intrinsic mechanisms have major roles in neuronal morphogenesis and connectivity. These mechanisms include transcription regulators, post-translational modifications, and inherited signaling pathways that are cell autonomous and orchestrate neuronal responses to extrinsic cues. Invertebrate model organisms have led the study of the cell-intrinsic mechanisms that regulate neuronal morphogenesis. Studies in Drosophila have been invaluable to the discovery of in vivo functions for transcription factors in diverse aspects of neuronal morphogenesis. In particular, studies of the da sensory neurons in the fly peripheral nervous system have defined roles for different transcription factors in distinct aspects of dendrite development, from growth and branching to tiling [23].

Several observations also highlight the importance of cell-intrinsic mechanisms in the control of neuronal morphogenesis and connectivity in mammalian neurons. For example, the in vivo programs of neuronal development, including polarization, migration, axon and dendrite growth, and synapse formation, can be recapitulated in different populations of neurons dissociated in primary culture [24, 25]. Extrinsic cues and cell-intrinsic mechanisms do not cooperate in isolation. Although neuronal morphogenesis is influenced by extrinsic signals, neurons often seem to carry a memory or intrinsic potential that is not altered by a new and different environment. Transplantation studies have suggested that neuronal precursors of the cerebral cortex that give rise to later-born upper layer neurons are restricted in their developmental potential and do not give rise to earlier-born deep-layer neurons when placed in the subventricular zone (SVZ) of younger hosts undergoing deep layer neurogenesis [26, 27]. Likewise, transplantation studies have revealed that dendrite patterning of granule

neurons in the rat olfactory bulb appear to be specified from the moment that neurons were born in the SVZ [28]. These studies are consistent with the idea that developmental programs for different populations of neurons are determined by cell intrinsic mechanisms. The intrinsic programing is retained even in new environments. This intrinsic identity may also influence how neurons respond to extrinsic cues. Application of the same neurotrophic factor to neurons located in distinct cerebral cortical layers shows differential effects on dendrite morphology [29, 30], suggesting that neurons inherit distinct developmental programs that dictate their responses to extrinsic signals. Purified rat embryonic retinal ganglion neurons cultured in a variety of conditions grow axons much faster than ganglion neurons from postnatal animals [31]. In addition, with maturation, retinal granule neurons undergo a switch from preferential axon growth to preferential dendrite growth [31]. Collectively, these observations suggest that neurons harbor developmentally inherited cell-intrinsic mechanisms that determine, in large part, neuronal morphogenesis.

Transcriptional control of gene expression represents a major mechanism of cell-intrinsic regulation of neuronal development. Transcription factors can govern entire developmental programs, directing distinct stages of neuronal development as well as altering the competency and response of cells to extrinsic cues. Accordingly, often the expression of one or a set of transcription factors is sufficient to direct the subtype specification of distinct neuronal populations and thus their morphology and projection patterns [32-35]. The current challenge is to understand cell intrinsic regulation by identifying the transcription factors responsible for different aspects of neuronal morphogenesis, their direct targets, and the interplay with extrinsic cues.

Cerebellar granule neurons as a model to study neuronal development

The rodent cerebellar cortex provides an excellent model system for the study of mechanisms that shape neurons [36-39]. Cerebellar cortex has a simple and well-defined anatomy, and more than 100 years worth of detailed anatomical characterization throughout development. The cerebellar cortex itself has only 3 or 4 layers of structures, depending on the developmental stage examined. In addition to its classical motor coordination function, an increasing body of evidence indicates that the cerebellum is also involved in psychological alterations and in the modulation of affective, cognitive, and perceptual functions [40-43].

There are two major neuron types in the cerebellar cortex, the Purkinje cells and the granule cells. In the intact cerebellar cortex, cerebellar granule neurons (CGNs) begin life as proliferative precursors in the external granular layer (EGL). They exit the mitotic cycle, and migrate through the molecular layer (ML), past the soma of the Purkinje cells in the Purkinje cell layer, and take up residence in the internal granular layer (IGL), where they remain for the lifetime of the organism. During the migratory phase, granule neurons extend a long, T-shaped axon. Once in the internal granular layer, dendrites undergo growth, arborization, pruning and dendritic claw formation to establish synaptic connections [36, 37] (Figure1-1).

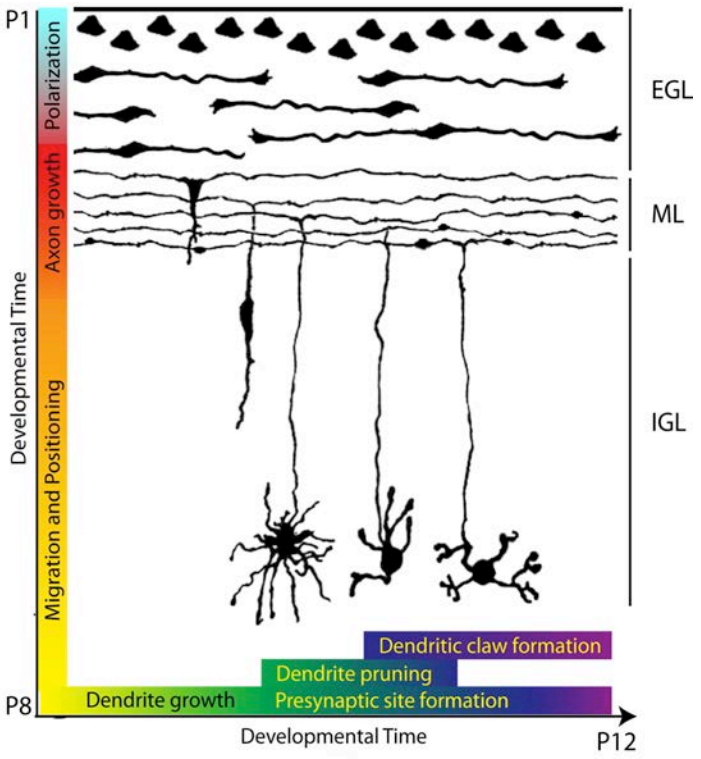

Figure 1-1 Development of cerebellar granule neurons.

Cerebellar granule neurons (CGNs) begin life as proliferative precursors in the external granular layer (EGL). They exit the mitotic cycle, and migrate through the molecular layer (ML), past the soma of the Purkinje cells in the Purkinje cell layer, and take up residence in the internal granular layer (IGL), where they remain for the lifetime of the organism. During the migratory phase, granule neurons extend a long, T-shaped axon. Once in the internal granular layer, dendrites undergo growth, arborization, pruning and dendritic claw formation to establish synaptic connections (Adapted from [8])

CGN development is elaborated through the interaction of both cell-intrinsic and extrinsic mechanisms. Pure cultures of dissociated granule neurons faithfully recapitulate not only these morphological changes [24, 44], but also accompanying changes in gene expression, as demonstrated by recent genome-wide analyses [45]. These suggest that the basic set of instructions to shape granule neurons is intrinsically encoded. Meanwhile, like all other neurons, cerebellar granule neurons require depolarizing electrical activity or trophic factors to develop and form the neuronal circuit. Cell division and migration within the EGL are regulated by proteins produced by neighboring cells, including sonic hedgehog (Shh) derived from Purkinje neurons and stromal cell-derived factor 1(SDF-1) released from the meninges[46-48]. Membrane depolarization is critical for dendrite growth and branching in cerebellar granule neurons[49, 50]. Membrane depolarization activates voltage-sensitive calcium channels, leading to the entry of calcium into neurons and the activation of calcium-dependent signaling molecules, including Ca^{2+}-calmodulin-dependent protein kinases and the phosphatase calcineurin [51-55]. Calcium-dependent signaling pathways also regulate the gene expression profile of cerebellar granule neurons [54] [56].

The granule neurons are the most abundant neuronal type in cerebellum, and easily purified from neonatal rodents and maintained in culture [57]. This permits a molecular analysis of a homogenous population of developing neurons. The expression of particular genes can be manipulated by transfection into CGNs, allowing one to characterize the gene function in a given aspect of neuronal morphogenesis and connectivity. Most importantly, granule neurons follow a stereotypical, cell autonomous maturation program both in vivo and in vitro. Cerebellar granule neurons have emerged as a popular in vitro

model system to study neuronal morphogenesis. CGNs assist to elucidate how various stages of neuronal development are regulated, including the functions of specific genes as well as how the expression of various genes is controlled at different developmental steps.

Neuron dendritic patterning is regulated by transcription factors

Dendrite development is one of the critical steps of the process in neuronal morphogenesis. Dendrite development in CGNs consists of a series of events beginning with the initiation of growth and branching, leading to the formation of an exuberant arbor, followed by pruning, and culminating in the formation of postsynaptic structures termed dendritic claws at the ends of the remaining few dendrites. Dendritic claws house sites of connectivity with mossy fiber terminals and Golgi neuron axons [36, 37, 39] (Figure 1-1).

Dendritic arborization patterns determine the way a neuron integrates inputs. It is not surprising that alterations in dendrite morphology or defects in dendrite development contribute to several neurological and neurodevelopmental disorders, such as autism spectrum disorders (ASDs), Alzheimer's disease (AD), schizophrenia, Down syndrome (DS), Fragile X syndrome (FXS), Rett syndrome (as reviewed in [58]), anxiety, and depression [59] [60] (Figure 1-2).

Dendrite growth and maturation are under transcriptional control in granule neurons. Transcriptional regulators CREB and CREST have been identified to promote dendritic growth and branching [61, 62]. Intriguingly, transcription factors in these developmental

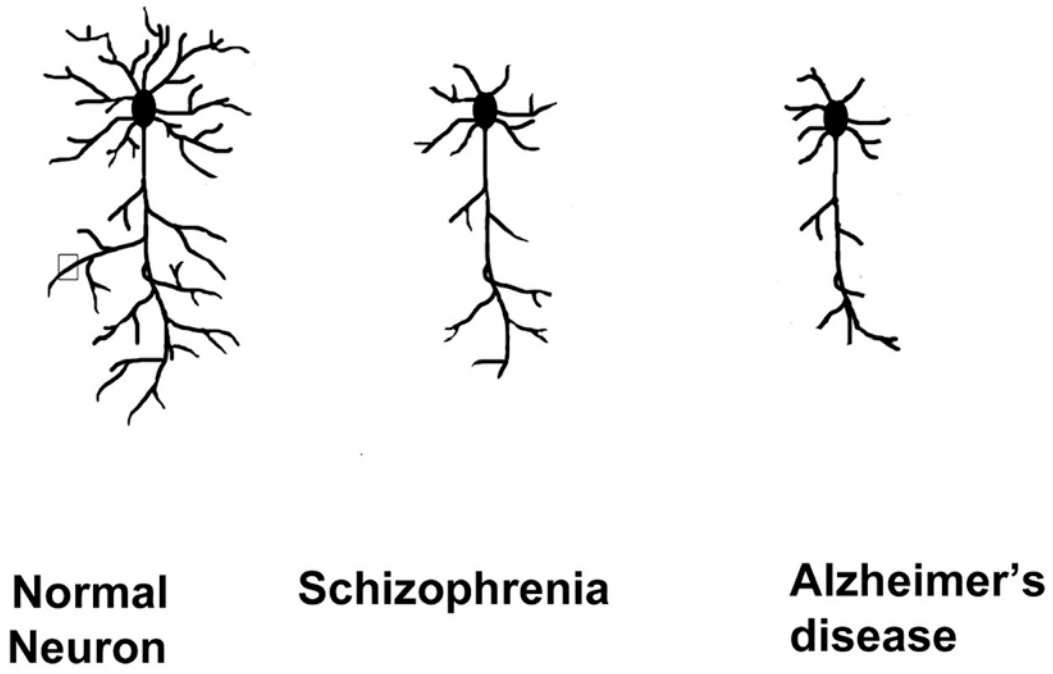

Figure 1-2 Schematic representation of neuron depicting atrophies in dendrite morphology in brains of individuals with the neurological disorders.

Schizophrenia: neurons show reduced dendritic arbor. Patients with positive symptoms show smaller dendrite branches in CA3 region of the hippocampus[2-4]. Alzheimer's disease (AD): substantial alterations in the dendritic arbor are seen in individuals with AD. Significant reductions in the total dendrite lengths of apical and basal trees of CA1a and CA1b subdivisions of CA subregion of the hippocampus are observed. Dentate gyrus granule cells of the hippocampus show significant decreases in dendrite length of apical trees. (Adapted from [9])

steps are strongly influenced by neuronal activity and calcium signaling. The bHLH transcription factor NeuroD promotes dendrite growth in response to activation of L-type voltage sensitive calcium channels (VSCCs) [49]. In a later phase of development, the sumoylated repressor form of the transcription factor myocyte enhancer factor 2A (MEF2A) drives postsynaptic dendritic claw differentiation in a manner that is also regulated by VSCC activation [63]. These studies suggest that activity-dependent calcium signaling regulates dendrite growth and maturation at least in part through changes in gene expression governed by transcription factors.

Sp4 regulates neuron dendritic patterning

Transcription factor Sp4 is part of the cell-intrinsic mechanism that determines dendritic morphology through gene expression programs. The previous study in our lab demonstrated that Sp4 controls dendritic patterning during cerebellar development by limiting branch formation and promoting pruning [64]. Knockdown of the transcription factor Sp4 led to an increased number of highly branched dendrites during maturation of cerebellar granule neurons in dissociated and organotypic cultures. Time-course analysis revealed that depletion of Sp4 led to persistent generation of dendritic branches and a failure in resorption of transient dendrites. Depolarization induced a reduction in the number of dendrites, and knockdown of Sp4 blocked depolarization-induced remodeling. Furthermore, overexpression of Sp4 wild type, but not a mutant lacking the DNA-binding domain, was sufficient to promote dendritic pruning in nondepolarizing conditions. These findings indicate that the transcription factor Sp4 controls dendritic patterning during cerebellar development by limiting branch formation and promoting activity dependent pruning.

Sp4 is a transcription factor in neurons

Sp4 is a member of the Sp family of transcription factors that is characterized by three conserved Zinc fingers for DNA-binding. Among this family, Sp1 was first identified in humans. It has contiguous zinc finger motifs and binds to the GC- (GGGGCGGGG) and GT- (GGTGTGGGG) boxes [65]. In 1992, Hagen performed recognition site screening for DNA binding factors, which bind to the uteroglobin promoter sequence GT box, and cloned cDNAs encoding the DNA-binding proteins Sp3 and Sp4, thus revealing the existence of a multigene family of Sp proteins [66]. GC and GT boxes are recurrent elements in regulatory sequences such as promoters, enhancers and CpG islands in many genes. GC/GT boxes are important for the expression of different ubiquitous as well as tissue-specific cellular and viral genes [67]. In addition, these elements are involved in the maintenance of the methylation-free status of the CpG islands [68, 69].

There are at least 8 Sp proteins in the Sp family. Sp4 is closely related to Sp1 and Sp3 structurally. In addition to the highly conserved C2H2-type zinc finger DNA binding domain close to the C terminus, all three proteins contain serine/threonine-rich regions and two glutamine-rich regions in the N-terminal part of the protein [70]. Glutamine-rich regions have been identified in Sp1 as transactivation domains (Figure 1-3). The N-terminal inhibitory domain of Sp1 has been suggested to involve protein-protein interactions with corepressors SMRT, NCoR and BCoR [71]. The inhibitory domain of Sp3 under certain conditions has been mapped between the second glutamine-rich activation domain and the first zinc finger [72]. One mechanism involved in the repressive activity of Sp1 and Sp3 is post-translational modification by the small

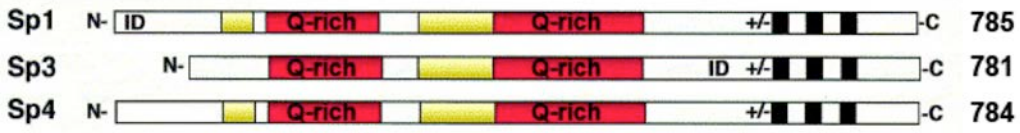

Figure 1-3 Structural motifs in Sp factors.

Structural motifs in Sp factors. serine/threonine-rich (yellow), glutamine-rich(red) and highly charged regions(+/-), and zinc fingers(black) are indicated, as well as inhibitory (ID) domains. (Adapted from [6])

ubiquitin-related modifier (SUMO) [73-75]. In terms of transcriptional properties, Sp1 and Sp3 function as both transcriptional activators and repressors [15, 76-78]. Similarily, Sp4 represses NT3 and activates the TrKC promoter in cerebellar granule neurons [79]. It is not known whether Sp4 protein has the same homologous activation and repression domains as Sp1 and Sp3 or whether post-translation modification in certain domains is involved in Sp4 activity. I have carried out an intial study of to map the functional domains of Sp4. A novel Sp4 activation domain was mapped between the second glutamine-rich activation domain and the first zinc finger, the data shown in appendix II.

Sp1, Sp3 and Sp4 regulate gene transcription by binding to the GC/GT box, but individual Sp factors have different functional properties and fulfill specific biological roles. What determines their specificity in gene regulation? Besides the DNA context in promoters with various cis-regulatory elements and different protein machinery recruited by specific gene and other mechanisms, tissue specific expression of Sp proteins is also one of the events that determine the specific control of gene transcription.

In contrast to Sp1 and Sp3, which are ubiquitously expressed, Sp4 is abundantly expressed in the central nervous system, highly enriched in neurons [66, 80, 81]. Sp4 is abundantly expressed in brain, also detectable in heart, skeletal muscle and other tissues [66, 80]. These results were demonstrated by northern blot and in situ hybridization [66, 80] in mice and lacZ staining of $Sp4^{lacz}$ allele in heterozygous Sp4 mutant mice [82].

The central nervous system consists of neurons and glial cells. Neurons constitute about half the volume of the CNS and glial cells make up the rest. Glial cells provide support and protection for neurons. Astrocytes are one of the major types of CNS supporting cells. As assayed by immunofluorescence in rat tissue and mixed hippocampal

neuron-glia cultures, RT-PCR analysis of mRNA in cultures of astrocytes or neocortical neurons, western-blot analysis and antibody-dependent ‘‘supershift’’ of DNA-binding assays in cultured astrocytes, neocortical neurons, neuronal cell line, microglial cell line, Mao demonstrated that Sp1 were enriched in astrocytes, whereas Sp4 and Sp3 were enriched in neurons [83]. That is, Sp4 is mainly expressed in neurons, whereas Sp1 is predominantly expressed in glial cells. Similar results were also found in our lab.

Transcription factor Sp4 is expressed in cerebellar granule neurons. In transgenic mice containing multiple copies of a modified BAC in which an EGFP reporter gene is inserted immediately upstream of the coding sequence of Sp4 gene, EGFP was detected in the cerebellar granule neurons both in EGL and IGL at postnatal Day7 mice (Figure 1-4). This demonstrated that Sp4 is expressed in cerebellar granule neurons under the control of its promoter. The expression patterning of Sp4 in cerebellum also provides a solid basis for using CGNs to study Sp4’s function.

The restricted expression pattern explains and predicts the specific biological function of Sp4 in central nervous system and its contribution to central nervous system diseases (see below).

Sp4 target genes

Transcription factor Sp4 has an essential role during dendritic development in cerebellar granule neurons [64]. Knockdown of Sp4 in organotypic slices and dissociated cultures led to an increased number of highly branched dendrites. Time course analysis

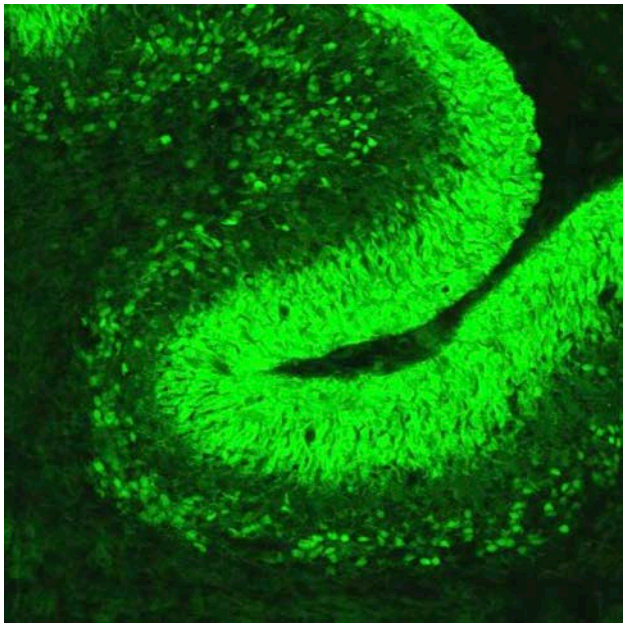

Figure 1-4 Sp4 is expressed in cerebellar granule neurons.

Using a BAC transgenic mice, EGFP was introduced upstream of the ATG start codon of Sp4 gene on a selected BAC clone. The inserted gene has its own ATG start codon and polyadenylation signal. This modification results in the expression of EGFP under the control of the promoter and regulatory elements of Sp4 gene that are present on the BAC clone. The EGFP is expressed in the cerebellar granule neurons in both EGL and IGL at postnatal Day7 mice [5].

revealed that upon Sp4 knockdown, removal of dendrites was blocked and new dendritic branches continued to be added at later times. Furthermore, over-expression of Sp4 was sufficient to promote dendritic pruning in non-depolarizing conditions. These studies revealed that Sp4 is required to limit addition of new branches and promote activity-dependent pruning of dendrites. Like the related Sp1 and Sp3 proteins, Sp4 binds to specific promoter elements to regulate transcription [15, 67, 84, 85], but the number and nature of Sp4 target genes that mediate dendritic patterning have not been described.

Our lab found neurotrophin-3 (NT3) as a target gene important for Sp4-dependent dendritic morphogenesis [79]. Sp4 overexpression reduced NT3 promoter activity whereas knockdown of Sp4 increased NT3 promoter activity and mRNA. Moreover, Sp4 bound to the NT3 promoter in vivo, supporting a direct role for Sp4 as a repressor of NT3 expression. Addition of exogenous NT3 promoted dendritic branching in cerebellar granule neurons. Furthermore, sequestering NT3 by a soluble form of its receptor blocked the continued addition of dendritic branches observed upon Sp4 knockdown, but had no effect on dendrite pruning. These findings demonstrate that, during cerebellar granule neuron development, Sp4-dependent repression of neurotrophin-3 is required to limit dendritic branching and thereby promote acquisition of the mature dendritic pattern.

Analysis of mutant mice expressing low levels of Sp4 revealed an age-dependent decrease of NT3 mRNA in the hippocampus [1]. When Sp4 was knocked out specifically in neural crest, NT3 levels were not affected, although a reduction in levels of TrkC mRNA was observed [86]. These studies suggest cell type and/or developmental time dependent regulation of NT3 and TrkC by Sp4.

Analysis of the Sp4 target gene NT3 showed that increased neurotrophin-3 results in increased dendritic branching, but not in an increased number of primary dendrite numbers that happens upon Sp4 depletion in cerebellar granular neurons. Thus there are additional Sp4 target genes regulating dendritic formation and patterning that remain to be determined.

Sp4 and neuropsychiatric disorders

Sp4 has been associated with the incidence of neuropsychiatric disorders. Genome wide association studies, identification of rare copy number variants (CNV) and targeted analysis of single nucleotide polymorphisms (SNPs) have revealed Sp4 locus associated with major depressive disorder, schizophrenia and bipolar disorder [87-90].

A case-control genome-wide association study of recurrent early-onset major depressive disorder (MDD) using single nucleotide polymorphism (SNP) array technology and a meta-analysis with other data sets supported the association of SNPs within the Sp4 gene and recurrent early-onset MDD [90].

10 SNPs selected from the human Sp4 genomic locus were tested in case-control populations. 2 out of the 10 were associated with bipolar disorder both in European Caucasian bipolar families and Chinese bipolar case control families. One of the two SNPs is also associated with Chinese trio families for schizophrenia[88]. A large-scale genome-wide CNV scan has been conducted in schizophrenia patients and controls. SP4 deletion was found in two sporadic patients. The two SP4 deletions excised the first three exons as well as the 5'-flanking regulatory region of SP4 gene, constituting loss-of-

function mutations of the Sp4 gene [91]. It was concluded that Sp4 is a susceptibility gene for bipolar disorder and schizophrenia.

Beyond the genome wide association study, in collaboration with the Gill lab, a group in Spain examined the Sp4 protein level in postmortem brains from 10 BP patients and 10 controls. They found that Sp4 protein level was reduced in post-mortem tissue cerebellum and prefrontal cortex of bipolar disorder patients, but not the mRNA[92]. SP4 protein and mRNA levels were also reduced in the prefrontal cortex. This study supported a possible role of transcription factor SP4 in the pathogenesis of Bipolar Disorder. Sp4 protein was significantly decreased in a relatively small sample of patients (n=10), compared to 2 sporadic Sp4 deletion patients from a large schizophrenia patient population in Zhou's study. That is, the Sp4 protein level may be defective even when the Sp4 gene is intact in bipolar patients. This suggests Sp4 is an important component in maintaining the normal function of neurons, and deficits in Sp4 increase the risk of neuropsychiatric disorders.

Neuropsychiatric disorders significantly impair the ability of the affected individual to communicate effectively, have clear mentation, and behave in a socially appropriate and consistent manner, which negatively impacts the ability to conduct a normal lifestyle. Schizophrenia related disorders affect approximately 2.4 million people, or 1.1% of the USA population >18 years old each year [93, 94], while the spectrum of bipolar disorders affect approximately 5.7 million people, or 2.6% of the USA population >18 years old each year [95]. These disorders place a profound emotional, financial, and social burden on the individual, their families, and society at large.

To date, the majority of medications receiving FDA approval for neuropsychiatric disorders are modifications of drugs that were developed several decades ago based upon clinical observations, as well as trial and error. Unfortunately, bona fide targets for neuropsychiatric and neurodegenerative disorders are relatively scarce, and there is a paucity of proof-of-concept studies for novel drugs in psychiatric and neurological practice. Clearly, there is an urgent need to identify relevant neurobiological mechanisms that can be translated into treatments for amelioration of psychotic disorders.

Impaired Sp4 function contributes to the formation of neuropsychiatric disorders. The study of the mechanisms by which Sp4 regulates gene transcription in the central nervous system may provide insights into the understanding of pathogenesis of neuropsychiatric disorders and guide the development of new drugs to treat neuropsychiatric disorders.

Animal models for study of Sp4 function

In order to study the biological function of Sp4, several groups have generated Sp4 gene targeted mice. Strategies to generate Sp4 null mice included targeting the DNA binding domain [80], replacing the start codon by a LacZ reporter gene [96] and deleting the poly-glutamine region [82]. About 2/3 of Sp4 null mice die of sudden cardiac death because of defects in conduction system, since Sp4 is also expressed and plays a critical role in the heart conduction system [96].

Sp4 null mice are smaller in size and reproductively sterile. Homozygous Sp4 mutant males do not copulate, despite the fact that they have histologically intact testes containing mature sperm and no abnormalities in the hypothalamus or the vomeronasal organ were observed. Pheromone receptor genes in the vomeronasal organ also appear

unaffected. These findings raise the possibility that Sp4 is required for normal male reproductive behavior [80, 82].

Sp4 null mice display impaired postnatal development of hippocampal dentate gyrus [97]. Cell proliferation is reduced in the hippocampus, and dendritic growth and arborization of dentate granule cells was decreased in the cultured hippocampal granule neurons.

In 2005, a group at the University of California, San Diego generated Sp4 hypomorphic mice. The LacZ gene with a splicing acceptor (5') and 2 additional transcription terminators (3') (flanked by loxP sites) was inserted into the first intron of Sp4 gene. Following that, a rat sp4 coding sequence was fused in frame [1] (Figure 1-5). The Sp4 expression level in the brain of homozygous Sp4 mutant mice was reduced to about 2-5% of the wild-type Sp4 level, as shown in Figure 2-1. The viability of these Sp4 mutant mice is improved, although the Sp4 hypomorphic mice are still underrepresented in the weaned litter population. About 20% of Sp4 hypomorphic mice die in the first week after birth, according to my experience with breeding this strain. Similar to Sp4 null mice, Sp4 hypomorphic mice are smaller in size and reported to be reproductively sterile.

Sp4 hypomorphs display behavioral deficits in sensorimotor gating, contextual and spatial memory, and Sp4 hypomorphic mice have reduced long-term potiationation (LTP) in hippocampal CA1 region and reduced NMDAR1 protein level in the whole brain, which properties are all related to some psychiatric disorders, for example, schizophrenia [91, 98].

Prepulse inhibition (PPI), a measure of sensorimotor gating, is reduced in schizophrenia and other neuropsychiatric disorders. Sp4 hypomorphic mice showed less

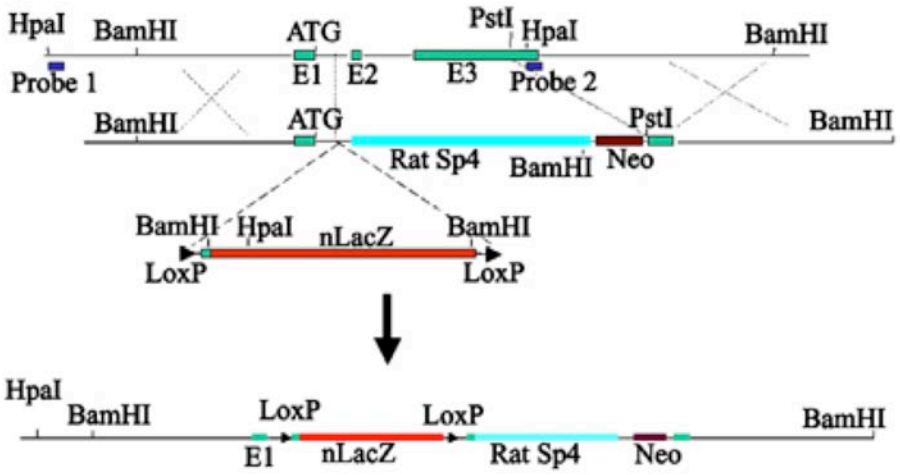

Figure 1-5 Gene targeting strategy used to generate Sp4 hypomorphic mice.

A nuclear LacZ gene with splicing acceptor flanked by two loxP sites was inserted into the first intron of the Sp4 gene. A rat Sp4 coding sequence with mouse Sp4 3'UTR was fused in frame into the second exon of mouse Sp4 gene. (Adapted from [1])

inhibition of the startle response after prepulses, which means that the sensorimotor gating is impaired in Sp4 hypomorphic mice. Sp4 hypomorphic mice displayed learning/memory deficits in both contextual fear conditioning and passive avoidance tests [1], and also displayed impaired spatial learning/memory in the Barnes maze, mimicking a cognitive deficit seen in schizophrenia [99, 100]. Impaired NMDA neurotransmission, supported by the decrease of NMDAR1 protein level and electrophysiological observations about reduced LTP in the Sp4 hypomorphic mice hippocampus, provided a plausible molecular mechanism for the behavioral deficits in both memory and sensorimotor gating in Sp4 hypomorphic mice. In humans, LTP in the hippocampal CA1 has an important role in the formation of memory [101]. Impaired N-methyl-D-aspartate (NMDA) neurotransmission has been suggested to cause cognitive deficits in schizophrenia [102]. Sp4 hypomorphic mice thus emerge as a model to study neuropsychiatric disorders.

We used the Sp4 hypomorphic mice to observe whether Sp4 play roles in cerebellum gross anatomy (shown in Appendix III). We extracted mRNA from cerebellum tissue to compare the gene expression profiles between WT and Sp4 hypomorphic mice, in order to identify Sp4 target genes (data in Chapter 2). We also validated the expression of interesting Sp4 targets in mouse cerebellum tissue and cultured CGNs (data in Chapter 3). Sp4 hypomorphic mice gave us a valuable platform to study Sp4's function and its target genes.

NMDA receptor in neuropsychiatric disorders

NMDA receptor and its regulation

N-methyl-d-aspartate receptors (NMDARs) are glutamate-gated ion channels that are pivotal in brain function. On the one hand, these receptors are critical for glutamate-mediated excitatory signaling, participating in synaptic transmission and triggering the synaptic plasticity that is thought to underlie learning and memory. If left uncontrolled, however, NMDARs can destroy neurons and initiate several forms of neuronal death. To keep these dual effects in balance, NMDARs are highly regulated by a host of mechanisms, including the action of ions such as magnesium, zinc, protons and calcium, and amino acids such as glutamate, aspartate, glycine and d-serine, and among others, receptor phosphorylation, intramembranous receptor movement and intracellular receptor trafficking. (Reviewed by [103])

NMDA neurotransmission and neuropsychiatric disorders

NMDAR activation provides intracellular calcium signals that initiate several forms of synaptic plasticity including long-term potentiation (LTP) and long-term depression (LTD). LTP and LTD are leading mechanisms thought to underlie the synaptic changes associated with learning.

The involvement of NMDARs in synaptic plasticity and excitotoxicity has implications for the pathophysiology of neurological and psychiatric disorders including mental retardation, autism, schizophrenia, mood disorders and epilepsy. Many of these disorders are associated with impaired learning and memory, and defects in synaptic plasticity are likely to play key roles in cognitive dysfunction.

Impaired NMDA neurotransmission has been suggested to cause prefrontal cognitive deficits in schizophrenia [104]. This theory is based on the observation that acute

administrations of non-competitive NMDA receptor antagonists, such as Phencyclidine and ketamine, induce schizophrenia-like symptoms in healthy people and worsen some symptoms in schizophrenia [105-107]. Recently, patients with anti-NMDA-receptor encephalitis (anti-NMDAR1) were found to display many schizophrenia-like symptoms and/or loss of memory[108]. Mouse hypoglutamatergic models with the deletion of nmdar1 gene display deficient sensorimotor gating and memory [109-111].

NMDAR assembly and trafficking

NMDA receptors are tetrameric ion channels consisting of two NR1 subunits and two NR2 /3 subunits. There are eight splice variants of NR1. The NR2 subunit has NR2a, NR2b and NR2c and NR2d homologs. There are two NR3 subtypes that are expressed at highest levels during development and appear to negatively modulate channel function. There are also developmental changes in the expression of NR2A and NR2B subunits; NR2B predominates early in development and NR2A increases with maturation, although both are expressed into adulthood.

After NMDA receptor subunits are translated, NMDA receptors are assembled in the endoplasmic reticulum (ER), processed by the Golgi network and transported to the neuronal surface. The subunits that are not assembled are degraded by the proteasome. The NMDA receptors on the neuron surface are kept in dynamic balance. NMDA receptors are internalized by endocytosis and sorted by the early endosome. Some of them go to the lysosome to be degraded and some of them are transferred into the recycling endosome and integrated back into the plasma membrane (Figure 1-6).

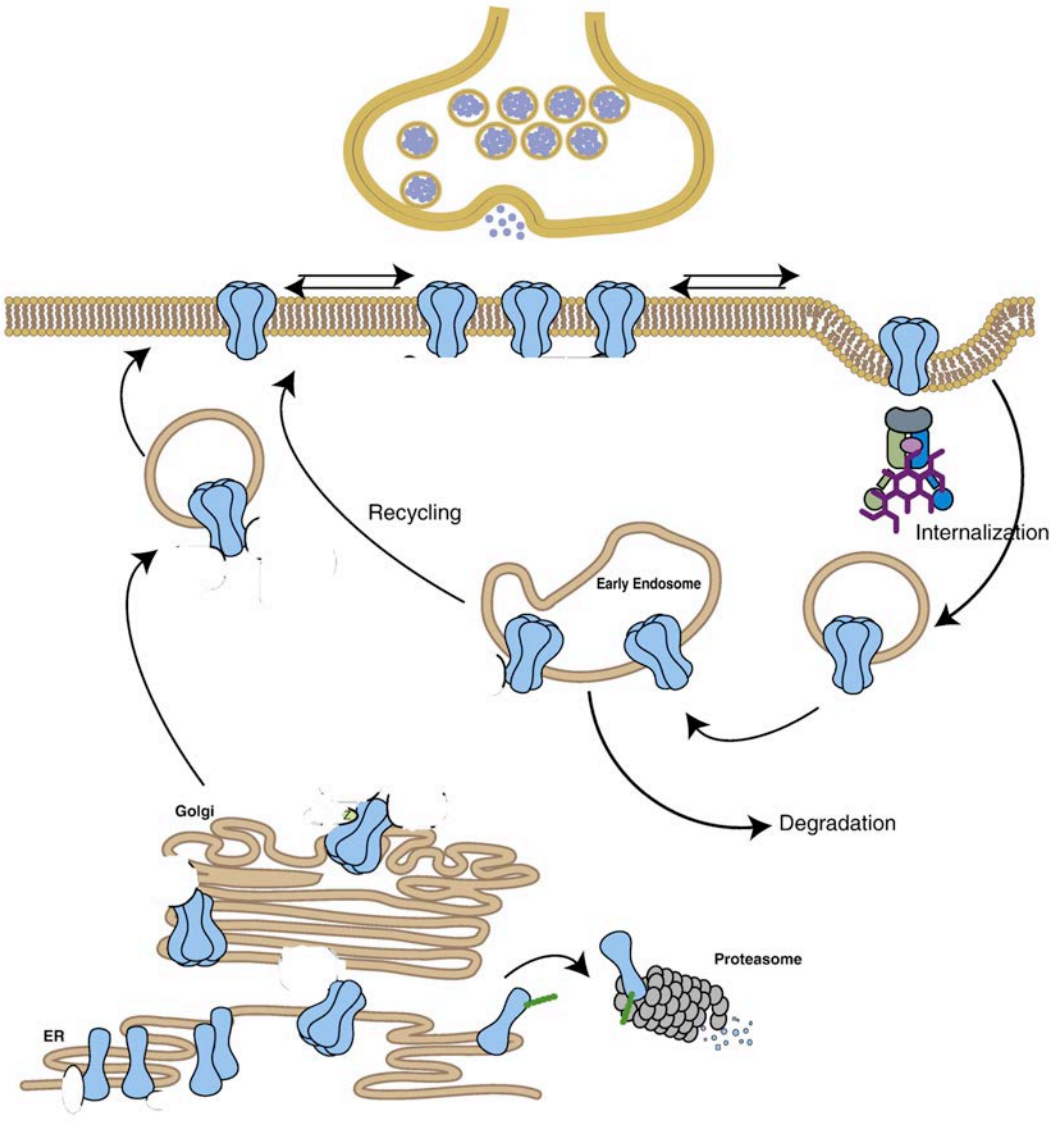

Figure 1-6 NMDAR assembly, transport and trafficking

After NMDA receptor subunits are translated, NMDA receptors are assembled in the endoplasmic reticulum (ER), processed by the Golgi network and transported to the neuronal surface. The subunits that are not assembled are degraded by the proteasome. The NMDA receptors on the neuron surface are kept in dynamic balance. NMDA receptors are internalized by endocytosis and sorted by the early endosome. Some of them go to the lysosome to be degraded and some of them are transferred into the recycling endosome and integrated back into the plasma membrane. (Figure dapted from [7])

Sp4 and NMDAR

A recent study showed NMDAR1 (NR1) protein level, but surprisingly not its mRNA, is reduced in the Sp4 hypomorphic mouse [91], which provides a plausible molecular mechanism for the impaired spatial learning and memory and reduced LTP found in the hypomorphic mice.

We also see reduced NMDAR1 protein level in Sp4 hypomorphic and Sp4 depleted CGNs (Data in Chapter 3). Several possible mechanisms have been proposed by Zhou [91]. First, Sp4 may regulate NMDAR1 RNA expression only in a small number of specific types of neurons (e.g. interneurons), which may subsequently result in down-regulation of NMDAR1 (e.g. NMDAR1 turnover) in a large number of pyramidal neurons via abnormal NMDA neurotransmission networking. Second, since mouse Sp4 gene expression starts around E9 during embryogenesis, subtle developmental abnormalities (although not detected yet) cannot be ruled out during the development of Sp4 hypomorphic mice, which might indirectly contribute to down-regulation of NMDAR1 protein expression. Third, the reduced expression of Sp4 gene may cause aberrant expression of many downstream target genes, which somehow results in down-regulation of NMDAR1 proteins. Fourth, Sp4 transcription factors may be directly involved in post-transcriptional regulation of NMDAR1 expression, such as Y-box-binding proteins, which function as both DNA and RNA binding proteins, and directly affects DNA repair, RNA splicing and exon skipping in a transcription-independent manner [91].

Here we show in chapter 3 a role for Sp4 target gene, Nwk2, in NMDAR1 protein level regulation, which supports the third model about regulating NMDAR1 by the Sp4 target genes.

Nervous Wreck 2 (Nwk2)

We identified Nervous Wreck 2 (Nwk2) as an activation target of Sp4 that regulates dendritic patterning and NMDAR protein levels.

Nwk2 protein structure

Nwk2 is a homolog of nervous wreck (Nwk) in Drosophila. Nwk exists in various species including Drosophila, C.elegans, moth, mouse, and human, suggesting it has conserved functions across evolution. There are 2 homologs of Nwk in human and mice, Nwk1 and Nwk2. Based on functional domains in the protein, Nwk1 is also known as Fchsd2 (FCH and double SH3 domains 2), and Nwk2 as Nwk2 (FCH and double SH3 domains 1).

Nwks are characterized by the presence of an FCH-BAR (FES-CIP4 Homology and Bin/Amphiphysin/Rvs) domain, 2 SH3 domains and a proline-rich domain (Figure 1-7). FCH-BAR domains can detect, induce and be regulated by membrane curvature and have essential roles in endocytosis, and regulation of actin cytoskeleton and signaling. SH3 domains bind to proline-rich ligands, and commonly link transmembrane signaling molecules with cytoskeleton-associated effectors in the cytoplasm. The proline-rich domain binds SH3, and plays a role in transcription activation and endocytosis.

Nwks belongs to a F-BAR protein family with the archetypal feature of an FCH-BAR domain. The F-BAR-domain-containing proteins (F-BAR proteins) are a group of adaptor proteins, members of which are found in all eukaryotes except plants. They have essential roles in fundamental biological processes, such as endocytosis, exocytosis and cell motility. There are at least 21 F-BAR-protein-encoding genes in the human genome,

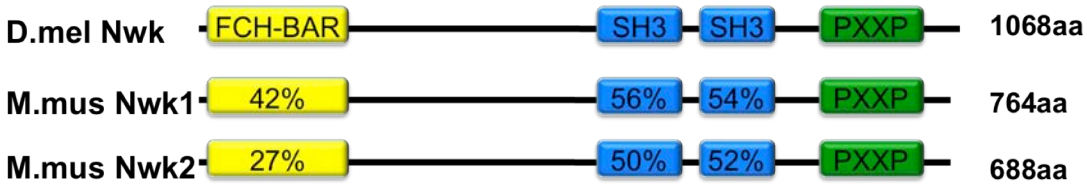

Figure 1-7 A schematic diagram of Nwk protein structure.

Nwks are characterized by the presence of an FCH-BAR (FES-CIP4 Homology and Bin/Amphiphysin/Rvs) domain, 2 SH3 domains and a proline-rich domain. Comparison of Nwk to 2 homologs in *M. mus* was shown in this figure. Percentages indicate the identity each domain shares with its counterpart in *Drosophila* Nwk; for example, FCH-BAR domain in *M.mus* Nwk1 is 42% identical to FCH-BAR domain in Nwk.

which encode approximately 36 proteins. The C-terminus of these proteins includes various combinations of RhoGAP, kinase, SH2 and SH3 domains, and they can be divided into 6 subfamilies: FES/FER tyrosine kinases (with a tyrosine kinase domain at the C-terminus), the PACSIN/syndapin subfamily (with one SH3 domain at the C-terminus), the CIP4 subfamily (with one SH3 domain), the SRGAP subfamily (with one RhoGAP and one SH3), the PSTPIP subfamily (with SH3 domain) and the FCH-domain-only (FCHO) subfamily (reviewed by [112]).

Nwk1 and 2 belong to the SRGAP subfamily, whose members typically have one RhoGAP domain and one SH3 domain besides the F-BAR domain. Nwks don't have a RhoGAP domain; instead, they swap the RhoGAP domain with another SH3 domain. The SRGAP subfamily is important in Slit-Roto signaling during mouse neurogenesis [113]. Another member of SRGAP subfamily, MEGAP, is functionally inactivated in patients who have deletion 3p syndrome [114], a severe mental retardation syndrome caused by impaired neuronal migration and axonal connectivity [115].

Several other members of the F-BAR protein family have been found to play critical roles in the central nervous system. CIP4 and PACSIN can bind to Huntington protein. CIP4 is upregulated in the brain of patients with Huntington's disease (HD) [116]. PACSIN1 is localized to neurites and synapses, but it is progressively lost in HD patients, beginning in presymptomatic and early stages of HD [117]. FBP17 (also named Rapostlin) in the CIP4 family was expressed in the developing rat brain, and knockdown of FBP17 led to a decrease in spine density in cultured hippocampal neurons [118].

With common functional domains common to other family members, Nwk2 might also play an important role in neuronal development and central nervous system diseases.

Nwk functions

There are no studies performed in mammalian system focused on Nwk2, but studies have been done in drosophila about the Nwk2 homolog Nwk.

Nwk is important for synaptic growth. The mutant of Nwk in Drosophila displays a temperature-sensitive paralytic phenotype due to increased synapse number and hyperbranched boutons in neuromuscular junctions (NMJs). Nwk is abundantly and restrictively expressed in neurons. Nwk localizes to the periactive zone of synaptic boutons, which is proposed to be specialized for synaptic growth regulation [119].

The underlying mechanism of Nwk is likely reflected in the following obervations:

(1) Nwk activates Wasp-Arp2/3 mediated endocytic actin assembly via interacting with dynamin, Dap160 and Cdc42 [119-121].

(2) Nwk interacts with the BMP receptor Thickveins and endocytic machinery to attenuate retrograde BMP signaling during synaptic growth [121].

(3) Nwk functions in endosomal trafficking, mediated by SNX16 and the ESCRT complex to control synaptic growth signaling [120, 122].

In summary, drosophila Nwk controls synapse morphology by regulating actin dynamics, endosomal trafficking and retrograde BMP growth signaling. There is no published study about Nwk2 in mammals yet. Thus the study of Sp4 target gene Nwk2 might provide us new insights into the mechanisms underlying human neuron morphogenesis.

Hypothesis and Aims of study

Hypothesis

The nervous system is a well-coordinated network that depends on the formation of proper connections among diverse types of neurons. Dendritic arborization patterns determine the way a neuron integrates inputs. Defects in this process are associated with neurodevelopmental disorders such as schizophrenia [2-4]. Previous studies from our laboratory indicated that transcription factor Sp4 controls dendritic patterning during cerebellar development by limiting branch formation and promoting activity-dependent pruning [64].

Sp4 null mice display impaired postnatal development of the hippocampal dentate gyrus, which plays a central role in learning and memory [124]. Mice with reduced expression of Sp4 (2%-5% of wild-type level) display behavioral deficits in sensorimotor gating and contextual and spatial memory, and Sp4 hypomorphic mice show reduced long-term potiationation (LTP) in hippocampal CA1 and reduced NMDAR1 protein level in whole brain, which are all related to some neuropsychiatric disorders, for example, schizophrenia [91, 98]. Genome wide association studies, identification of rare copy number variants and targeted analysis of single nucleotide polymorphisms (SNPs) have revealed Sp4 locus associated with major depressive disorder, schizophrenia and bipolar disorder [87-90]. Thus, Sp4 plays an important role in neuronal development and neuropsychiatric disease. Understanding of fundamental transcription regulatory mechanisms by Sp4 in neurons, especially its control of dendritic morphology may provide insights into the pathogenesis of diverse neurodevelopmental disorders.

We hypothesize that Sp4 activates transcription of specific neuronal target genes, which are critical for neuron development and altered in neuropsychiatric disorders. Recent studies in our lab revealed that Sp4 activates TrKC and represses NT3

to limit dendritic branching [79]. However, little is known about other target genes of Sp4 involved in various aspects of dendritic development and NMDA neurotransmission.

Specific aims

In order to identify important target genes of Sp4 in neuronal development, we proposed the following specific aims.

Aim I: Identify target genes of Sp4

1. Screen for candidate Sp4 target genes by comparing gene expression profiles in Sp4 WT and hypomorphic mouse cerebellum.
2. Validate the expression of candidate Sp4 target genes by real-time PCR in cerebellum and CGNs.
3. Determine neuron-specific direct target genes of Sp4 by ChIP (Chromatin Immunoprecipitation) assay

These experiments will help to find the direct target genes of Sp4 that have potential role in neuronal development and neuropsychiatric disorders.

Aim II: Functional study of Sp4 and its target genes in neurons.

1. Confirm expression pattern of selected Sp4 target genes in WT and Sp4 mutant mouse tissue
2. Test the role of Sp4 target genes in CGN dendritic patterning.
3. Test the role of Sp4 target genes in NMDA neurotransmission.

These experiments will provide insights into novel pathways through which Sp4 functions in neuron development and neuropsychiatric diseases.

We identified a set of Sp4 regulated genes with roles in neuron development or altered expression in neuropsychiatric disorders. In particular, we identified Nervous Wreck 2 (Nwk2) as an activation target of Sp4 that regulates dendritic patterning and NMDAR protein levels. Our studies suggest that Sp4-Nwk2-NR1 as a new pathway that contributes to normal neuronal morphogenesis and may be disrupted in neuropsychiatric diseases.

Chapter 2. Global gene expression profiling of Sp4 hypomorphic mouse cerebellum to identify candidate Sp4 target genes

Abstract

Transcription factor Sp4 controls dendritic patterning during cerebellar development by limiting branch formation and promoting activity-dependent pruning. Sp4 is associated with neuropsychiatric disorders such as major depressive disorder, schizophrenia and bipolar disorder. However, little is known about the target genes of Sp4 in dendritic patterning and neuropsychiatric disorders. A previous study in our lab showed that NT3 is a repression target of Sp4 [125]. Sp4-dependent repression of neurotrophin-3 is required to limit dendritic branching. But increased NT3 does not result in the increased primary dendrite numbers that was observed in Sp4 depletion CGNs. There must be other Sp4 targets that control the primary dendrite numbers.

We used a gene expression profiling analysis to find the candidate target genes of Sp4 critical for neuronal development. We used microarray-based methods to identify Sp4-regulated genes by comparing global gene expression in the cerebella of wild type and Sp4 hypomorphic mouse cerebellum. We ran GSEA, DAVID and IPA analysis of the microarray data, and tried to find Sp4 target genes or pathways with biological functions relevant to neuronal development and central nervous system diseases. We validated a subset of target genes by RT-qPCR in 2 mouse strains and we investigated whether they are direct neuron specific Sp4 target genes by ChIP assay in cultured CGNs.

We identified Nwk2 and other potentially interesting target genes of Sp4 involved in neural development and function. These provide us a valuable resource for future studies of Sp4's transcriptional regulatory role in neuronal development and central nervous system disease.

Introduction

Sp4 is an important transcription factor in neuronal development and a contributor to neuropsychiatric disorders. Transcription factor Sp4 controls dendritic patterning during cerebellar development by limiting branch formation and promoting activity-dependent pruning. Sp4 is associated with neuropsychiatric disorders such as major depressive disorder, schizophrenia and bipolar disorder.

The pattern of dendrites elaborated by a neuron determines integration of inputs. Defects in dendritic patterning are characteristic of many neurodevelopmental and mental retardation disorders[2-4]. Dendritic development is a highly dynamic process that includes stages of addition, growth, branching, and stabilization or elimination of dendrites. Dendritic arborization patterns are regulated during development by the coordinated action of extracellular signals and intracellular gene expression programs [126, 127]. Sp4 is not only an essential effector for activity-dependent remodeling during cerebellar development, but also an important cell intrinsic factor that controls diverse aspects of dendritic morphology through the regulation of its target genes. However, little is known about the target genes of transcription factor Sp4 involved in regulating neuron dendritic patterning.

Gene expression profiling analysis in postmortem patient brains or animal models has been widely used to reveal gene expression changes in neuropsychiatric disorders. Major depressive disorder, schizophrenia and bipolar disorder are posited to be multigenic, multi-factorial diseases. Although Sp4 has been associated with multiple neuropsychiatric disorders from a population-based perspective, and Sp4 protein is reduced in bipolar

patients, little is known about Sp4 regulated genes or pathways that contribute to the altered gene expression changes in neuropsychiatric disorders.

A previous study in our lab showed that NT3 is a repression target of Sp4[125]. Sp4-dependent repression of neurotrophin-3 is required to limit dendritic branching. But increase of NT3 does not result in the increased primary dendrite numbers that are observed in Sp4 depleted CGNs. Accordingly, there must be other Sp4 targets that control the primary dendrite numbers.

We utilized a microarray approach to elucidate the global changes of gene expression between cerebellum of WT and Sp4 hypomorphic mice. We used a gene expression profiling analysis to find the candidate target genes of Sp4 in dendritic patterning and neuropsychiatric disorders.

Materials and methods

Mouse strains and breeding

The Sp4 hypomorphic mice were a generous gift from Dr. Zhou, UCSD [123] and maintained in Black Swiss background. Black Swiss is an outbred strain. Homozygous Sp4 hypomorphic mice were bred from heterozygous littermates. Due to the dystocia and poor nursing performance in the heterozygous Black Swiss female, the Black Swiss strain was outcrossed to CD1 mice (Charles River). The microarray analysis was performed in the F1 and N1 Black Swiss x CD1 background. Mice were maintained in American Association for Accreditation of Laboratory Animal Care approved animal facilities at Tufts University. This facility meets all Federal and State requirements for animal care. All protocols for the use of vertebrate animals were approved by the committee for the humane use of animal at Tufts University School of Medicine. All mice were maintained on a 12-hour light/dark cycle with *ad libitum* access to food and water.

RNA source, purification, quality control, labeling and bead array processing

RNA was extracted from 3 pairs of WT and Sp4 hypomorphic mouse cerebella at postnatal Day 21 mice. At Day 21, granule neurons have finished their migration and most of their dendritic development.

Total RNAs were extracted by Trizol (invitrogen) and RNeasy Kit (QiAGEN). Each pair of mice was composed of gender-matched littermates at age of postnatal Day21. Samples were run on an Agilent 2100 bioanalyzer to determine quality. 3 biological replicates were sent to the Keck Microarray Facility (Yale University) for RNA amplification, cRNA production, labeling and hybridization to MouseWG-6 v2.0

Expression BeadChip. Illumina Genome Studio software was used to export both raw data and background corrected, quantile-normalized data.

Microarray data analysis

P<0.05 and fold change>1.5 was used as the cutoff after quantile normalization.

Ingenuity pathway analysis (IPA) was performed on the filtered gene set to summarize the networks, biological functions and canonical pathways changed in Sp4 hypomorphic mice.

Filtered lists were also input into the DAVID (**D**atabase for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery) functional annotation-clustering tool. DAVID default settings were used for this analysis. The DAVID analysis tool treats all gene input equally, that is, magnitude of fold change or expression value from the Illumina array is not taken into account for the analysis. Enrichment of a particular annotation cluster indicates that this annotation is over-represented, or enriched in the gene list for the given population compared to the background level expected based on the frequency of occurrence of genes with the same annotation in the mouse genome. Resulting enrichment clusters were considered for further analysis if the enrichment score was >1.3 (as recommended by the database designer).

RT-qPCR analysis

RNA was extracted and purified using Trizol (Invitrogen) and RNeasy Kit (QiAGEN) according to the manufacturer's protocol. For quantitative RT-qPCR, first strand cDNA synthesis from 2 μg total RNA was carried out with Oligo dT primer using Superscript III reverse transcriptase (Invitrogen). cDNA were analyzed by quantitative PCR (Bio-Rad) using the indicated primers. Expression of genes was normalized to that of Gapdh and

Hprt1. For RT-qPCR, at least 3 biological replicates were conducted and within each experiment every condition was measured in triplicate. Student's t-test was used to determine the significance between groups.

Table 2-1 Primers used for RT-qPCR.

PRIMER	SEQUENCE
ATP6V1D-F	CTGATGGGTGAAGTGATGAG
ATP6V1D-R	GATAACTGTGGTGCTGAAGTC
BMP8A-F	CCTTTCATGGTAACCTTCTTCAG
BMP8A-R	GTGGCCATCATCAAAGATCC
BTG4-F	TGTGCTGTAGGTATGGTGAG
BTG4-R	ATCAGAAGATGTGCCAGAGG
CCL21A B C-F	CTGCAAGAGAACTGAACAGAC
CCL21A B C-R	TTTCTAGCTCCCTCTTTGCC
CDK5RAP1-F	GGCTTTACCACCAACTATAAACC
CDK5RAP1-R	AGCTGAAGAACCTCATCAGG
CITED4-F	ATCTCTCCGTACCCCCTACCT
CITED4-R	TCTGCAGGGGTTAGGTGTAAA
CNKSR1-F	GCTTGGACAGACTCTACCTC
CNKSR1-R	ACCTTTAGATTTCTTCCGACC
CNTFR-F	ATCATCCCAGGAAGACTTTGG
CNTFR-R	ACAGAAGCAGCCATCTCGTTA
COMMD2-F	GATGTACAGCTTGCAAGCAG
COMMD2-R	CTGCTGAACCAAATGAAGCA
COMP-F	TGCTCAATCAGGGAATGGAG
COMP-R	TGTTTACATGGAATGTGCCC
DHCR24-F	CTGCGAGTCGGAAAGTACAAG
DHCR24-R	ATAGACACCAAGGGCTCCACT
EFHA1-F	TGGATGTGGATGGGAATGAG
EFHA1-R	TGTTGGGTCCTGGTATTCTG
ESD-F	GACGAGTTTCTTTCAAATGGG
ESD-R	TGATCATAACCCTCTTGTAGTC
GAPDH-F	CTGAGGACCAGGTTGTGTCC
GAPDH-R	CATTGTCATACCAGGAAATGAGC
GCNT1-1 2 3-F	TCTAAACGTGATATCTTGTCCC
GCNT1-1 2 3-R	AAAGAACATTCAGAGGCTTCC
GNG7-F	GAAGCCTTTCTATTGGAACCTC
GNG7-R	TGTTAGTACCTGACATCATCGG
GOT1L1-F	ATTCCCATTCTTTGACATTCCC
GOT1L1-R	CACTCCTTCATCATAAATGCCA
GRB7-F	CAATACCCTAGTGGCTATGGA
GRB7-R	GGTTTGTCTTCTTCCTCCAG
HPCA-F	CTTCAGCATGTATGACCTGGA
HPCA-R	GAAACCATCTTGTAAATGGCCT
HPRT1-F	CTCTCGAAGTGTTGGATACAG
HPRT1-R	ACAAACGTGATTCAAATCCC
IL17RE-F	GACGCTAGACCTCATCATCC
IL17RE-R	TCTATGGGAGACATCAGGAC

IRF6-F	GTCATTAATCCAGGATCCACAG
IRF6-R	GGAGAACCGTTGATGTTCAG
MINA-F	AGAGATATAAGGATGAGCTGTG
MINA-R	ATCATCATAATGTGGAGGGAG
MKKS-F	TCAGACACAAGGTTCATCAC
MKKS-R	ATCATGTTCCAAAGAGCCAG
MYD88-F	CTATACCAACCCTTGCACCAA
MYD88-R	GTAGACAGGACGGCATCAGAG
NWK2-F	TGAGCTGTCAGAATACTTGAG
NWK2-R	GTTCCCAGTTTACCTGAGAC
PCDH20-F	ATATGGGGCATCTACATCGTC
PCDH20-R	GCAGTCCCTCATTTAGGCTGT
PCDH9-F	CTCCTCCACTAGTTCAGATCAC
PCDH9-R	GCTGTATGAGTTACACTGTCTG
PDRG1-F	AGATGTGATGGTTTGCTTTGG
PDRG1-R	GGTGCTCTTGATCTTTCTGG
PLEKHF2-F	CGCTGTCAGAAAGCAAAATTC
PLEKHF2-R	AGAAGACTGGCTGGGAAGAAG
PNLIP-F	GGGTCACTTAGATTTCTTTCCA
PNLIP-R	AAAGTTTCGAGTTCCTTCCCA
PRKAG2-1 2 3-F	CTTTGTAGGAATGCTCACGA
PRKAG2-1 2 3-R	GGTTTCTTGTAAGTACAACTCCC
RAMP3-F	CTTCCACTGTTGTTGCTGCTT
RAMP3-R	ACTTCCAGACAGCCACCTTCT
RASSF7-F1	ATAGGCCAGACAGGTCGATTT
RASSF7-F2	CAAGTACCCAGGACAGGGATG
RASSF7-R1	AACATCATTGGCAAACTGTCC
RASSF7-R2	GCAGGTGGTCTGTTCTGAGAC
RBBP4-F	ACTGCCAAGATATCTGACTTCTC
RBBP4-R	GTAAATGTTCTCCGCCATCTG
SERPINB2-F	TCCTAGAACTTCCGCATACTG
SERPINB2-R	TTCACTTTCCAGCAATTCCA
SPATA7-F	CGAGAAATGGTGCTGAAGAC
SPATA7-R	TTACAGGATGCCCTTTCTGAG
TMEM159-F	CCATATTATTGGAAGGTCTGGTC
TMEM159-R	TAGGACATCATGGCTATCCC
TSPAN17-F	ATCCGAGACCAACTTAATCTC
TSPAN17-R	CACAGCAAGACCAGTATTCC

Chromatin Immunoprecipitation

Chromatin Immunoprecipitation was performed following Upstate Biotechnology assay protocol with modifications. 2×10^7 cerebellar granule neurons at DIV (Day in vitro) 6 were cross-linked with 1% formaldehyde for 15 min at 37 °C. Chromatin was sonicated to between 500 and 1000 bp and immunoprecipitated with 3 μg of anti-Sp4 (Santa Cruz) or normal rabbit IgG. Immunoprecipitated DNA was amplified by real-time PCR using specific primers as indicated. The real-time PCR was performed as described by the manufacturer using the SsoFast™ EvaGreen Supermix (Bio-Rad). 10% of total starting material was precipitated and used as input. Fold enrichment of Sp4 antibody was normalized to IgG.

Table 2-2 Primers used for ChIP.

CITED4(-139-122)-F1	AGCTACTCCCATACCCCCTCT
CITED4(-139-122)-F2	CCATACCCCCTCTTAACCAGT
CITED4(-139-122)-R	GTCGTGAACCTGTCCAAGAAG
CITED4(-34)-F	ATTCATCCAGCCAATGACAGA
CITED4(-34)-R	AAAGAAACAGACAGCCCAAGG
CITED4(+211)-F	GGTGAGACAGTGAACGAATCC
CITED4(+211)-R	ACCTGTAGCAGGCAGTAGCC
CITED4(3UTR)-F	ATCTCTCCGTACCCCCTACCT
CITED4(3UTR)-R	TCTGCAGGGGTTAGGTGTAAA
NWK2(-203)-F	TTGACTCAGAAGCTCGAGGAG
NWK2(-203)-R	TACTTGTACCCCGGAAAGGTC
NWK2(-71)-F	ACAGGTTGAGTTCCACTGCTG
NWK2(-71)-R	CAAAGGCTGCCAGACACTCTA
NWK2(3UTR)-F	CTGGTAACCCATCAGCACAGT
NWK2(3UTR)-R	TGGAGGTGGGTGAACTGTAAG
MINA(-233)-F	ATGCCTTAGCTGGGTCAACTT
MINA(-233)-R	TCCAGAGCTGCACTTCTCAG
MINA(-349)-F	TGTAGCTGTAGGCAGGAGCAG
MINA(-349)-R	GCAAGTTGACCCAGCTAAGG
MINA(-66)-F	CTGAGAAGTGCAGCTCTGG
MINA(-66)-R	CAACCTACGCTCCAAGTCC
MINA(+49)-F	GGAGCGTAGGTTGCCTTTTTA
MINA(+49)-R	ATGACCGCTGGTAGACTTCCT
MINA(3UTR)-F	TGGATGCTCTGAAGCAAATCT
MINA(3UTR)-R	CAGAGAGACAGTGCCAGGTTC
RASSF7(-119)-F	CCGGCCACTACCTAACTCAG
RASSF7(-119)-R	ATTAGCTCGGAGGCGTGTGT
RASSF7(-145-79)-F	TTCCGCATTGATTAACAGAGG
RASSF7(-145-79)-R	CCACAGCATCCCATTAGCTC
RASSF7(-39)-F	CCTCCGAGCTAATGGGATG
RASSF7(-39)-R	CCTGTCCTGGGTACTTGCTG
RASSF7(-619-548)-F	GCTTCAGGGAGAGGAGGACT
RASSF7(-619-548)-R	GAGGGAGGTGGAGTTTAGTGG
RASSF7(-619)-F	CAGCAGAGAAGCCATTAGCC
RASSF7(-619)-R	TGTCTGAAGCTCCATCCTCTC
RASSF7(+628)-F	AAAAGGGTAGTGGCCTTTGAG
RASSF7(+628)-R	CTCCTTCACCAGCCTTCTCTT
RASSF7(3UTR)-F	AGCTCCTGGAGGTAGCAGTTC
RASSF7(3UTR)-R	GATAACAGTTGCCAGCACTCC
TSPAN17(-130-106-81-14)-F	CCAAGAAGACCGAGCCTTG
TSPAN17(-14)-F	GGTGTGGACTGACACTGTGG

TSPAN17(-14)-R	TTCATGGTCAAAGCCACACA
TSPAN17(-162)-F	GTTAGCTAGGGCAGAGCCTTG
TSPAN17(-162)-R	AAGGCTCGGTCTTCTTGGA
TSPAN17(-299-224-205)-F	CAGAAGTTAGCTAGGGCAGAGC
TSPAN17(-299-224-205)-R	CCCAGTCAAGTTCAGTCCTCA
TSPAN17(-340)-F	CTCCAAGAAGGCCGACTGT
TSPAN17(-340)-R	GGCTCTGCCCTAGCTAACTTC
TSPAN17(-81)-F	AGAAGACCGAGCCTTGCTG
TSPAN17(-81)-R	GTCAAAGCCACACAGCTCAC
TSPAN17(3INTRON)-F	CTCGTAAGTGCACCTGGAGTC
TSPAN17(3INTRON)-R	ATGGAGAGGTCTGAGACAGCA

Results

Sp4 protein is greatly reduced in Sp4 hypomorphic mouse cerebellum

Sp4 is ubiquitously expressed in brain. It is highly expressed in cerebellum, hippocampus and olfactory bulb according to the data from the Allen Brain Atlas (http://mouse.brain-map.org/). Sp4 protein level in cerebellum was greatly reduced in Sp4 hypomorphic mice. Compared to cortex, hippocampus and olfactory bulb, the biggest difference of Sp4 protein level between WT and Sp4 hypomorphic brain at postnatal day21 was in cerebellum (Figure 2-1). So we decided to use cerebellum as the region for gene expression profiling analysis to find potential Sp4 target genes.

Sp1 protein level was increased in the Sp4 hypomorphic brain, such as cerebellum and cortex. The increase of Sp1 might reflect the compensation for loss of Sp4 between Sp family members or be a secondary response to Sp4 loss.

Genome-wide expression profile change in mouse cerebellum

Using MouseWG-6 v2.0 Expression BeadChip which profiles 45,200 transcripts, we identified 161 genes decreased, 112 genes increased in expression in Sp4 hypomorphic

mouse cerebellum at postnatal Day21, if we take the cutoff of $p<0.05$ and fold change>1.5 after quantile normalization. The genes are listed in Appendix I. The genes with decreased expression level are candidate activation targets, and the genes with increased expression level are candidate repression targets.

We applied the following criteria to prioritize Sp4 target genes for future analysis

(1) Genes related to neuronal development

(2) Genes validated by RT-qPCR in 2 mouse strains and purified neurons

(3) Direct target genes as determined by ChIP in neurons.

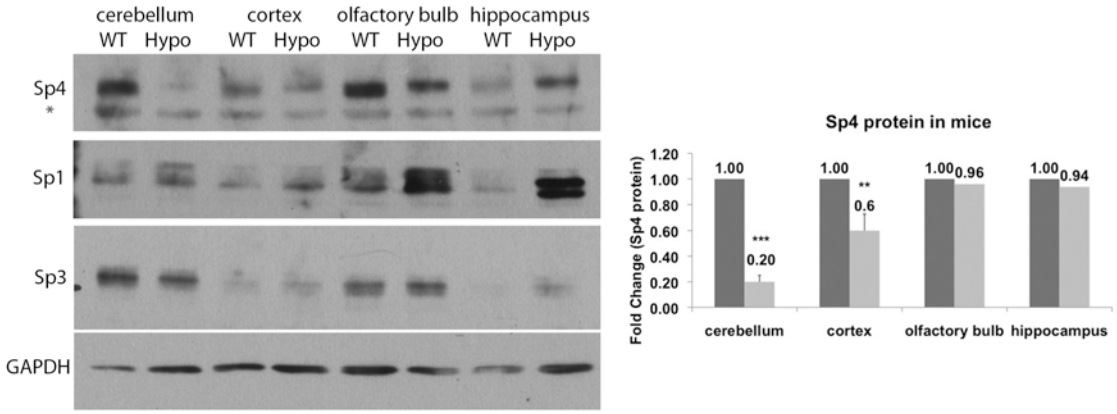

Figure 2-1 Sp4 protein level in WT and Sp4 hypomorphic brain.

Western blots were done in postnatal day 21 mouse tissues. *, non-specific band of Sp4 antibody. 2 isoforms of Sp1 are evident in the figure. ImageJ was used to quantitate the intensity of bands. The expression of Sp4 was normalized to GAPDH and Sp4 expression in WT was set as 1 to get the relative expression in Sp4 hypomorphic brain regions. Left panel shows the representatives of Sp protein level in mice. Right panel is from 3 experiments. **, $p<0.05$; ***, $p<0.005$.

Predicted biological functions changed in Sp4 hypomorphic mice.

Ingenuity pathway analysis (IPA) is software that delivers a rapid assessment of the signaling and metabolic pathways, molecular networks, and biological processes that are most significantly perturbed in the dataset of interest. Ingenuity pathway analysis was performed on the microarray identifying differentially expressed genes in Sp4 hypomorphic cerebellum. Of the top biological functions changed between WT and Sp4 hypomorphic mice summarized by IPA, one of the top differentially expressed biological networks was associated with Nervous System Development and Function. This matched the major role of Sp4 in central nervous system. By IPA, the candidate activation targets are more related with nervous system development and the candidate repression targets are more related with cell survival, inflammatory response and oxidative stress (Table 2-3). The genes in the highlighted nervous system network are listed in Table 2-2.

Table 2-3 Biological functions and pathways changed in Sp4 hypomorphic mice.

	Top network	Top physical system development and functions	Top canonical pathways
Both increased and decreased genes	1. Cellular Function and Maintenance, Cellular Movement, Hematological System Development and Function 2. **Nervous System Development and Function**, Tissue Morphology, Cell-To-Cell Signaling and Interaction 3. Gastrointestinal Disease, Hepatic System Disease, Hereditary Disorder	1. Hematological System Development and Function 2. Immune Cell Trafficking 3. Humoral Immune Response,	1. IL-1 Signaling 2. CD40 Signaling 3. TWEAK Signaling 4. Type I Diabetes Mellitus Signaling 5. IL-10 Signaling
Decreased genes	1. Cardiovascular Disease, Skeletal and Muscular System Development and Function, Cellular Assembly and Organization 2. Cancer, Gastrointestinal Disease, Hepatic System Disease 3. Cell-To-Cell Signaling and Interaction, Hematological System Development, **Nervous System Development and Function**	1. **Nervous System Development and Function** 2. Tissue Development 3. Visual System Development and Function	1. TWEAK Signaling 2. Pyrimidine Deoxyribonucleotides 3. De Novo Biosynthesis I 4. IL-1 Signaling 5. Activation of IRF by Cytosolic Pattern Recognition Receptors 6. CD40 Signaling
Increased genes	1. Hematological System Development and Function, Inflammatory Response, Tissue Morphology 2. Carbohydrate Metabolism, Cell Death and Survival, Developmental Disorder 3. Tissue Development, Cell Morphology, Cellular Compromise	1. Hematological System Development and Function 2. Tissue Morphology 3. Cell-mediated Immune Response	1. Glutathione Redox Reactions I 2. Glucocorticoid Receptor Signaling 3. Acute Phase Response Signaling 4. NRF2-mediated Oxidative Stress Response 5. Formaldehyde Oxidation II (Glutathione-dependent)

Combining the results from IPA analysis and GSEA, we generated a short list of interesting genes from microarray data that are related to dendritic morphology and central nervous system diseases (Table 2-4).

We compared our Sp4 gene list with a shared gene set with altered expression in schizophrenia and bipolar disorder [128]. The overlapping genes are genes with common functions under Sp4's regulation and altered in SZ and BD (Table 2-5). These genes may indicate pathophysiology under the control of Sp4 that contributes to neuropsychiatric disorders.

Table 2-4 Candidate Sp4 target genes related with neuron morphogenesis and CNS disease.

Biological process	Candidate Genes
Quantity of neurites (from IPA)	GJB1, MAP1B
Growth of neurites (from IPA)	CNTFR, GJB1, MAP1B, NELF, NINJ2, RBBP4, RET, STAT3, TNFRSF12A
Depression (from GSEA)	PTHR1, PPFIA4, COMP
Alzheimer's disease (from IPA)	PSEN2, CCL2, CNKSR1, CNNM1, CNTFR, CYP46A1, DHCR24, DRD4, EMILIN2, EML1, FSTL5, MAP1B, MTMR10, PCDH9, PVRL2, RAB3B, RORA, SERPINA3, SLC22A5, TUFT1
Alzheimer's disease (from GSEA)	SERTAD3, SSH3, CYP11A1, CLIC1, PTHR1, ZNF24

Table 2-5 Sp4 candidate genes overlapped with shared altered genes in schizophrenia and bipolar disorder by functions.

Molecule Name	Entrez Gene ID for Human
CACNB4	785
CCL19	6363
CCL21	6366
CD40	958
CD200	4345
Cd59a	966 for Cd59
CDH15	1013
CNTFR	1271
GJB1	2705
IFNGR1	3459
MAP1B	4131
MARCKSL1	65108
MKKS	8195
OPTN	10133
PSEN2	5664
RB1	5925
RET	5979
ROM1	6094
SCT	6343
SERPINA3	12
SERPINI1	5274
SLC30A3	7781
SP4	6671
STAT3	6774
TNFRSF12A	51330
UNC119	9094
ZNF24	7572
ZNF41	7592

Significantly enriched annotation clusters in Sp4 candidate target genes

To characterize the different types of genes changed in expression when Sp4 is lost, significantly regulated gene identifiers were input into DAVID database for functional annotation clustering. This tool compares the gene ontology classification for the experimental gene identifiers to a normal control list of mouse genes. The top enriched clusters are listed in table 2-6. The enrichment of Ras-associated genes is not surprising because Ras-Mapk signaling has been reported to mediate the BNDF-Trk activation to regulate neuron survival and neural patterning [129].

Table 2-6 Functional annotation clusters in candidate Sp4 target genes by DAVID analysis

Ras-associated genes	Annotation cluster 1, enrichment score 1.8 Ras association (RalGDS/AF-6) domain family (N-terminal) member 7 Ras association (RalGDS/AF-6) domain family member 4 Growth factor receptor bound protein 7 ral guanine nucleotide dissociation stimulator-like 1
Tetraspanin	Annotation cluster 2, enrichment score 1.67 rod outer segment membrane protein 1 tetraspanin 11 tetraspanin 17 tetraspanin 18
DNA metabolic process	Annotation cluster 3, enrichment score 1.3 CTF18, chromosome transmission fidelity factor 18 homolog (S. cerevisiae) DNA topoisomerase 1, mitochondrial GINS complex subunit 2 (Psf2 homolog) N-methylpurine-DNA glycosylase eyes absent 2 homolog (Drosophila) polymerase (DNA directed), iota retinoblastoma binding protein 4 ribonucleotide reductase M1

The microarray results are reproducible.

The RNA for microarray was obtained from mice with Black Swiss and CD1 mixed background. We validated the expression of candidate target genes by RT-qPCR. In order to identify the most robust target genes of Sp4, we also tested the candidate target genes in mice with only Black Swiss background (Table 2-7).

75% of decreased genes and 56% of increased genes show changes consistent with microarray data in Black Swiss x CD1 background by RT-qPCR. 40% of decreased genes and 11% of increased genes have the same trend of changes in Black Swiss mice. Figure 2-2 shows some examples of RT-qPCR validation of candidate target genes in both mouse strains. Rassf7 was increased in Sp4 hypomorphic mice in both backgrounds, and Tspan17 was decreased in Sp4 hypomorphic mice in both backgrounds. CDK5RAP1 was increased in mice with CD1 background, but not in Black Swiss mice.

We validated more candidate activation targets than repression targets, which indicated that Sp4 functions more robustly as a transcription activator.

Table 2-7 Top 20 increased and decreased genes in sp4 hypomorphic mice and validation in 2 mouse genetic backgrounds

Increased genes			Decreased genes		
	Strain1 (B.S.xCD1)	Strain2 (B.S.)		Strain1 (B.S.xCD1)	Strain2 (B.S.)
ZFP35			COMP	✔	✔
PDRG1			CCL21A,B,C	✔	
SERPINB2			CCL21A,B,C	✔	
BTG4			ZNF24		
MKKS			GCNT1	✔	✔
PNLIP			NWK2	✔	✔
CNKSR1	✔		PRKAG2		
CNTFR	✔		SPATA7		✔
GOT1L1	✔		COMMD2		
RBBP4			BMP8A		
PCDH9	✔		DHCR24	✔	
ATP6VLD			TSPAN17	✔	✔
PLEKHF2	✔		IL17RE	✔	✔
CDK5RAP1	✔		TMEM159	✔	✔
RASSF7	✔	✔	CITED4	✔	✔
PCDH20	✔		HPCA	✔	
EFHA1			GNG7	✔	
ESD			MINA	✔	✔
GRB7	✔		IRF6	✔	
MYD88	✔		RAMP3		✔

Top 20 increased and decreased genes in sp4 hypomorphic mice are listed in this table. All the genes listed in this table are tested by RT-qPCR. ✔means confirmed gene expression by RT-qPCR with the same trend of change in Microarray.

We identified candidate genes by functional analysis of differential expression profiles between WT and Sp4 hypomorphic mice, and then we validated these expression effects by RT-qPCR in 2 mouse strains. Finally we narrowed the choice of direct target genes by ChIP for Sp4 in CGNs. For example, after ChIP analysis in purified CGNs, only Nwk2 and Mina are potential direct target genes of Sp4 (Figure 2-3).

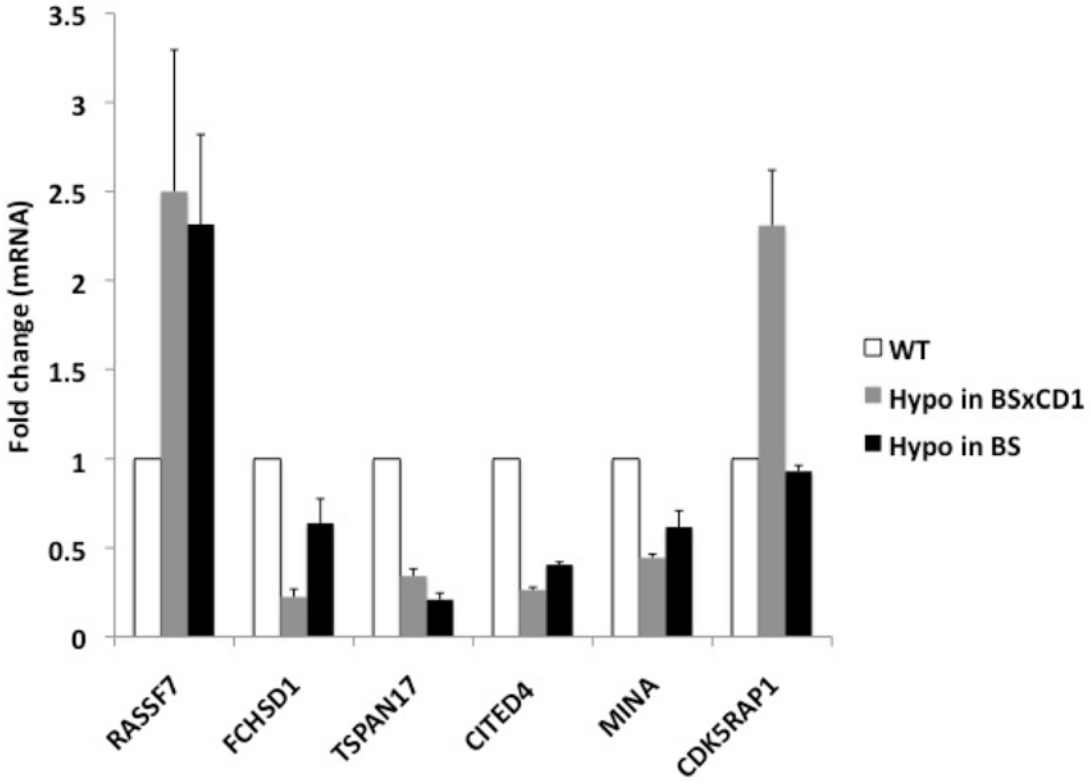

Figure 2-2 Validation of microarray data by RT-qPCR.

RT-qPCR was performed on total cerebellum mRNA from 3 pairs of gender-matched littermates of Sp4 WT and hypomorphic mice both with Black Swiss x CD1 background and Black Swiss background at postnatal Day 21. Each PCR has 3 technical repeats. Error bar represent SEM from 3 biological repeats. Gene expression was normalized to GAPDH and WT was set as 1. Fchsd1 is another name for Nwk2.

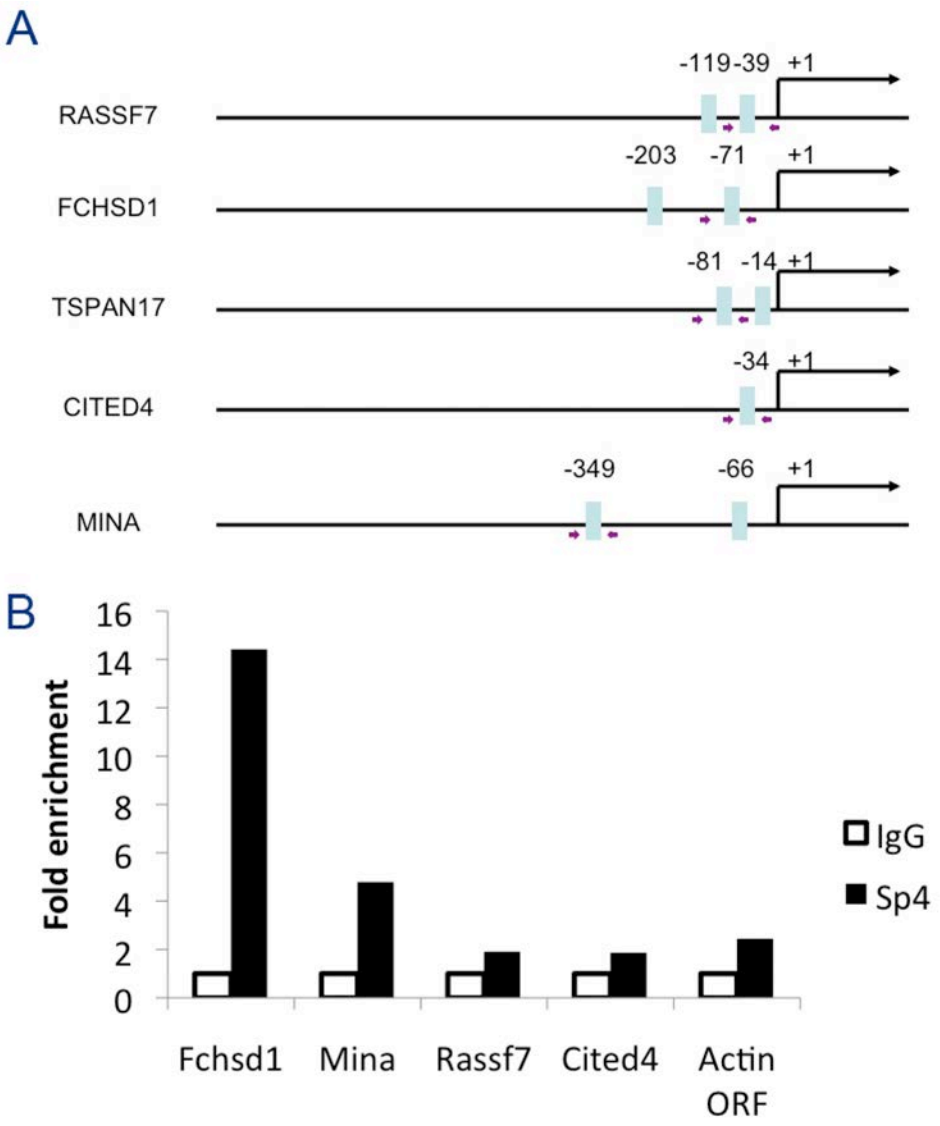

Figure 2-3 Direct target genes identified by ChIP assay.

A, the green boxes represent predicted Sp4 binding sites. The purple arrows indicate the primers for ChIP assay. B. ChIP was performed in cultured mouse cerebellar granule neurons from postnatal Day5 at Day in vitro 6 with anti-Sp4 antibody and negative control IgG. Primers amplying actin ORF worked as a control to show the specific binding of Sp4 to promoter regions. Fchsd1 is another name for Nwk2.

Discussion

In this study, we identified a set of genes with differential expression in WT and Sp4 hypomorphic mouse cerebellum. Their expression level is directly or indirectly under the control of Sp4. The list of genes provides candidates that may mediate Sp4's function in neuron development and CNS diseases.

Functional clustering of Sp4 target genes matches Sp4's role in neuron development

In order to elucidate what pathways and processes are regulated by Sp4, we performed IPA and DAVID analysis with the microarray data. Functional clustering of Sp4 target genes matches the function of Sp4 in mice. The functional characterization of genes with differential expression by IPA analysis showed that pathways in nervous system development and function changed in Sp4 hypomorphic mice. This is consistent with Sp4's role in neuronal development and psychiatric disorders.

Several pathways in immune response and oxidative stress response are also among the top regulated pathways in Sp4 hypomorphic mice. It is possible these are secondary responses with the loss of Sp4. In the future, it would be interesting to determine whether there is loss or death of neurons, or indications of neuron damage.

Gene expression changes in cerebellum show strain specificities

For the top 20 increased and decreased genes, we validated 75% of the decreased genes and 56% of the increased genes in the same mouse background as the microarray. The different results with microarray and RT-qPCR might come from sensitivity of techniques and the process of RNA sample preparation. We validated an even smaller percentage in the second mouse strain, suggesting the expression change has strain

specificity. For example, CDK5RAP1 was increased in the Black Swiss x CD1 background, but not changed in the Black Swiss background. The possible explanation for the strain specificities is that both Black Swiss and CD1 are outbred strains. We did not backcross Black Swiss mutant mice for generations to CD1 background before we ran the microarray. Accordingly some of the differential expression calls from the microarray might come from variability of expression in outbred mouse backgrounds. That is also the reason we decided to study the genes with the same trend of changes in both backgrounds.

In the future, backcrossing the mice to pure background would help to improve the efficiency and efficacy of gene identification by this microarray analysis.

Direct target genes were identified in neurons

There are 3 potential sources of RNA for microarray: First, RNA directly from the whole cerebellum of WT and Sp4 hypomorphic mice. Second, RNA from primary granule neurons purified from WT and Sp4 hypomorphic mice and maintained in culture after they differentiate in vitro. Third, RNA from WT and Sp4 hypomorphic granule cells harvested immediately after purification at postnatal Day 6. The gene expression profiles are reported to be similar in the first and second method, while granule cell precursors are enriched by purification in the third method[45]. We chose the first method, because we wanted to identify Sp4 target genes critical for dendritic patterning, which happens at a later stage after granule cell precursors migrate. There are less external factors affecting the experiment in this way, such as culture conditions. The disadvantage of this method is that the gene expression profile is not from a pure population of neurons. Thus an additional step is required to determine if the gene expression changes observed are in

neurons or in other cell types such as glial cells. In the future, we may validate candidate target genes in purified neurons culture by RT-qPCR to check whether the expression change is neuron cell autonomous, for example, as we did for Nwk2 in chapter 3.

ChIP assay with Sp4 antibody in purified neurons also facilitated identification of neuron-specific genes under the direct control of Sp4.

From five candidate genes with validated expression change, 2 of them were confirmed as the direct target genes of Sp4 by ChIP assay. Possible reasons that we saw expression changes of some genes at mRNA level but we did not "ChIP" their promoters include (1) they are largely enriched in other cell types instead of neurons. (2) the genes are expressed in neurons, but the expression changes come from indirect cell responses, instead of direct transcription control of Sp4. (3) they might be direct target genes, but we did not design proper primers amplifying the right GC box where Sp4 localizes.

We chose one neuron-morphogenesis related gene, NWK2, from the list of candidate genes for further analysis, after validating its expression level in cerebellum and confirming Sp4 binds to its promoter in CGNs. However, the list of differentially expressed genes provides us a great resource for future study of Sp4's function. As a transcription factor, Sp4 may play various roles in different developmental stage and different brain regions. In the future, it would be interesting to compare the spatial and temporal specific targets of Sp4 in CNS development.

Chapter 3. Transcription factor Sp4 regulates expression of Nervous Wreck 2 (Nwk2) to control dendritic patterning and NMDA receptor level

Abstract

Dendritic patterning and N-methyl-D-aspartate (NMDA) neurotransmission play important roles in neuronal plasticity, cognition and memory and alterations in these processes may underlie the pathophysiology of multiple psychiatric disorders. Transcription factor (TF) Sp4 controls dendritic patterning during cerebellar development by limiting branch formation and promoting activity-dependent pruning. Studies in mice have also shown that Sp4 regulates NMDA receptor 1 (NMDAR1, NR1) protein level. However, little is known about the target genes of Sp4 involved in regulating neuron dendritic patterning and NMDA neurotransmission. Here we report that RNA and protein expression of the nervous wreck homolog, Nwk2, were decreased in Sp4 hypomorphic mice. Sp4 localized to the Nwk2 promoter region by ChIP assay in cerebellar granule neurons (CGNs). Knockdown of Nwk2 in CGNs produced increased number of primary dendrites, which phenocopied the effect of Sp4 knockdown. Importantly, exogenous expression of Nwk2 rescued the dendrite morphology of Sp4 knockdown neurons. Furthermore, we found that the Sp4-Nwk2 pathway acted post transcriptionally to regulate NMDA receptor protein level. Overexpression of NR1 in Sp4 or Nwk2 knockdown neurons partially rescued the dendrite number. The data from our study show that Nwk2 is a direct activation target of Sp4, which regulates dendritic patterning and NMDAR level downstream of Sp4 in cerebellar development. As dendritic patterning and NMDA signaling are altered in diverse neuropsychiatric disorders, these studies may provide insights into the pathogenesis of these disorders.

Introduction

The nervous system is a well-coordinated network that depends on the formation of proper connections among diverse types of neurons. Dendritic arborization patterns determine the way a neuron integrates inputs. Defects in this process are associated with neuropsychiatric disorders such as schizophrenia [130] and are also hallmarks of neurodegenerative diseases such as Alzheimer's disease [123]. Dendritic development of neurons is under the regulation of both cell extrinsic and intrinsic cues. Transcription factors compose a major part of cell intrinsic programming of dendritic patterning, and also mediate coordination between the extrinsic signaling and the intrinsic program.

NMDAR activation provides intracellular calcium signals that initiate several forms of synaptic plasticity including long-term potentiation (LTP) and long-term depression (LTD), which are thought to underlie the synaptic changes associated with learning and memory. The involvement of NMDARs in synaptic plasticity and excitotoxicity has implications for the pathophysiology of neurological and psychiatric disorders including mental retardation, autism, schizophrenia, and mood disorders with impaired learning and memory. Impaired NMDA neurotransmission has been suggested to cause prefrontal cognitive deficits in schizophrenia [104]. This theory is based on the observation that acute administrations of non-competitive NMDA receptor antagonists, such as Phencyclidine and ketamine, induce schizophrenia-like symptoms in healthy people and worsen some symptoms in schizophrenia [105-107]. Recently, patients with anti-NMDA-receptor encephalitis (anti-NMDAR1) were found to display many schizophrenia-like symptoms and/or loss of memory [108]. Mouse hypoglutamatergic models with the deletion of nmdar1 gene display deficient sensorimotor gating and memory [109-111]. In

summary, impaired NMDA neurotransmission is one of molecular mechanisms underlying neuropsychiatric disorders.

Previous studies from our laboratory indicated that Sp4 controls dendritic patterning during cerebellar development by limiting branch formation and promoting activity-dependent pruning [64]. Sp4 is a transcription factor of the Sp family, which binds GC box sequences in gene promoter regions. In contrast to Sp1 and Sp3 in the same family, Sp4 is abundantly expressed in the central nervous system, and is highly enriched in neurons [80]. Mice with reduced expression of Sp4 (2%-5% of wild-type level) have reduced protein expression of NMDA receptor 1 in the brain [91], which could underlie the phenotypes displayed in the Sp4 hypomorphic mice including behavioral deficits in sensorimotor gating and contextual and spatial memory, and reduced long-term potiationation (LTP) in hippocampal CA1. Genome wide association studies, identification of rare copy number variants and targeted analysis of single nucleotide polymorphisms (SNPs) have revealed that the Sp4 locus is associated with major depressive disorder, schizophrenia and bipolar disorder [87-90]. Thus, Sp4 plays an important role in neuronal development and neuropsychiatric disease.

However, the underlying molecular mechanisms by which Sp4 regulates dendritic patterning and NMDA neurotransmission are barely understood. Little is known about target genes of Sp4 that are involved in dendritic development and NMDA neurotransmission.

Nervous Wreck 2 (Nwk2) is a member of the F-BAR protein family, which is characterized by an FCH-BAR domain. Its Drosophila homolog Nwk is abundantly and restrictively expressed in neurons. Nwk localizes to the periactive zone of synaptic

boutons and is important for synaptic growth. The mutant of Nwk in Drosophila displays a temperature-sensitive paralytic phenotype due to the increased synapse number and hyperbranched boutons in neuromuscular junction (NMJ). The possible mechanisms lie on the Nwk's regulation of endocytic actin assembly, endosomal trafficking and attenuating retrograde BMP signaling.

Here we report that Nwk2, as a direct activation target gene of Sp4, mediates Sp4's role in control of dendritic patterning and NMDAR1 level. Identifying the target genes of Sp4 will contribute to the understanding of fundamental transcription mechanisms by Sp4 in control of dendritic morphology and NMDA neuron transmission. This may provide insights into the pathogenesis of diverse neurodevelopmental and neurodegenerative disorders and guide the discovery of new drugs for central nervous system diseases.

Materials and methods

Mouse strains and breeding

The Sp4 hypomorphic mice were a generous gift from Dr. Zhou, UCSD [123] and maintained in Black Swiss background. Black Swiss is an outbred strain. Homozygous Sp4 hypomorphic mice were bred from heterozygous littermates. Due to the dystocia and poor nursing performance in the heterozygous Black Swiss female, the Black Swiss strain was outcrossed to CD1 mice (Charles River). The microarray analysis was performed in the F1 and N1 Black Swiss x CD1 background. Mice were maintained in American Association for Accreditation of Laboratory Animal Care approved animal facilities at Tufts University. This facility meets all Federal and State requirements for animal care.

All protocols for the use of vertebrate animals were approved by the committee for the humane use of animal at Tufts University School of Medicine. All mice were maintained on a 12-hour light/dark cycle with *ad libitum* access to food and water.

Plasmids

Nwk2-Flag was expressed from the CMV promoter in the pcDNA vector. Short hairpins RNAs were expressed from the U6 promoter in the pll3.7 vector. Sequences targeted by the short hairpin RNAs were: Scrambled control (gacgacccgcaggcgatacgt); GFP control (ggcgatgccacctacggcaag), Sp4 (gggtgctgcgggtgttcaagt) as described in [131];Nwk2#1(gcaatgagtacctgctaaatt for human and mouse, gcaacgagtacctgctaaatt for Rat); Nwk2#2(gctttgtccctgagcgatatc for human and mouse, gctttgttcctgaacgatatc for Rat). Silent mutations resistant to Nwk2 shRNA#1 were introduced to Nwk2-Flag by PCR mutagenesis, and nucleotides were mutated to gcaatgagtacTtATtGaatt.

Cerebellar granule neuron culture and transfection

Cerebellar granule neurons were obtained from postnatal day 6 rat pups or postnatal Day5 mouse pups as described [123]. The Animal Experimentation Protocol was approved by Tufts University Institutional Animal Care and Use Committee. Briefly, neurons were maintained in Basal Minimum Essential medium supplemented with 10% fetal calf serum, 25 mM KCl, penicillin (50 U/ml), streptomycin (50 μ /ml) and 2 mM glutamine. Cytosine arabinofuranoside was added. Cells were transfected by lipofectamine 2000 immediately after they were purified. A vector expressing the antiapoptotic protein Bcl-xL was included in morphometric studies.

Immunofluorescence and immunoblotting

At the indicated times, cells were fixed in 4% paraformaldehyde. Neurons were immunolabelled with Chicken anti-GFP (Molecular Probes), Mouse monoclonal anti-NMDAR1 (BD Pharmingen), followed by Alexa Fluor® Dyes conjugated secondary antibodies (Molecular Probes), and mounted by ProLong® Gold Antifade Reagent with DAPI (Molecular Probes). Lysates from tissue was prepared using Tissue extraction reagent II (invitrogen). Neuro2A cells were plated at 1.8×10^4 cells/cm^2 24 h before transfection with Lipofectamine 2000(Invitrogen). 72h post transfection, Neuro2A extracts were prepared in lysis buffer 50 mM Tris pH 7.4, 150 mM NaCl containing 1% NP-40 for 30 min on ice, followed by sonication and centrifugation at 15000 g for 15 min. Lysates were resolved by SDS/PAGE and immunoblotted with polyclonal anti-Sp4 antibody (Santa Cruz) or polyclonal antibody against Gapdh (Chemicon International) and polyclonal anti-Nwk2 antibody (Abcam).

NMDAR1 was stained without permeablization by detergent. The fluorescence intensity was measured by the software Volocity. Intensity of transfected neurons was normalized to the mean intensity of all the surrounding neurons in one field after substraction of background. The relative fluorescence among groups was compared.

Immunohistochemistry

For mouse immunohistochemical staining, frozen sections (10 μm) were cut from mouse brains fixed by transcardial perfusion of 4% formaldehyde buffered in PBS and cryo-protected. Polyclonal anti-Nwk2 antibody (Abcam) and monoclonal anti-NeuN (Millipore) were used as primary antibody with 1:100 dilutions. The VECTASTAIN elite

ABC kit (Vector Laboratories) was used for signal amplification, and NovaRed (Vector Laboratories) was used for color reaction.

Morphometric analysis of dendrites

Images of individual transfected neurons with no overlapping processes from other transfected neurons were captured randomly in a blinded manner at 400× magnification using a Nikon A1R confocal microscope with a CCD camera. Statistical differences were determined by ANOVA and a post hoc Tukey test or two-tailed t-test. For every condition, quantitation was performed in at least 3 independent experiments with similar results.

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed following Upstate Biotechnology assay protocol with modifications. 2×10^7 cerebellar granule neurons were crosslinked with 1% formaldehyde for 15 min at 37 °C. Chromatin was sonicated to between 500 and 1000 bp and immunoprecipitated with 3 μg of anti-Sp4 (Santa Cruz) or normal rabbit IgG. Immunoprecipitated DNA was amplified by real-time PCR using specific primers as indicated. The real-time PCR was performed as described by the manufacturer using the SsoFast™ EvaGreen Supermix (Bio-Rad). 10% of total starting material was precipitated and used as input.

Primers for ChIP:

Nwk2 promoter-F: ACAGGTTGAGTTCCACTGCTG

Nwk2 promoter-R: CAAAGGCTGCCAGACACTCTA

Nwk2 3'UTR-F: CTGGTAACCCATCAGCACAGT

Nwk2 3'UTR-R:TGGAGGTGGGTGAACTGTAAG

RNA extraction and reverse transcription-qPCR analysis

RNA was extracted and purified using Trizol (Invitrogen) and RNeasy Kit (QiAGEN). Each pair of mice was gender-matched littermates at postnatal Day21. For quantitative RT-qPCR, first strand cDNA synthesis from 2 μg total RNA was carried out with Oligo dT primer using Superscript III reverse transcriptase (Invitrogen). cDNAs were analyzed by quantitative PCR (Bio-Rad) using the indicated primers. Expression of genes was normalized to that of Gapdh and Hprt1. For RT-qPCR, at least 3 biological replicates were conducted and within each experiment every condition was measured in triplicate. Student's t-test was used to determine the significance between groups.

Nwk1-F: CTCACAGTCCTTTAAACGCC

Nwk1-R: CTTGTGTAACTTTCACCTTCCTC

Nwk2-F: TGAGCTGTCAGAATACTTGAG

Nwk2-R: GTTCCCAGTTTACCTGAGAC

Nr1-F: GTACCCATGTCATCCCAAAT

Nr1-R: TCTGGTGGACATCTGGTATC

Nr2A-F: GCAGAGAATAGGACCCACTCCCTAA

Nr2A-R: TGGCATGTGGCCCGGCTTGA

Nr2B-F: CAACGGGACCTGGAACGGCAT

Nr2B-R: AAGGGCACAGAGAAGTCAACCACC

Gapdh-F: CTGAGGACCAGGTTGTGTCC

Gapdh-R: CATTGTCATACCAGGAAATGAGC.

Hprt1-F: CTCTCGAAGTGTTGGATACAG

Hprt1-R: ACAAACGTGATTCAAATCCC

Results

Nwk2 is a direct activation target of transcription factor Sp4 in neurons

We found gene Nwk2 from gene expression profiling comparing WT and Sp4 hypomorphic mice. Nwk2 is a homolog of Nervous Wreck in Drosophila, which plays a critical role in synaptic growth. Nwk2 expression was examined both in cerebellar tissue and primary cultured CGNs. In the primary culture, 99% of cells were CGNs, which helped us to find neuron specific factors controlling dendritic development. Nwk2 expression was greatly reduced in Sp4 hypomorphic mouse cerebellum and CGNs at mRNA level, compared to WT mice (Figure3-1).

There are 2 homologs of Nwk in mouse, Nwk1 and Nwk2. Nwk1 mRNA level did not change significantly between WT and Sp4 hypomorphic neurons (Figure3-2). This suggests that Sp4 regulate the transcription of Nwk2 specifically, but not Nwk1.

We also tested the protein level of Nwk2 in WT and Sp4 hypomorphic cerebellum tissue and cultured CGNs by Western Blot. Nwk2 expression was greatly decreased in Sp4 hypomorphic mouse cerebellum and CGNs at the protein level, compared to WT mice (Figure3-3). The change of Nwk2 protein level was more obvious in CGNs. Sp4 is enriched in neurons, so it makes sense that the purified neurons magnified the difference of Nwk2 in WT and Sp4 hypomorphic mice.

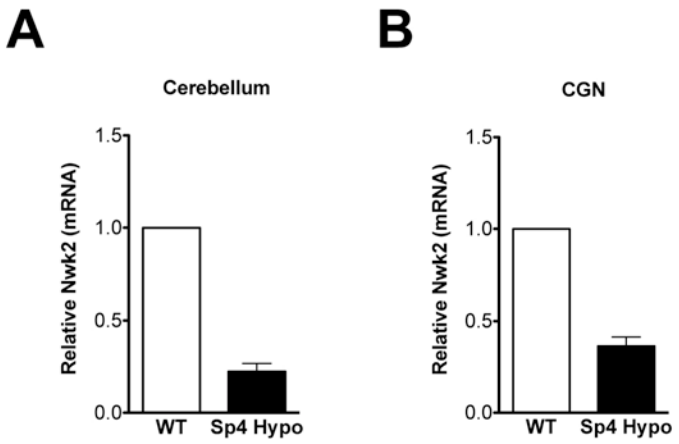

Figure 3-1 Nwk2 mRNA level is reduced in Sp4 mutant cerebellum tissue and CGNs.

A. Total RNA was extracted from postnatal Day21 WT (open bar) and Sp4 hypomorphic (shaded bar) mouse cerebellum and subject to RT-qPCR with primers for Nwk2, Gapdh and Hprt1. Values represent mean±SEM generated from experiments performed in triplicate and normalized to Gapdh and Hprt1 expression levels. The relative expression of Nwk2 was normalized to WT mice, which was taken as 1. Student's t-test was used to determine the significance between groups. The P value was <0.05 between WT and Sp4 hypomorphic mice.
B. CGNs were purified from postnatal Day5 WT (open bar) and Sp4 hypomorphic (shaded bar) littermate cerebellum. CGNs were cultured in vitro for 6 days. RNA extraction and RT-qPCR was performed as above.

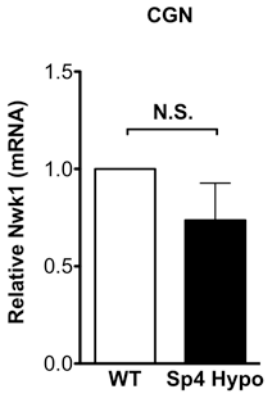

Figure 3-2 Nwk1 mRNA level in WT and Sp4 mutant CGNs.

CGNs were purified from postnatal Day5 WT (open bar) and Sp4 hypomorphic (shaded bar) littermate cerebellum. CGNs were cultured in vitro for 6 days. Total RNA was extracted and subject to RT-qPCR with primers for Nwk1, Gapdh and Hprt1. Values represents mean±SEM generated from experiments performed in triplicate and normalized to Gapdh and Hprt1 expression levels. The relative expression of Nwk1 was normalized to WT mice, which was taken as 1. Student's t-test was used to determine the significance between WT and Sp4 hypomorphic mice. N.S. means not significant.

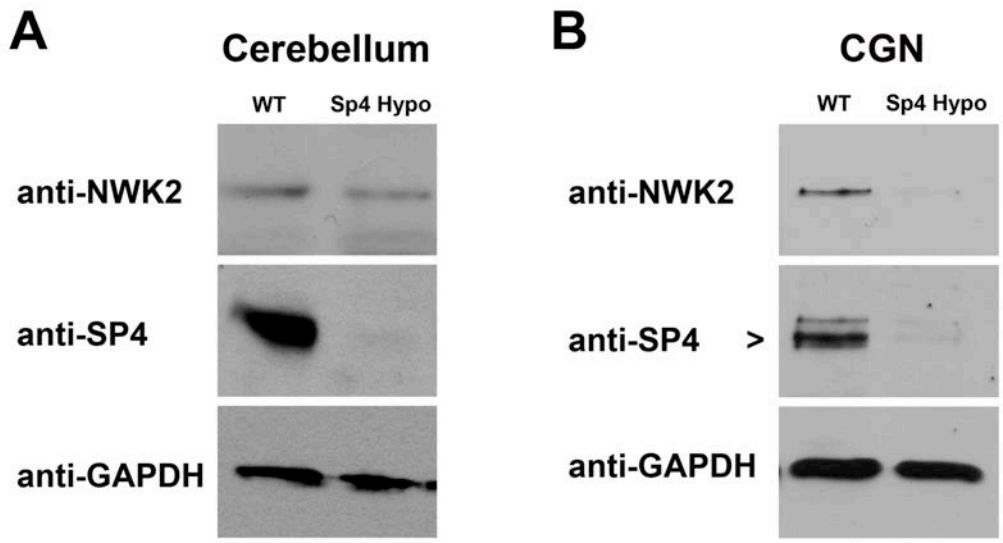

Figure 3-3 Nwk2 protein level is reduced in Sp4 mutant cerebellum tissue and CGNs.

A. Protein lysates from postanatal Day21 cerebellum were immunoblotted for Nwk2, Sp4 and GAPDH.
B. CGNs were purified from postnatal Day5 WT and Sp4 hypomorphic littermate cerebellum. CGNs were cultured in vitro for 6 days. Protein lysates from cultured CGNs were immunoblotted for Nwk2, Sp4 and GAPDH.

Immunohistochemistry of Nwk2 in postnatal Day7 mouse cerebellum sections showed that Nwk2 protein level was greatly reduced in Sp4 hypomorphic mice compared to the WT mice (Figure3-4). The IHC in the WT mice showed that Nwk2 was enriched in the neuron layer. The pattern of expression is similar to the pattern of NeuN, which is a neuron specific nuclear protein.

Immunofluorescence of Nwk2 in cerebellum sections in postnatal Day21 mice also showed that Nwk2 expression is greatly reduced in Sp4 hypomorphic mice. The distribution pattern of Nwk2 is similar to neuron marker Map2 (Figure3-5).

At both mRNA and protein level, we demonstrated that Nwk2 expression is decreased in Sp4 hypomorphic mice. We conclude that Sp4 regulates Nwk2 at the transcriptional level. However, we did not know whether Nwk2 transcriptional change is a direct or indirect effect of loss of Sp4. Therefore, ChIP analysis was performed in primary cultured cerebellar granule neurons from WT mice to test the physical contact of Sp4 to the Nwk2 promoter. Utilizing ChIP assay, we were able to confirm that Sp4 occupied Nwk2 promoter, but not 3'UTR region (Figure 3-6). We conclude that Nwk2 is a direct activation target gene of Sp4 in CGNs.

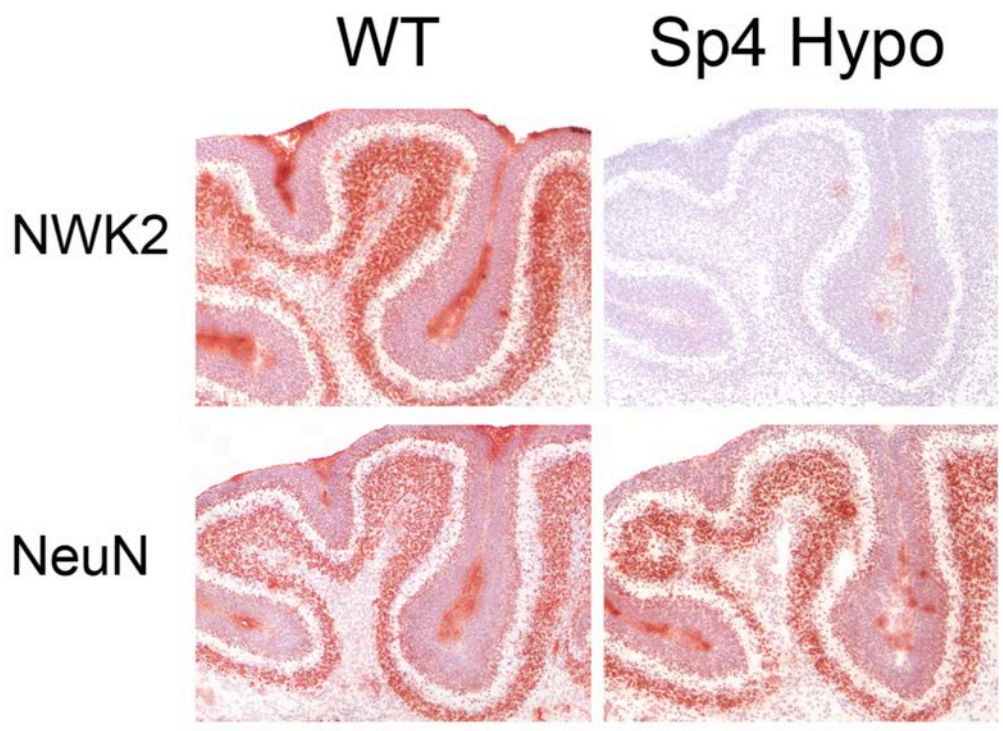

Figure 3-4 Nwk2 in postnatal Day7 WT and Sp4 mouse cerebellum.

Immunohistochemical staining of Nwk2 and NeuN were performed in frozen section of gender-matched postnatal Day7 WT and Sp4 hypomorphic mouse cerebellum.

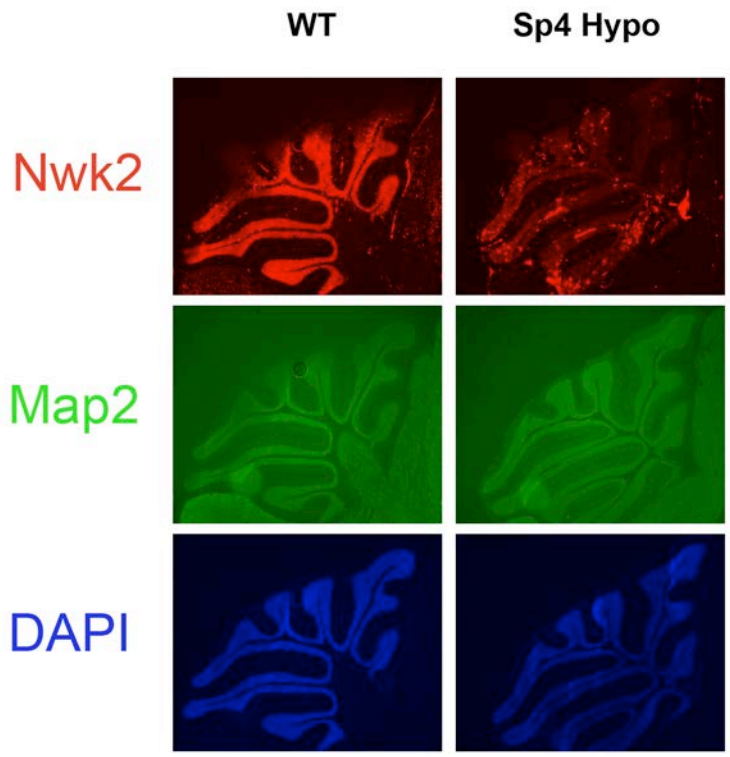

Figure 3-5 Nwk2 in postnatal Day21 WT and Sp4 mouse cerebellum.

Immunofluorescence staining of Nwk2 and Map2 were performed in frozen sections of gender-matched postnatal Day21 WT and Sp4 hypomorphic mouse cerebella.

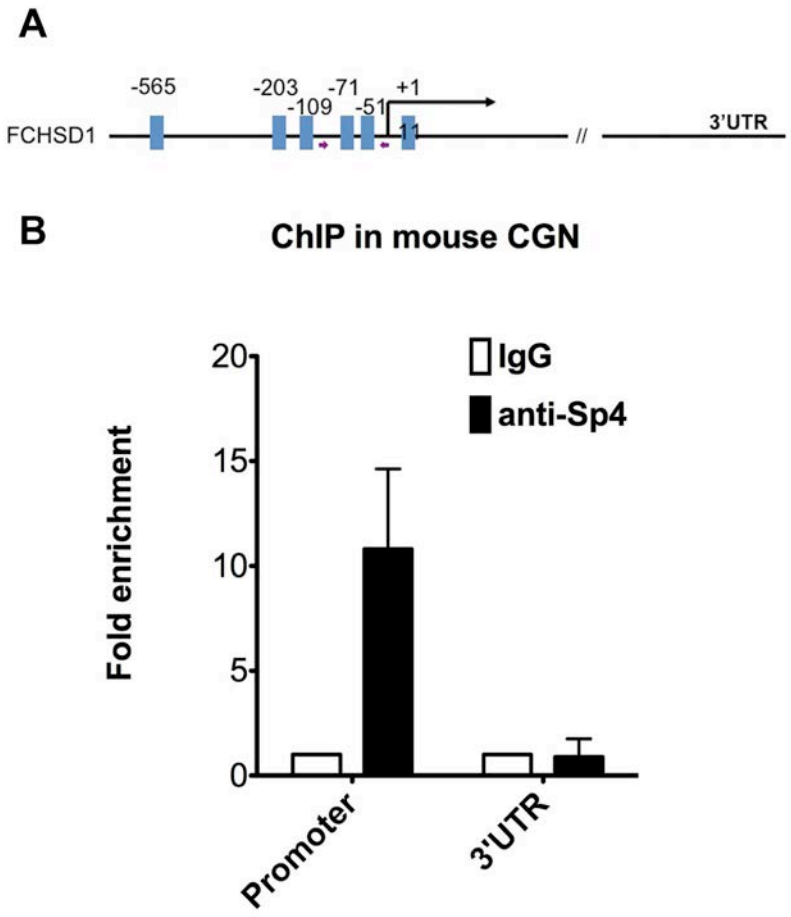

Figure 3-6 Sp4 localizes to the promoter region of Nwk2 gene.

A.The schematic diagram of Nwk2 gene promoter and 3'UTR. The blue boxes represent the predicted Sp4 binding site in the promoter region. The purple arrows indicate the position of PCR primers.

B. CGNs were purified from postnatal Day5 WT mouse cerebellum and cultured in vitro for 6 days. ChIP assays were performed, using rabbit antibody for Sp4, or normal rabbit IgG as control. Immunoprecipitated DNA was analyzed by real-time PCR with primers that amplified a fragment near the transcription start site in Nwk2 promoter or a control fragment in the 3'UTR of Nwk2. The signal for DNA precipitated by Sp4 antibody was normalized to the DNA precipitated by IgG.

Knockdown of Nwk2 in cerebellar granule neurons leads to increased dendrite number

Knockdown of Sp4 led to an increased number of highly branched dendrites during maturation of CGNs [64] (Figure3-9). We asked whether Nwk2 mediates the ability of Sp4 to control dendritic patterning.

We generated two shRNAs targeting Nwk2. Nwk2 expression level was strongly knocked down by each of the two shRNAs (Figure3-7A). We also generated an expression plasmid encoding a rescue form of Nwk2 that was resistant to the shRNA by introducing four silent mutations. We confirmed that Nwk2 shRNA failed to effectively induce knockdown of Nwk2-Res (Figure 3-7B).

First we tested the effect of endogenous Nwk2 depletion in CGN using shRNAs that induced specific knockdown of Nwk2. Nwk2 knockdown increased the number of primary dendrites of CGN (Figure 3-8). To determine the specificity of the Nwk2 shRNA-induced dendritic phenotype, we performed a rescue experiment. Expression of Nwk2Res rescued the morphology of Nwk2-deficient CGNs (Figure3-8). In summary, knockdown of Nwk2 in rat CGN produced an increased number of primary dendrites, which phenocopied the effect of Sp4 knockdown on primary dendrites.

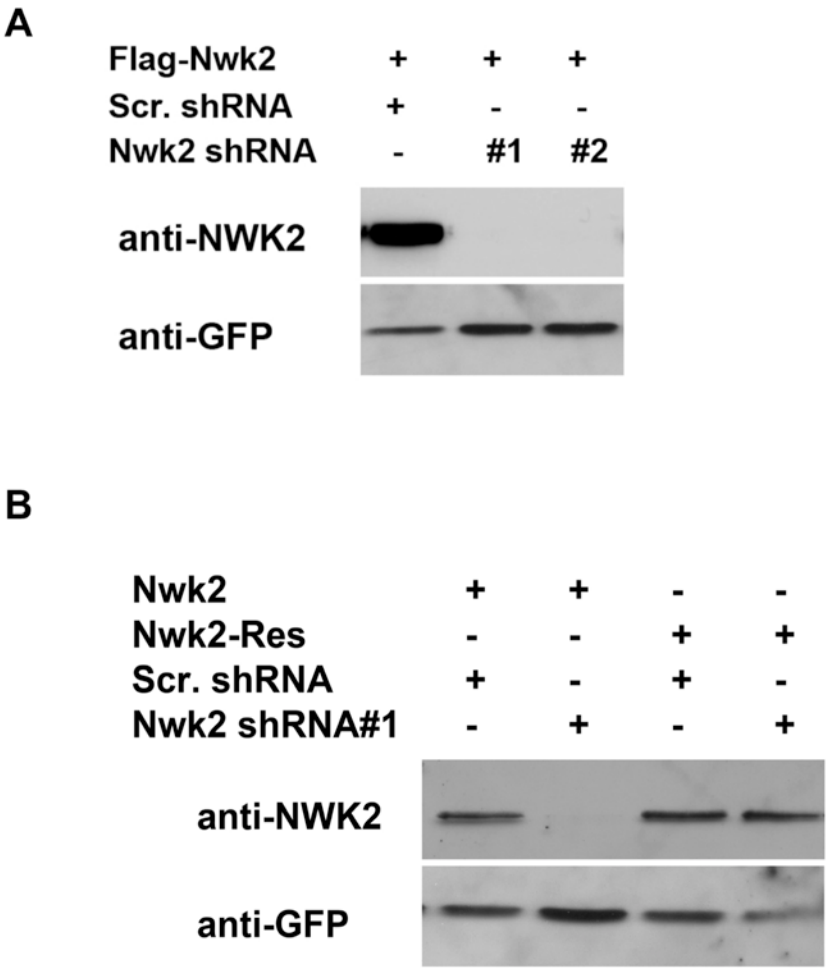

Figure 3-7 Validation of specific Nwk2 knockdown and rescue.

A. Lysates from Neuro2A cells cotransfected with Flag-Nwk2 plasmid and the indicated shRNA plasmid that also encodes GFP, were immunoblotted with antibodies against Nwk2, GFP and GAPDH.
B. Lysates from Neuro2A cells cotransfected with indicated shRNA plasmid that also encodes GFP, together with an expression vector encoding WT Nwk2 or an RNAi-resistant Nwk2 protein (Nwk2-Res), were immunoblotted with antibody against Nwk2 and GAPDH.

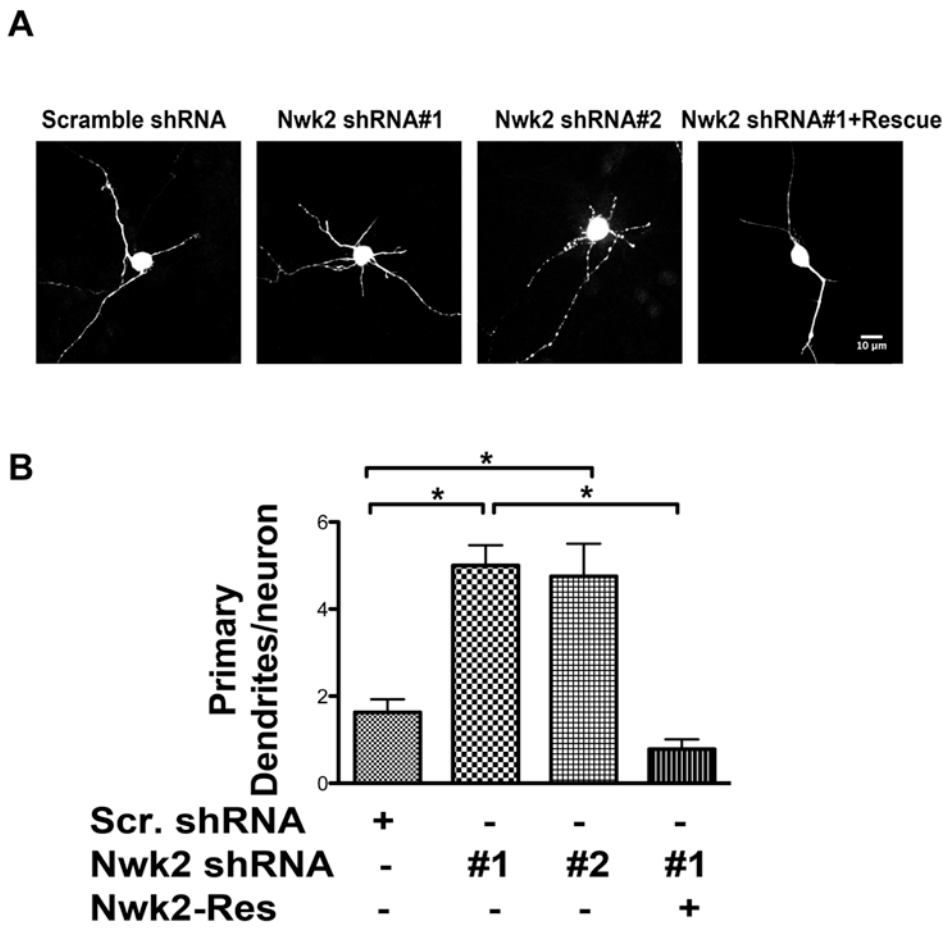

Figure 3-8 Knockdown of Nwk2 in CGNs leads to increased primary dendrite number.

Cerebellar granule neurons were transfected with Scrambled control shRNA plasmid or the indicated Nwk2 shRNA plasmid that also encodes GFP, and/or an expression vector encoding RNAi-resistant Nwk2 protein immediately after neurons were purified. Then neurons were immunostained with antibody against GFP 6 days post-transfection.

A.Representative images of neurons. The white bar in the lower right corner is a scale bar:10um.

B.Quantification of primary dendrite numbers. The experiment was repeated 3 times. (ANOVA; *P<0.05) Values represent mean ± SEM, *n* = 48-63 per condition.

Sp4 regulates dendritic patterning through the function of Nwk2

In order to investigate whether Sp4 and Nwk2 function in a common pathway to reduce dendrite numbers, we knocked down both Sp4 and Nwk2 in CGNs. Although Sp4 knockdown and Nwk2 knockdown each significantly increased the number of primary dendrites, the combination of Sp4 and Nwk2 knockdown did not further increase the number of dendrites (Figure 3-9). These results suggested that Sp4 and Nwk2 operated in a common pathway to control neuronal dendrite numbers, instead of 2 independent pathways.

If Sp4 activates the transcription of Nwk2 and inhibits dendrite numbers through Nwk2, we predicted that overexpression of Nwk2 would reverse the phenotype of Sp4 depletion in CGNs. We tested this by expression of exogenous Nwk2 in Sp4 knockdown neurons. We found that the expression of Nwk2 substantially restored the morphology of Sp4 knockdown neurons (Figure 3-10). Thus, the expression of Nwk2 suppressed the effect of Sp4 knockdown on dendritic number in primary CGNs. These data support the idea that Sp4 regulates the transcription of Nwk2 to control dendrite numbers.

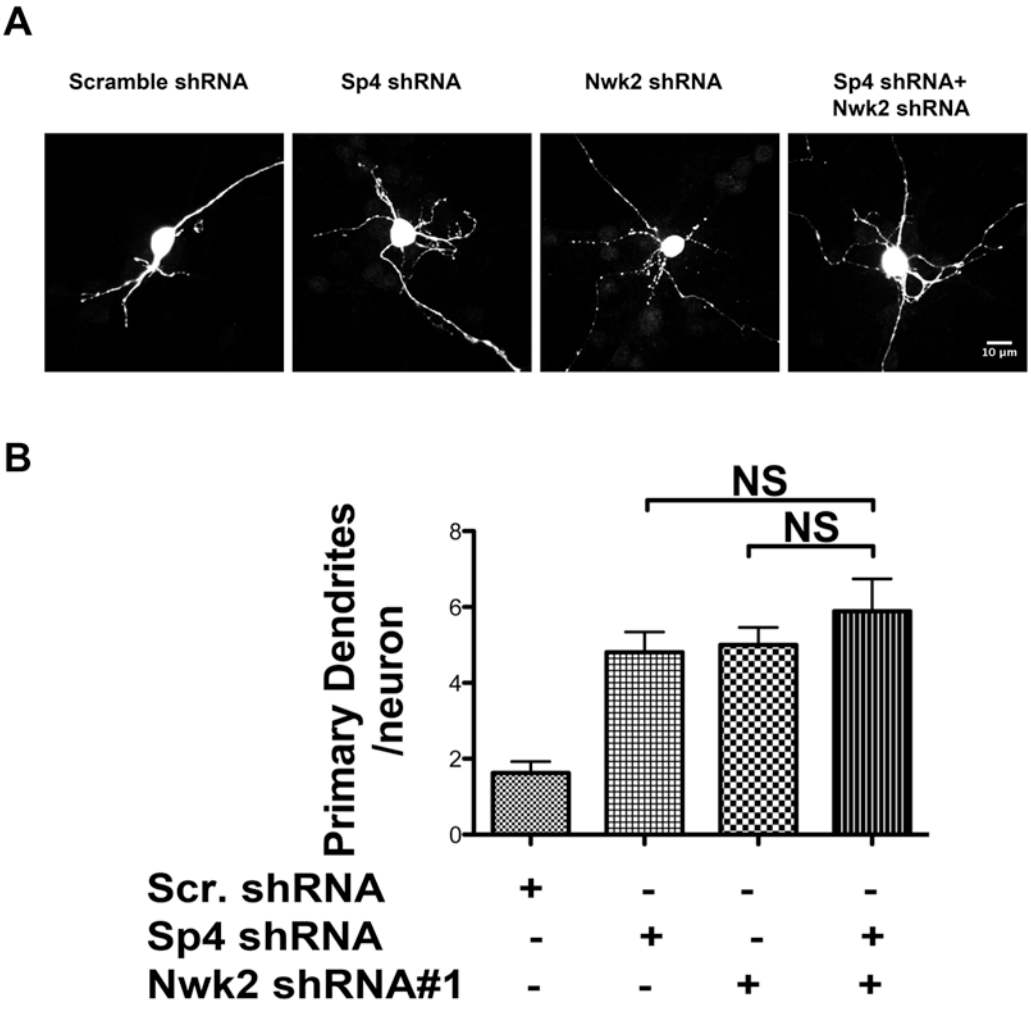

Figure 3-9 Knockdown of both Sp4 and Nwk2 does not increase the number of dendrites additively.

Cerebellar granule neurons were transfected with Scrambled control shRNA plasmid or the indicated Sp4 or/and Nwk2 shRNA plasmid that also encode GFP immediately after neurons were purified. Then neurons were immunostained with antibody against GFP 6 days posttransfection.
A.Representative images of neurons. The white bar in the lower right corner is a scale bar: 10um.
B.Quantification of primary dendrite numbers. The experiment was repeated 3 times. (ANOVA; NS: not significant). Values represent mean ± SEM, *n* = 48-63 per condition.

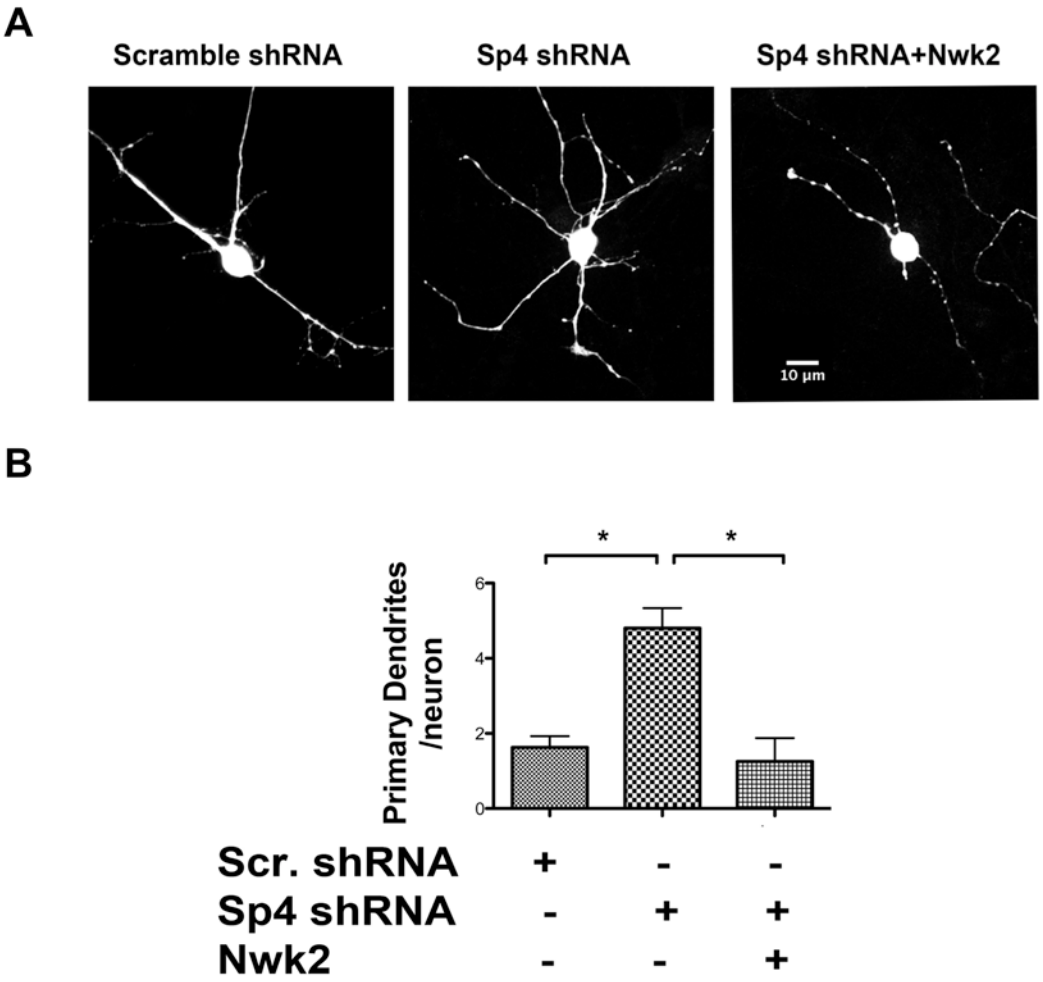

Figure 3-10 Expression of Nwk2 rescues dendrite number phenotype of Sp4 knockdown in CGNs

Cerebellar granule neurons were transfected with Scrambled control shRNA plasmid or the indicated Sp4 shRNA plasmid that also encode GFP, together with an expression vector encoding a WT Nwk2 protein, immediately after neurons were purified. Then neurons were immunostained with antibody against GFP 6 days posttransfection.

A.Representative images of neurons. The white bar in the lower right corner is a scale bar: 10um.

B.Quantification of primary dendrite numbers. The experiment was repeated 3 times. (ANOVA; *$p<0.05$). Values represent mean ± SEM, n = 48-63 per condition.

Sp4-Nwk2 pathway regulates NMDAR levels

NMDAR1 protein level was reported to be reduced in the hippocampus of Sp4 hypomorphic mice[91]. Reduced NMDAR was suggested to contibute to impaired spatial learning/memory and hippocampal long-term potentiation (LTP). Impaired NMDA neurotransmission has been implicated in schizophrenia and other neuropsychiatric disorders. We therefore tested the NMDAR levels in cultured CGNs from WT and Sp4 hypomorphic mice. The NR1, NR2a and NR2b protein levels were decreased in Sp4 mutant CGNs. We did not observe differential expression of other glutamate receptors such as GluR2 (Figure3-11). There are predicted Sp4 binding sites in the promoter region of several NMDA receptor genes, and the Sp family of transcription factors had been suggested to regulate Nmdar1 gene expression by binding to the GC-rich region in promoters [132, 133]. To test whether the NMDA receptors were regulated by Sp4 at transcription level, we performed RT-qPCR in cultured CGNs. The mRNA level of NMDARs did not change in cultured CGNs between WT and Sp4 hypomorphic neurons (Figure3-11), which was similar to what was found in hippocampus[91]. We concluded that NMDARs level were modulated by Sp4 post-transcriptionally.

Since Drosophila Nwk regulates synaptic growth factor recepters by endosomal trafficking [122], we chose to investigate whether Nwk2 contributes to the reduction of NMDARs in Sp4 deficient neurons. Following knockdown of Sp4 in cultured CGNs, the cell surface NR1 protein level as detected by immunofluorescence with NR1 antibody was decreased. The decrease of NR1 was a specific result of Sp4 knockdown, since the

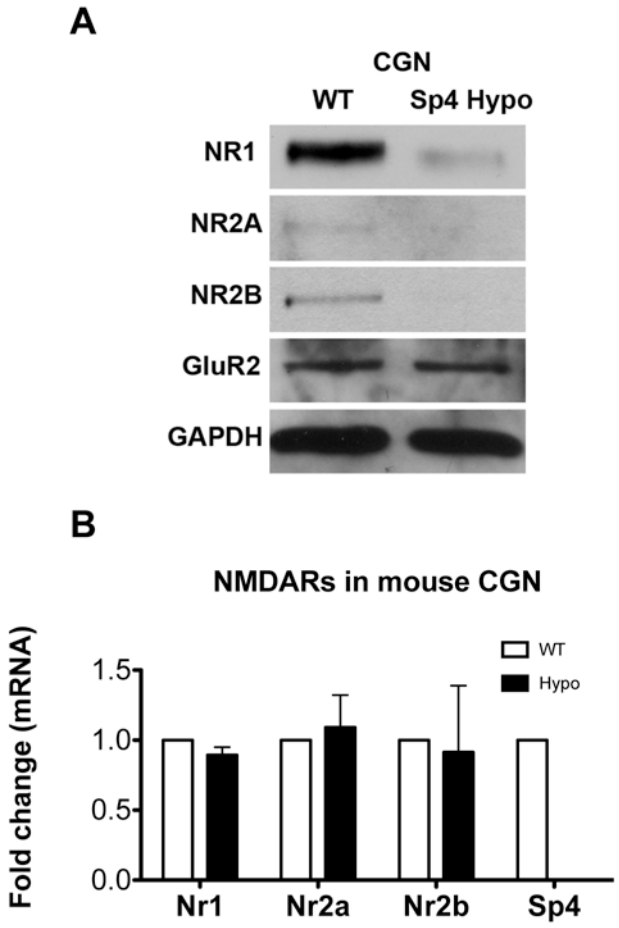

Figure 3-11 NMDAR protein level is reduced in CGNs from Sp4 hypomorphic mice.

CGNs were purified from postnatal Day5 WT and Sp4 hypomorphic littermate cerebellum. CGNs were cultured in vitro for 6 days.

A. Protein lysates from cultured CGNs were immunoblotted NR1, NR2A, NR2B, GluR2 and GAPDH.
B. Total RNA was extracted from cultured neurons, and RT-qPCR was performed with primers amplifying NR1, NR2A, NR2B and Sp4. Values represent mean±SEM generated from experiments performed in triplicate and normalized to Gapdh and Hprt1 expression levels. WT expression was normalized as 1. A Student's t-test was used to determine the significance between and Sp4 hypomorph.

decrease of NR1 was rescued by an Sp4 shRNA resistant expression plasmid. CGNs served as an in vitro model to study Sp4's role in regulation of NMDAR. Thus, Nwk2 was knocked down in CGNs and cell surface NR1 was measured. We found that the NR1 level was decreased in Nwk2 knockdown neurons. The loss of NR1 was rescued by Nwk2-shRNA-resistant Nwk2 expression plasmid (Figure3-12). Moreover, expression of Nwk2 in Sp4 depletion neurons partially restored the cell surface NMDAR1 protein. The quantification of NR1 fluorescence level is shown in Figure3-13. We conclude that Sp4 modulated cell surface NMDAR1 through the function of Nwk2. By shRNA knockdown of Sp4 in isolated CGNs, we observed reduced cell surface NR1 with the "acute" loss of Sp4 in neurons. This means the decrease of NR1 is not a consequence of absence of Sp4 during early development in mice, but is a direct effect of Sp4-mediated control of Nwk2 or perhaps other gene targets.

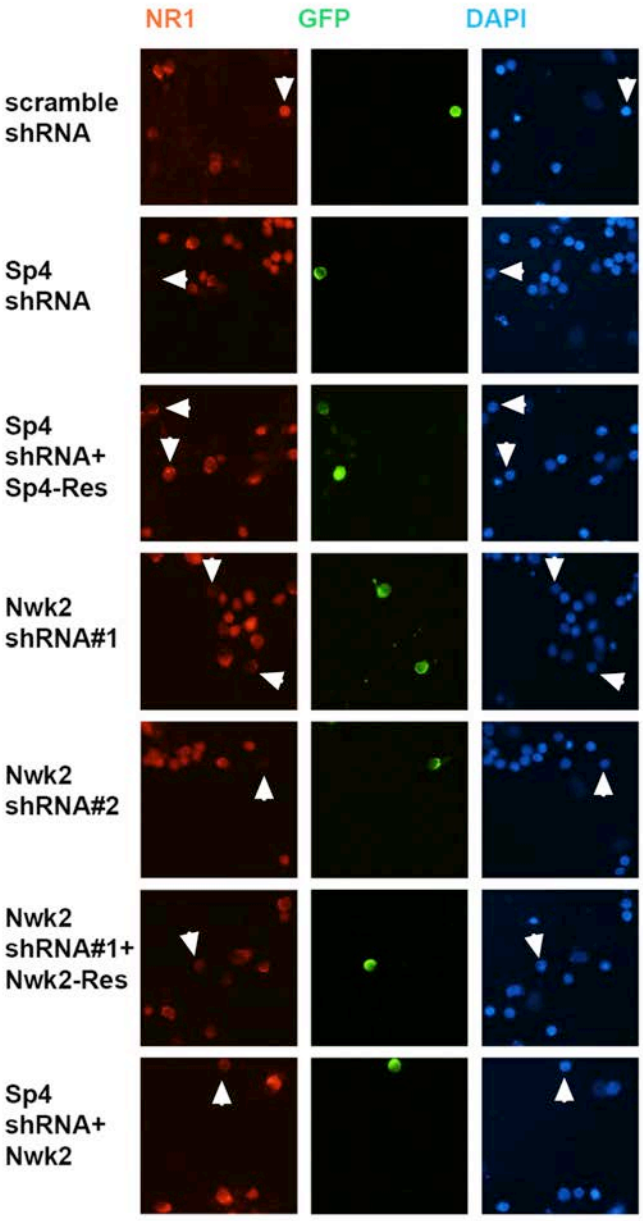

Figure 3-12 Sp4-Nwk2 pathway modulates the cell surface NMDAR1 protein level.

Cerebellar granule neurons were transfected with shRNA plasmids that also encode GFP and/or overpression plasmids one day after neurons were purified. Then neurons were immunostained with antibody against NR1 5 days posttransfection without permeablization. Representative images of neurons are shown. Arrowheads indicate the transfected neurons.

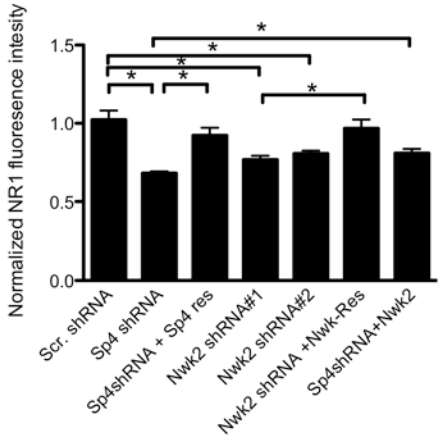

Figure 3-13 Quantification of cell surface NR1 level under the control of Sp4-Nwk2 pathway.

Cerebellar granule neurons were transfected with the indicated Nwk2 shRNA plasmids one day after neurons were purified. Then neurons were immunostained with antibody against NR1 5 days posttransfection without permeablization.
Quantification of fluorescence intensity of NR1. The experiment was repeated 3 times. (ANOVA; *P<0.05) Values represent mean ± SEM, *n* = 48-63 per condition.

NMDAR1 acts as a downstream effector of Sp4-Nwk2 pathway in dendritic patterning

We have demonstrated that the Sp4-Nwk2 pathway control the primary dendrite formation. NMDAR1 protein level is also regulated by the Sp4-Nwk2 pathway. We wondered whether the NMDAR1 protein is a mediator of dendritic patterning. To test this, we expressed NMDAR1 in Sp4 or Nwk2 knockdown neurons. NMDAR1 overexpression rescued the dendritic number phenotype of Sp4 or Nwk2 depletion (Figure 3-14). We conclude that NMDAR1 plays a major role downstream of Sp4-medulated Nwk2 expression to control dendritic patterning.

Discussion

In this study, we have elucidated a pathway by which Sp4 regulates dendritic patterning and NMDAR protein level. We identified Nwk2 as a direct activation target of Sp4 in cerebellar granule neurons. By epistatic genetic analysis, we confirmed that the Sp4-Nwk2 pathway played an important role in dendritic patterning and NMDAR post-transcriptional regulation. Overexpression NMDAR1 suppressed the primary dendrite phenotype of Sp4 or Nwk2 loss. Our results revealed a novel molecular pathway, Sp4-Nwk2-NMDAR1, which regulates dendritic patterning and NMDAR levels in neurons. As discovered by previous studies that Sp4 and NMDAR levels are changed in neuropsychiatric diseases, our finding may provide new insight into the pathogenesis and treatment of diverse neuropsychiatric disorders.

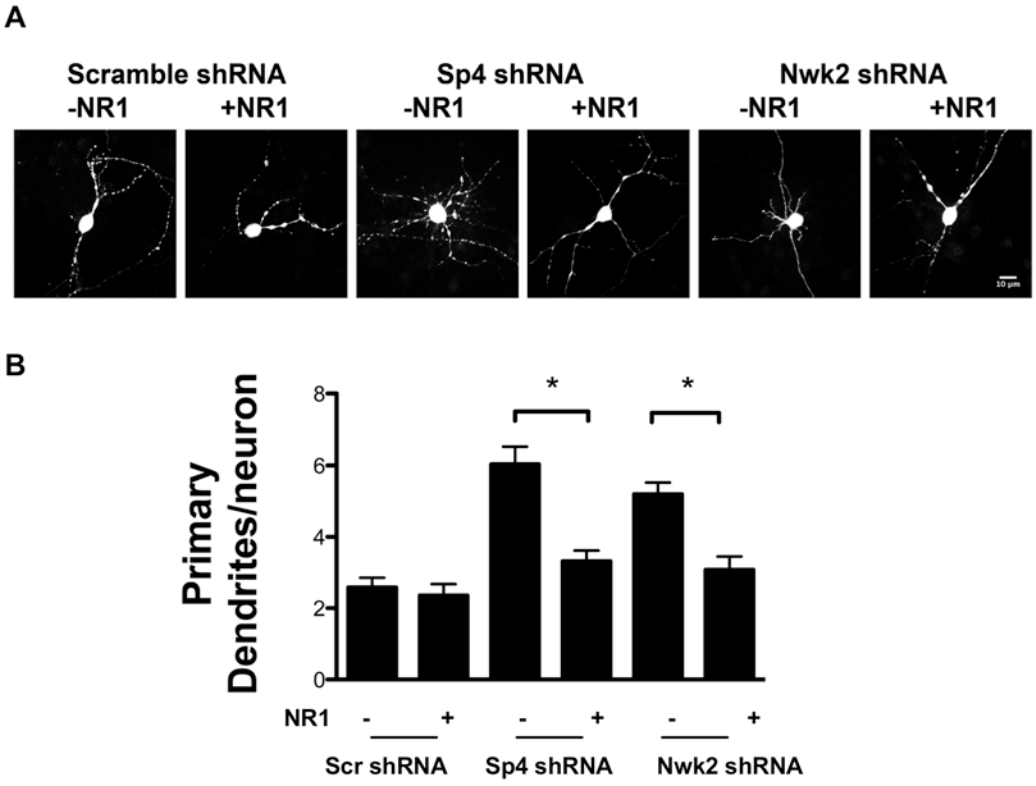

Figure 3-14 NR1 rescues Sp4 or Nwk knockdown dendritic phenotype.

Cerebellar granule neurons were transfected with Scramble control shRNA plasmid or the indicated Sp4 shRNA or Nwk2 shRNA plasmid that also encode GFP, together with or without an expression vector encoding NMDAR1 protein immediately after neurons were purified. Then neurons were immunostained with antibody against GFP 6 days posttransfection.

A.Representative images of neurons. The white bar in the upper left corner indicates a scale bar, 10um.

B.Quantification of primary dendrite numbers. The experiment was repeated 3 times. (ANOVA; *P<0.05) Values represent mean ± SEM, *n* = 48-63 per condition.

Sp4-Nwk2-NMDAR1 pathway in dendritic patterning

Transcription factor Sp4 controls dendritic patterning during cerebellar development by limiting branch formation and promoting activity-dependent pruning[64]. Little is known about the target genes of Sp4 in neuron development and dendritic patterning. NT3 is a repression target of Sp4. Sp4-dependent repression of neurotrophin-3 is required to limit dendritic branching [79]. But increased NT3 does not explain the increased primary dendrite numbers in Sp4 depletion CGNs. There must be other Sp4 target genes that regulate primary dendrite numbers. Nwk2 was investigated as one of the Sp4 target genes that control primary dendrite numbers.

Nwk2 expression decreased not only in Sp4 hypomorphic cerebellum tissue, but also in isolated Sp4 mutant CGNs. Sp4 localized to the Nwk2 promoter, so Nwk2 is likely a direct activation target gene of Sp4. Nwk2 knockdown increased primary dendrite number. The increased primary dendrite number is due to the specific knockdown of Nwk2, because it was rescued with shRNA resistant expression of Nwk2. Nwk2 knockdown phenocopies Sp4 deletion in the aspect of primary dendrite number. The double knockdown of Sp4 and Nwk2 did not further increase the primary dendrite number, suggesting these two proteins do not work in parallel or that knockdown of one of them alone had maximized the neuron's capacity to respond. More importantly, overexpression of Nwk2 suppressed the phenotype of Sp4 knockdown, which confirmed that Nwk2 acts downstream of Sp4 in the same pathway. (Figure 3-15 right)

NMDA receptor protein level was regulated by Sp4 post- transcriptionally in CGNs. Transient knockdown of Sp4 or Nwk2 led to the loss of NMDAR1 on the neuron surface, which was consistent with the observation of NMDAR1 loss in Sp4 hypomorphic CGNs.

The loss of NMDAR1 by Sp4 knockdown was partially rescued by overexpression of Nwk2, this demonstrates that Sp4-Nwk2 pathway is a cell intrinsic mechanism underlying NMDAR protein regulation. Previous work in Sp4 hypomorphic mice showed that NMDAR1 is reduced in mouse brain [91]. However, it is not known whether the reduced NMDAR protein is the consequence of loss of Sp4 in other cell types in brain or the consequence of Sp4 loss from a very early stage of development. In our study, we used transiently transfected shRNA to knockdown Sp4 or Nwk2, and observed loss of NMDAR1 in neurons with depletion of Sp4 or Nwk2. This experiment clearly showed that Sp4-Nwk2 pathway is a cell intrinsic mechanism that regulates NMDAR protein level on neuron surface (Figure 3-15 left).

We demonstrated that Sp4-Nwk2 pathway regulates dendritic patterning and NMDAR1 level. We wonder whether there is relationship between NR1 and dendritic patterning. People have been interested of the role of NMDA neurotransmission in dendritic patterning for a long time. In the Xenopus retinotectal system, NMDAR antagonists inhibit dendritic arborization of tectal neurons during development [134]. Excess glutamate decreases dendrite growth through NMDA receptor activation in cortical neurons [135]. Since differential NMDAR subunit combination confers distinct functional properties, including the regulation of conductance, binding affinity, and and gating and desensitization kinetics, each combination may play different role in dendritic patterning. In the cortex-specific NR1 knockout, individual layer 4 stellate cells lose oriented arborization and grow exuberant dendrites and spines [136]. A study in the rodent trigeminal principal nucleus (PrV) showed that NR1KD mice have widespread terminal arbors, increased branch tips in axon and longer dendrites [13] The knockout of

NR2B subunit in NMDA receptors leads to an increase of primary dendrite number in hippocampal dentate gynus granule neurons [137]. Overexpression of NR3B, a putative dominant-negative subunit in glutamate-induced ionic currents, promotes the addition of branch segments in developing motor neurons [138]. However, little is known about the role of NMDA neurotransmission in dendritic pattering in CGNs. In our study, overexpression of NMDAR1 in Sp4 or Nwk2 depletion neurons rescued the dendrite number in CGNs. This experiment linked the NMDAR1 protein with dendritic development in CGNs. Sp4-Nwk2-NR1 is identified as a new pathway that contributes to normal neuronal morphogenesis (Figure 3-15).

Working mechanisms of Nwk2

Nwk2 is a homolog of *nwk* in Drosophila. Nwk is characterized by an F-BAR domain, which has essential roles in regulation of actin cytoskeleton and endosomal trafficking signaling. Members of F-BAR family have a critical role in the central nervous system diseases and neurite morphogenesis. MEGAP, CIP4 and PACSIN have been demonstrated to correlate with severe mental retardation and Huntington's disease. Knockdown of FBP17 led to a decrease in spine density in cultured hippocampal neurons [118]. The F-BAR protein CIP4 inhibits neurite formation by producing lamellipodial protrusions [14]. F-BAR proteins of the syndapin family shape the plasma membrane and are crucial for neuromorphogenesis [139]. The mechanisms underlying Nwk2's role in dendritic patterning remain to be studied. Work needs to be done to examine whether there are actin cytoskeleton or endosomal trafficking or growth signaling deficits in Sp4 or Nwk2 mutant neurons.

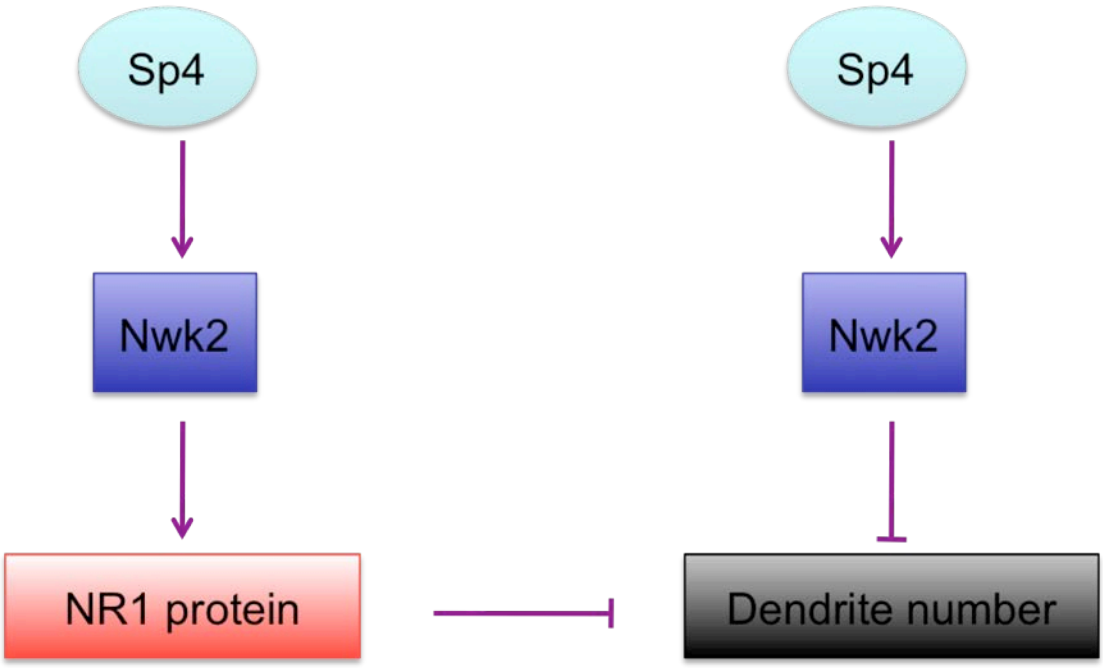

Figure 3-15 Sp4-Nwk2-NR1 is a new pathway that contributes to neuronal morphogenesis

We identified Nwk2 as a downstream activation target of transcription factor Sp4. Our study demonstrated the Sp4-Nwk2 pathway inhibits the formation of primary dendrites, and loss of the normal Sp4-Nwk2 pathway leads to the decrease of cell surface NMDAR1 protein level, and furthermore NMDAR1 inhibits the formation of primary dendrites. Sp4-Nwk2-NR1 is a new pathway that contributes to normal neuronal morphogenesis

We detected that cell surface NMDAR1 around soma was decreased when Sp4-Nwk2 pathway was disturbed. After NMDAR subunits are synthesized, they assemble in the endoplasmic reticulum (ER) and are modified in the Golgi. Then, assembled NMDA receptors are transported to plasma membrane by vesicles. NMDARs level on the cell membranes is in a dynamic balance. NMDA receptors are internalized by endocytosis and sorted by the early endosome. Some of them go to the lysosome to be degraded and some of them go to the recycling endosome and are integrated into the plasma membrane. Studies have demonstrated that schizophrenia patients have less NMDARs on their neuron membranes and the increased endocytosis results in more internalization of NMDAR [140, 141]. What might be the reasons for the reduced NMDAR1 in Sp4 mutant neurons? The following are possible reasons that lead to the reduced NMDA1 on neuron surface: (1) decreased protein synthesis of NMDAR1 subunits. (2) Impaired assembly of NMDAR. (3) Attenuated deliveries of NMDAR packets to membrane. (4) Increased internalization of NMDAR from the plasma membrane and reduced recycling of NMDAR to plasma membrane in the endosomal trafficking. Experiments need to be designed to test each possible step.

Nwk2 has a potential role in endosomal trafficking based on its protein structure similarity to NWK, which functions in Rab11-dependent endosomal trafficking with cdc42 to control synaptic growth signaling in Drosophila [122]. We co-stained Nwk2 and Rab11 in CGNs, and we did not see the colocalization of Nwk2 and Rab11. Nwk2 may have some working mechanism different from Drosophila Nwk.

Proteomic and genomic findings implicate endocytosis and endosomal trafficking act as convergence points in the development of schizophrenia [142, 143]. Two genes linked

to schizophrenia, DTNBP1 and MUTED, encode proteins that belong to the endosome-localized Biogenesis of Lysosome-related Organelles Complex-1 (BLOC-1). BLOC-1 plays a key role in endosomal trafficking and as such has been found to regulate cell-surface abundance of the D2 dopamine receptor, the biogenesis and fusion of synaptic vesicles, and neurite outgrowth [142]. More studies will be performed to investigate the role of Nwk2 in endosomal trafficking and the contribution of endosomal trafficking disturbance to Sp4 mutant phenotypes.

Taken together, Sp4-Nwk2-NMDAR1 is a novel pathway in dendritic morphogenesis. The altered function of this pathway may contribute to the formation of neuropsychiatric disorders. It is interesting to learn the working mechanisms of Nwk2 in mammalian system, for example, whether Nwk2 functions in endosomal trafficking.

Chapter 4. Summary and future directions

Summary

The nervous system is a well-coordinated network that depends on the formation of proper connections among diverse types of neurons. Dendritic arborization patterns determine the way a neuron integrates inputs. N-methyl-D-aspartate (NMDA) neurotransmission plays an important role in neuronal plasticity, cognition and memory, and altered NMDA neurotransmission may underlie the pathophysiology of multiple psychiatric disorders. Previous studies from our laboratory indicated that Sp4 controls dendritic patterning during cerebellar development by limiting branch formation and promoting activity-dependent pruning [64]. Sp4 hypomorphic mice display behavioral deficits in sensorimotor gating and contextual and spatial memory, and show reduced Long-term potentiation (LTP) in hippocampal CA1 and reduced NMDAR1 protein level in the whole brain, which are all related to some neuropsychiatric disorders, for example, schizophrenia [91, 98]. Recent studies in our lab revealed that Sp4 activates TrKC and represses NT3 to limit dendritic branching. However, little is known about other target genes of Sp4 involved in various aspects of dendritic development and NMDA neurotransmission.

We used microarray-based methods to identify Sp4-regulated genes by comparing global gene expression between wild type and Sp4 hypomorphic mouse cerebellum. We ran DAVID and IPA analysis of the microarray data, and tried to find Sp4 target genes or pathways with biological functions relevant to neuronal development and central nervous system diseases. We identified a set of genes regulated by Sp4 have overlapping function with the genes whose expression was altered in schizophrenia and bipolar disorder patients. We validated a subset of target genes by RT-qPCR in 2 mouse strains and we

investigated whether they are direct neuron specific Sp4 target genes by ChIP assay in cultured CGNs.

We identified Nwk2 as a direct activation target gene of Sp4 that mediates Sp4's role in control dendritic patterning and NMDAR1 level. RNA and protein expression of the nervous wreck homolog, Nwk2, were decreased in Sp4 hypomorphic mice. Sp4 localized to the Nwk2 promoter region by ChIP assay in cerebellar granule neurons. Knockdown of Nwk2 in CGNs produced an increased number of primary dendrites, which phenocopied the effect of Sp4 knockdown. Importantly, exogenous expression of Nwk2 rescued the dendrite morphology of Sp4 knockdown neurons. Furthermore, we found that the Sp4-Nwk2 pathway acted post-transcriptionally to regulate NMDA receptor protein level. Overexpression of NR1 in Sp4 or Nwk2 knockdown neurons rescued the dendrite number phenotype. Our studies identify Sp4-Nwk2-NR1 as a new pathway that contributes to normal neuronal morphogenesis and may be disrupted in neuropsychiatric diseases.

Neurons in Schizophrenia patients show reduced dendritic arborization in CA3 region of the hippocampus [2-4]. Sp4 is a susceptibility gene for SZ [88] and Sp4 deletions were found in SZ patients [91]. Impaired NMDA transmission is suggested to cause prefrontal cognitive deficits in SZ [104]. In our model, impaired Sp4-NMDAR1 pathway causes an increase of primary dendrite number. These contradictory effects of Sp4 or NMDAR can be explained by the following possibilities. First, we studied dendritic patterning in CGNs, which is a different cell type than hippocampal pyramidal neurons. As we discussed in the introduction, each population of neurons has their own developmental template specified by intrinsic cues. Second, CGNs were cultured in vitro, and we don't

know the dendritic patterning change in vivo with disturbance of Sp4 or NMDAR1. I observed the dendritic patterning of WT and Sp4 hypomorphic CGNs isolated from mice and maintained in culture, and I did not see the significant difference between WT and Sp4 mutant CGNs. Third, even if there is an increased number of primary dendrite in Sp4 or Nwk2 deficient neurons, we are not clear whether neurons have normal number of synapses that establish the functional neuronal network.

Future directions

Our study opens a series of questions:

(1) The neuronal development is a highly complex and dynamic process. Sp4 may play various roles at different developmental stages and in different brain regions. It will be interesting to identify spatial and temporal specific targets of Sp4 in CNS development. The conditional Sp4 knockout mice, microarray combined with ChIP-seq assay in different brain regions or cell types will help us to figure out the spatial and temporal specific targets of Sp4. It will also be interesting to know whether Sp4 has non-coding RNA targets.

(2) We demonstrated that Nwk2 limits primary dendrite number. We don't know whether Nwk2 limits adding of new dendrites or promotes pruning of dendrites. Previous work in our lab showed Sp4 promotes pruning. We wonder whether Nwk2 also limits dendrite number by promoter pruning. A time course analysis of dendritic patterning will give us some hints. Preliminary data in our lab have shown that Sp4 inhibits axon extension, it is interesting to know whether Nwk2 also has a role in axon morphogenesis. It is not known whether Nwk2 is important for depolarization-induced dendritic

remodeling. Knockdown of Nwk2 in depolarization and non-depolarization conditions will answer the question. It is not known whether Nwk2 functions in neuron plasticity in adult. Conditional Nwk2 knockout mice with specific deletion of Nwk2 in neurons will help us to understand Nwk2's role in a biological context.

(3) We mentioned possible reasons that may lead to the reduced NMDAR level on the cell surface of Sp4 or Nwk2 knockdown neuron. Experiments need to be designed to determine which step is defective. For example, detailed subcellular localization of Nwk2 will provide hints, inhibition of proteasome protein degradation or lysosome degradation will indicate by which pathways that NMDAR is more rapidly degraded, and a transferrin experiment and biotin labeling of NMDAR1 will be able to test whether the endosomal trafficking is defective in the Sp4 mutant neurons.

(4) We measured the NMDAR level on cell surface, mainly around soma. We also see the NMDAR1 existing along neurites, but we had technical obstacles to quantifying the NMDAR1 level on dendrites. We demonstrated the NMDAR1 level are important to determine primary dendrite number. We are just not clear which pool of NMDAR1 is active in determining dendrite numbers, in soma or in dendrites. It is also possible to study the role of synaptic NMDAR in dendritic patterning, including spine formation, by another neuron model system.

(5) Prelimitory data from Belen's group in Spain, in colaberation with our lab, showed that Nwk2 expression at mRNA level is decreased in bipoar disorder patients. This strongly predicts the Nwk2 expression corelates with Sp4 protein level. To support our proposal that a novel pathway Sp4-Nwk2-NMDAR1 contributes to the formation of neuropsychiatric disorders, it is necessary to test the NMDAR1 protein level in the same

group of samples. It is also interesting to know whether the Nwk2 expression, both at mRNA and protein levels, is changed in other study samples of neuropsychiatric patients.

Appendix I Microarray data

Table AI-1 Gene list with increased expression in Sp4 hypomorphic mouse cerebellum

REFSEQ ID	Gene SYMBOL	Fold change	P Value	ONTOLOGY PROCESS
NM_178939.2	Pdrg1	11.36	9.03E-03	protein folding
NM_011755.2	Zfp35	5.36	4.60E-03	transcription; spermatogenesis; cell differentiation; regulation of transcription, DNA-dependent; development
NM_026925.3	Pnlip	5.19	1.86E-02	lipid catabolism; cholesterol absorption
XM_001481025.1	LOC100043822	3.78	1.27E-04	
NM_021527.1	Mkks	3.64	2.01E-02	cellular protein metabolism; protein folding; flagellum biogenesis; photoreceptor maintenance
NM_009030.3	Rbbp4	3.59	3.77E-02	chromatin modification; chromatin remodeling; transcription; DNA replication; regulation of transcription, DNA-dependent; cell cycle
NM_001081377.1	Pcdh9	3.56	9.65E-05	homophilic cell adhesion
NM_019493.3	Btg4	3.24	4.59E-05	
NM_011111.3	Serpinb2	3.04	1.88E-02	
NM_023721.2	Atp6v1d	2.99	8.17E-04	ion transport; transport; ATP biosynthesis; ATP synthesis coupled proton transport; proton transport
NM_001081047.1	Cnksr1	2.63	8.32E-04	Rho protein signal transduction; Ras protein signal transduction
NM_175175.3	Plekhf2	2.60	6.86E-05	
NM_016673.1	Cntfr	2.52	2.25E-02	positive regulation of cell proliferation
NM_025876.2	Cdk5rap1	2.46	1.10E-04	brain development; cell proliferation; regulation of neuron differentiation
	4831403C07Rik	2.40	1.44E-03	
NM_016903.4	Esd	2.28	3.59E-04	
NM_028643.3	Efha1	2.23	3.37E-04	
	Rapgef5	2.23	5.85E-03	
XM_001479635.1	LOC100048020	2.22	2.84E-02	
NM_025579.1	Taf12	2.18	2.23E-02	transcription
NM_029674.1	Got1l1	2.07	3.37E-03	biosynthesis; amino acid metabolism
NM_030687.1	Slco1a4	2.01	1.28E-03	ion transport; transport
NM_001081642.1	Xlr4a	2.00	4.17E-03	
XM_001475552.1	LOC100046087	2.00	7.69E-03	

Table AI-1 Gene list with increased expression in Sp4 hypomorphic mouse cerebellum (continued)

NM_001099631.1	Sh2d5	1.99	1.33E-02	intracellular signaling cascade
NM_146206.4	Tpcn2	1.99	2.05E-02	ion transport; transport
NM_025886.3	Rassf7	1.98	1.73E-04	signal transduction
XM_355096.1	LOC381174	1.97	6.97E-03	
	Pcdh20	1.96	1.84E-04	
XM_001474596.1	2900016B01Rik	1.96	1.03E-02	
NM_024196.3	Tbc1d20	1.95	1.32E-02	
NM_031396.1	Cnnm1	1.93	1.56E-02	
	BC038167	1.93	3.77E-03	
	9330102E08Rik	1.92	1.83E-04	
NM_009250.1	Serpini1	1.91	1.26E-03	
NM_008161.2	Gpx3	1.90	3.25E-02	response to oxidative stress; response to lipid hydroperoxide; hydrogen peroxide catabolism; protein homotetramerization
XM_925223.2	9430038I01Rik	1.88	2.25E-04	
	C230027D02Rik	1.87	1.80E-03	
	Rwdd2	1.86	6.07E-04	
NM_001039543.1	Mlf1	1.86	1.15E-03	cell differentiation; development; hemopoiesis
NM_139306.1	Asah3l	1.83	1.34E-02	ceramide metabolism; lipid metabolism
NM_009415.1	Tpi1	1.80	7.33E-04	metabolism; pentose-phosphate shunt; fatty acid biosynthesis; lipid biosynthesis; gluconeogenesis; glycolysis
NM_201641.2	Ugt1a10	1.78	4.25E-02	metabolism
NM_010851.2	Myd88	1.78	5.41E-04	transmembrane receptor protein serine/threonine kinase signaling pathway; positive regulation of tumor necrosis factor-alpha biosynthesis; immune response; response to virus; cell surface receptor linked signal transduction I-kappaB kinase/NF-kappaB cascade ; signal transduction; inflammatory response
NM_001042557.1	Map2k7	1.76	3.34E-03	protein amino acid phosphorylation
NM_023537.4	Rab3b	1.75	1.26E-02	
XM_127882.3	Cln5	1.75	1.57E-03	
NM_019946.4	Mgst1	1.75	3.32E-02	glutathione metabolism
NM_133210.2	Sertad3	1.73	2.87E-03	negative regulation of cell growth; transcription; positive regulation of transcription; regulation of transcription, DNA-dependent
NM_146261.1	BC031748	1.72	4.67E-02	
XM_001481237.1	LOC100043918	1.72	4.33E-02	

Table AI-1 Gene list with increased expression in Sp4 hypomorphic mouse cerebellum (continued)

NM_009252.2	Serpina3n	1.72	2.11E-02	acute-phase response
NM_010346.2	Grb7	1.72	5.39E-04	signal transduction; intracellular signaling cascade
NM_008301.4	Hspa2	1.71	2.21E-03	response to heat; protein folding; response to unfolded protein
NM_177667.3	Ttc22	1.70	2.88E-03	
	Mycbp	1.69	4.86E-02	
XM_902085.2	BC048943	1.68	3.70E-02	
NM_029988.2	Pigh	1.66	1.40E-03	
NM_053099.1	Setbp1	1.66	1.61E-02	
NM_054079.1	Iltifb	1.66	4.67E-02	immune response
	mt-Nd5	1.66	1.47E-02	
XM_484933.5	Pcp4l1	1.63	1.95E-02	
NM_015754.2	Rbbp9	1.62	4.95E-02	
XM_203701.3	LOC280487	1.62	3.53E-03	
NM_011845.1	Mid2	1.62	1.44E-02	
NM_026039.2	Med18	1.61	1.19E-02	
NM_020566.1	Dnajc4	1.61	1.60E-03	protein folding
XM_988683.1	3200001K10Rik	1.61	2.22E-02	
NM_025735.1	Map1lc3a	1.60	3.16E-03	ubiquitin cycle; autophagic vacuole formation; autophagy
NM_001043335.1	Eml1	1.60	1.06E-03	
NM_177629.2	AU021034	1.60	1.40E-02	
NM_001012310.1	AI132487	1.59	1.97E-03	transport
NM_134122.2	Nrm	1.59	3.22E-03	
NM_011972.1	Poli	1.59	2.68E-02	DNA replication; response to DNA damage stimulus
NM_001024713.1	2610042L04Rik	1.59	1.24E-02	
NM_177342.3	Taf5	1.58	4.28E-03	regulation of transcription; transcription
	1700026D08Rik	1.58	1.97E-02	
NM_009606.2	Acta1	1.57	2.89E-03	muscle thin filament assembly; cytoskeleton organization and biogenesis
NM_030564.1	Rnf34	1.57	1.69E-03	ubiquitin cycle; apoptosis
NM_024452.1	Luzp1	1.57	2.15E-02	
NM_178856.1	Gins2	1.57	1.31E-02	DNA replication
XM_903197.2	Rora	1.57	2.14E-02	
NM_026083.2	Zc3h13	1.56	3.68E-02	
NM_183091.2	Nfkbil2	1.55	1.38E-02	

Table AI-1 Gene list with increased expression in Sp4 hypomorphic mouse cerebellum (continued)

NM_153076.1	Crygn	1.55	3.29E-03	
NM_145553.1	BC008163	1.55	2.53E-03	
XM_001474910.1	Shank1	1.54	5.34E-03	
	D030041G16Rik	1.54	3.72E-02	
XM_137041.6	Rshl3	1.54	3.22E-02	
NM_146123.2	Cacnb4	1.54	2.04E-02	ion transport; transport
NM_027865.1	Tmem25	1.54	7.67E-03	
XM_001474312.1	LOC100046068	1.54	1.36E-02	
XM_354782.1	LOC380876	1.53	1.54E-02	
	4933417E08Rik	1.53	4.46E-02	
NM_172662.1	Gtdc1	1.53	5.24E-03	biosynthesis; defense response
NM_145409.1	Chtf18	1.53	2.20E-03	
NM_029688.4	Srxn1	1.53	4.44E-02	response to oxidative stress
	4930522L14Rik	1.52	9.15E-03	
XM_147820.2	1700024G13Rik	1.52	5.62E-03	
XM_144101.1	LOC242703	1.52	1.41E-02	
NM_001013802.2	Macrod2	1.52	4.03E-03	
NM_001081203.1	Sbno1	1.52	2.80E-02	
NM_029852.2	Ccdc41	1.52	2.45E-03	
NM_198637.1	1700016K19Rik	1.51	3.48E-02	
NM_026743.2	Tspan11	1.51	3.97E-02	
NM_001033039.2	Klhdc9	1.51	1.62E-02	
NM_011486.4	Stat3	1.51	3.22E-03	eye photoreceptor cell differentiation; regulation of body size; transcription from RNA polymerase II promoter; cytokine and chemokine mediated signaling pathway; regulation of transcription from RNA polymerase II promoter; acute-phase response; glucose homeostasis; regulation of transcription, DNA-dependent; sexual reproduction; signal transduction; JAK-STAT cascade; transcription; thermoregulation; intracellular signaling cascade; eating behavior
NM_018760.1	Slc4a4	1.51	4.38E-02	sodium ion transport; bicarbonate transport; transport; regulation of pH; ion transport
NM_008634.1	Mtap1b	1.51	2.34E-02	microtubule-based process; microtubule bundle formation
	6430503K07Rik	1.51	1.77E-02	

Table AI-1 Gene list with increased expression in Sp4 hypomorphic mouse cerebellum (continued)

	A330021D07Rik	1.50	4.22E-02
XM_001479474.1	R3hcc1	1.50	2.16E-02

Table AI-2 Gene list with decreased expression in Sp4 hypomorphic mouse cerebellum

REFSEQ ID	Gene SYMBOL	Fold change	P Value	ONTOLOGY PROCESS
NM_173442.2	Gcnt1	-5.57	2.53E-05	
NM_011124.4	Ccl21b	-4.95	1.82E-02	immune response; lymph node development; chemotaxis; induction of positive chemotaxis; inflammatory response
NM_175684.4	Nwk2	-4.15	1.84E-04	endocytosis
NM_023052.1	Ccl21c	-3.91	1.49E-02	
NM_145401.1	Prkag2	-3.38	1.46E-02	lipid biosynthesis; response to stress
	Znf24	-3.33	8.16E-03	
	Comp	-3.28	3.78E-05	
NM_178914.3	Spata7	-3.18	2.53E-02	
XM_001473258.1	LOC100041504	-3.16	3.17E-02	
NM_053272.2	Dhcr24	-2.73	1.84E-02	skin development; sterol metabolism; tissue development; membrane organization and biogenesis; cholesterol biosynthesis; protein localization; amyloid precursor protein catabolism; cholesterol metabolism; electron transport
	1110067B18Rik	-2.63	2.36E-04	
NM_028841.2	Tspan17	-2.55	2.95E-04	
NM_021559.2	Zfp191	-2.44	2.82E-02	transcription
NM_145826.4	Il17re	-2.35	3.00E-03	
NM_175095.4	Commd2	-2.28	7.94E-03	
NM_145586.1	Tmem159	-2.28	3.56E-05	
NM_019511.1	Ramp3	-2.27	6.21E-04	transport; regulation of G-protein coupled receptor protein signaling pathway; intracellular protein transport; G-protein coupled receptor protein signaling pathway
NM_028404.1	Top1mt	-2.23	8.88E-04	DNA topological change
NM_145511.1	BC003331	-2.18	2.20E-03	
NM_011503.2	Stxbp2	-2.13	1.22E-03	
NM_019563.2	Cited4	-2.13	7.75E-05	transcription; regulation of transcription; positive regulation of transcription; regulation of transcription, DNA-dependent
NM_134138.1	Psmg2	-2.13	4.59E-03	regulation of apoptosis; mitotic spindle checkpoint; apoptosis
NM_009073.3	Rom1	-2.12	9.25E-04	visual perception; cell adhesion
NM_026728.1	Echdc2	-2.12	1.35E-02	metabolism
XM_913925.2	LOC638050	-2.11	3.62E-02	

Table AI-2 Gene list with decreased expression in Sp4 hypomorphic mouse cerebellum (continued)

NM_007652.2	Cd59a	-2.09	1.64E-02	
NM_016851.2	Irf6	-2.08	5.22E-04	regulation of transcription, DNA-dependent; transcription
NM_009239.2	Sp4	-2.08	7.60E-04	transcription; regulation of transcription, DNA-dependent
NM_007662.2	Cdh15	-2.06	6.73E-03	homophilic cell adhesion; cell adhesion
XM_913511.2	1810013D10Rik	-2.05	7.78E-04	
NM_010742.1	Ly6d	-2.05	7.59E-03	defense response; cell surface receptor linked signal transduction
NM_007558.1	Bmp8a	-2.00	1.85E-03	transforming growth factor beta receptor signaling pathway; development; growth; cell differentiation; ossification; spermatogenesis; cartilage development
NM_199197.1	1110032A13Rik	-1.98	4.41E-04	
NM_175558.3	Zfp446	-1.97	1.20E-02	
NM_030176.2	Spata2L	-1.97	9.29E-04	
NM_010319.3	Gng7	-1.96	2.18E-04	behavioral fear response; receptor guanylyl cyclase signaling pathway; G-protein coupled receptor protein signaling pathway; locomotory behavior; signal transduction
NM_015734.1	Col5a1	-1.95	4.64E-02	phosphate transport; cell adhesion
	1500035N22Rik	-1.95	2.72E-02	
NM_010471.2	Hpca	-1.95	1.89E-04	
	Rrm1	-1.94	4.70E-02	
NM_001018087.1	Ldoc1	-1.90	1.34E-02	
	1300011L04Rik	-1.88	1.14E-03	
XM_990406.1	EG667410	-1.88	1.25E-02	
NM_153543.1	Aldh1l2	-1.85	1.06E-03	10-formyltetrahydrofolate catabolism; biosynthesis; protein biosynthesis; one-carbon compound metabolism
NM_019924.1	Rps6ka4	-1.85	4.02E-03	protein kinase cascade; regulation of transcription, DNA-dependent; protein amino acid phosphorylation
NM_178045.3	Rassf4	-1.84	1.68E-03	cell cycle; signal transduction
XM_001000961.1	5830417I10Rik	-1.84	8.91E-03	
NM_144862.3	Lims2	-1.83	6.80E-03	
NM_026671.1	Lypd2	-1.83	3.15E-02	
NM_144806.1	Prpsap2	-1.83	1.94E-03	nucleoside metabolism; nucleotide biosynthesis
NM_029609.1	2310007H09Rik	-1.83	4.00E-04	metabolism
	Rnf40	-1.82	3.15E-03	
NM_016846.3	Rgl1	-1.82	2.71E-03	small GTPase mediated signal transduction; regulation of small GTPase mediated signal transduction; signal transduction
AK007474.1	scl0003949.1_31	-1.81	9.29E-03	
NM_025910.2	Mina	-1.79	3.86E-04	regulation of cell proliferation

Table AI-2 Gene list with decreased expression in Sp4 hypomorphic mouse cerebellum (continued)

NM_153541.3	Zbtb8	-1.78	4.73E-03	transcription; regulation of transcription, DNA-dependent
NM_027275.3	Ptcd3	-1.77	2.64E-02	
NM_172760.2	Elmo3	-1.77	4.98E-03	apoptosis; phagocytosis
NM_016765.2	Ddah2	-1.77	2.70E-02	arginine catabolism
	6330444G18Rik	-1.77	4.53E-02	
NM_028055.3	1500005I02Rik	-1.76	8.51E-04	
NM_054089.3	Tgs1	-1.76	1.14E-02	
NM_025623.2	Nipsnap3a	-1.75	1.58E-02	
NM_011379.3	Sipa1	-1.73	9.36E-04	negative regulation of cell adhesion; negative regulation of cell growth; induction of apoptosis; intracellular signaling cascade; cytoskeleton organization and biogenesis
NM_007878.2	Drd4	-1.72	2.28E-03	G-protein coupled receptor protein signaling pathway; signal transduction
NM_176987.3	4732471D19Rik	-1.72	2.36E-03	
NM_010822.2	Mpg	-1.72	7.76E-03	base-excision repair; DNA repair; response to DNA damage stimulus
NM_177564.4	BC022224	-1.71	1.64E-02	metabolism
NM_025882.3	Pole4	-1.71	3.87E-02	
NM_026855.3	Arv1	-1.70	1.03E-03	sphingolipid metabolism; lipid metabolism
NM_022325.3	Ctsz	-1.70	3.14E-02	
NM_026260.2	Tctn3	-1.69	5.36E-03	
	B430109P06Rik	-1.69	6.94E-03	
NM_145078.1	2610305D13Rik	-1.67	2.78E-02	regulation of transcription, DNA-dependent
NM_025912.3	2010011I20Rik	-1.67	5.72E-03	
NM_181848.3	Optn	-1.67	6.83E-03	
NM_172277.2	Snx8	-1.67	2.73E-03	transport; protein transport
NR_003107.1	LOC732482	-1.66	1.84E-03	
XM_129443.7	Ppfia4	-1.66	3.30E-02	
NM_001013808.1	LOC433801	-1.66	4.60E-02	
NM_013689.4	Tec	-1.65	1.07E-02	intracellular signaling cascade; protein amino acid phosphorylation
NM_033444.1	Clic1	-1.65	1.78E-03	
	C920004C08Rik	-1.64	2.14E-03	
NM_001080780.1	Ret	-1.64	1.72E-03	morphogenesis; cellular physiological process; neuron maturation; ureteric bud development; neural crest cell migration; homophilic cell adhesion; MAPKKK cascade; protein amino acid phosphorylation; embryonic epithelial tube formation
NM_133943.2	Hsd3b7	-1.64	1.45E-03	steroid biosynthesis; C21-steroid hormone biosynthesis
NM_013738.1	Plek2	-1.64	1.02E-02	intracellular signaling cascade

Table AI-2 Gene list with decreased expression in Sp4 hypomorphic mouse cerebellum (continued)

NM_010010.1	Cyp46a1	-1.63	3.22E-03	cholesterol metabolism; lipid metabolism; steroid metabolism; electron transport
NM_008227.1	Hcn3	-1.63	1.13E-03	transport; potassium ion transport; sodium ion transport
NM_011328.2	Sct	-1.63	3.27E-03	
NM_001081115.1	1190020J12Rik	-1.63	1.56E-02	protein catabolism
NM_053117.2	Pard6g	-1.62	3.52E-03	cell cycle; cell division
NM_145923.4	Rell1	-1.62	6.47E-03	
NM_026860.1	1190003M12Rik	-1.61	5.26E-03	
XM_907756.3	LOC633360	-1.61	3.34E-03	
NM_016865.2	Htatip2	-1.60	1.44E-02	positive regulation of transcription from RNA polymerase II promoter, mitotic; apoptosis; regulation of angiogenesis; regulation of apoptosis; cell cycle; nuclear import; angiogenesis; cell differentiation; positive regulation of programmed cell death; development
NM_008690.3	Nfkbie	-1.60	8.07E-03	
NM_025584.1	2410026K10Rik	-1.60	3.25E-03	
NM_022880.1	Slc29a1	-1.59	3.00E-03	nucleoside transport
NM_023053.2	Twsg1	-1.59	1.41E-02	tissue development;negative regulation of BMP signaling pathway; embryonic development; ossification; cell differentiation; forebrain development; eye development (sensu Mammalia) ; negative regulation of osteoblast differentiation; mesoderm formation; hemopoiesis; BMP signaling pathway
	Emilin2	-1.59	4.16E-02	
NM_172866.3	Rgp1	-1.59	1.67E-02	
NM_173738.2	BC027344	-1.59	4.89E-02	regulation of transcription, DNA-dependent
	BC065120	-1.59	2.33E-02	
NM_144923.2	Blvrb	-1.59	9.53E-03	
NM_172727.2	D330028D13Rik	-1.58	8.85E-03	
NM_030210.1	Aacs	-1.58	3.64E-03	
NM_011656.2	Tuft1	-1.58	4.12E-03	ossification; odontogenesis; bone mineralization
NM_008332.2	Ifit2	-1.58	3.60E-02	immune response
NM_016896.3	Map3k14	-1.58	1.81E-02	protein amino acid phosphorylation
NM_176999.3	Atp10b	-1.58	3.09E-02	cation transport
NM_175498.3	Pnma2	-1.57	2.05E-03	
	Cd200	-1.57	8.37E-03	
NM_172742.2	Mtmr10	-1.57	5.53E-03	phospholipid dephosphorylation
NM_175360.2	Obfc1	-1.57	1.76E-02	
NM_172288.1	Nup133	-1.56	1.24E-02	transport; protein transport; RNA transport
NM_175033.3	BC089491	-1.56	2.78E-03	cell redox homeostasis

Table AI-2 Gene list with decreased expression in Sp4 hypomorphic mouse cerebellum (continued)

NM_033314.2	Slco2a1	-1.56	1.65E-02	prostaglandin transport
NM_010511.2	Ifngr1	-1.56	1.33E-02	
NM_016718.2	Ninj2	-1.55	2.21E-02	tissue regeneration; cell adhesion
NM_008723.1	Npm3	-1.55	2.08E-02	protein folding; rRNA processing; rRNA transcription
NM_001039387.1	Nelf	-1.55	7.10E-03	
NM_011183.1	Psen2	-1.55	1.32E-02	cell fate specification; positive regulation of apoptosis; Notch receptor processing; Notch signaling pathway; amyloid precursor protein catabolism; anagen; membrane protein ectodomain proteolysis; positive regulation of enzyme activity; embryonic limb morphogenesis; intracellular signaling cascade; protein processing
NM_001033439.2	Lrch1	-1.55	2.42E-02	electron transport
NM_019686.3	Cib2	-1.55	2.91E-03	
NM_201368.1	Xkr8	-1.55	4.46E-02	
	Anxa7	-1.55	5.99E-03	
NM_026010.1	2610209A20Rik	-1.55	2.24E-02	
NM_009029.1	Rb1	-1.55	2.33E-02	negative regulation of transcription, DNA-dependent; regulation of transcription, DNA-dependent; striated muscle cell differentiation; cell cycle; cell division; G1/S transition of mitotic cell cycle; positive regulation of transcription from RNA polymerase II promoter; transcription; negative regulation of cell proliferation; negative regulation of transcription from RNA polymerase II promoter; cell cycle arrest
NM_009113.3	S100a13	-1.55	3.48E-02	
	9630007E23Rik	-1.54	4.31E-03	
NM_008990.2	Pvrl2	-1.54	1.73E-02	cell adhesion; homophilic cell adhesion
NM_008124.2	Gjb1	-1.54	3.88E-02	cell communication; cell-cell signaling
NM_173401.2	Fbxo44	-1.54	1.20E-02	ubiquitin cycle; protein catabolism
NM_026681.4	Ccdc88c	-1.54	1.48E-02	
NM_007452.1	Prdx3	-1.53	1.89E-02	
NM_016908.2	Syt5	-1.53	2.78E-02	transport
NM_054054.2	Brdt	-1.53	5.93E-03	regulation of transcription, DNA-dependent; chromatin remodeling; transcription; lipid transport
	D330025A21Rik	-1.53	1.01E-02	
NM_175092.3	Rhof	-1.52	2.92E-02	
NM_173751.3	Ilvbl	-1.52	4.68E-03	
NM_019731.1	Nme4	-1.52	2.67E-03	UTP biosynthesis; nucleotide metabolism; CTP biosynthesis; GTP biosynthesis
NM_183180.1	Tspan18	-1.52	2.29E-02	

Table AI-2 Gene list with decreased expression in Sp4 hypomorphic mouse cerebellum (continued)

NM_178673.3	Fstl5	-1.52	3.59E-03	
NM_011676.2	Unc119	-1.52	1.58E-02	visual perception
NM_010165.2	Eya2	-1.52	1.32E-02	metabolism; transcription; apoptotic program; regulation of transcription, DNA-dependent; development; protein amino acid dephosphorylation
XM_001476117.1	5430432N15Rik	-1.52	8.14E-03	
NM_001083936.1	Pthr1	-1.52	6.33E-03	skeletal development; ossification; calcium ion homeostasis; cell maturation; signal transduction; bone resorption; negative regulation of cell proliferation; G-protein signaling, coupled to IP3 second messenger (phospholipase C activating); bone mineralization; G-protein coupled receptor protein signaling pathway; positive regulation of cell proliferation; G-protein signaling, adenylate cyclase activating pathway
NM_030211.1	Kctd18	-1.52	1.12E-02	
NM_031874.4	Rab3d	-1.51	3.65E-02	transport; protein transport; regulation of exocytosis; small GTPase mediated signal transduction; exocytosis
NM_170702.2	Cd40	-1.51	2.48E-03	immune response; apoptosis; regulation of immune response; positive regulation of isotype switching to IgG isotypes; regulation of immunoglobulin secretion; positive regulation of B cell proliferation; signal transduction
NM_009106.1	Rtkn	-1.51	4.44E-02	Rho protein signal transduction; apoptosis; regulation of anti-apoptosis
NM_198113.2	Ssh3	-1.51	2.19E-02	protein amino acid dephosphorylation
NM_028015.2	Lass5	-1.51	2.79E-03	lipid biosynthesis; regulation of transcription; regulation of transcription, DNA-dependent
NM_011396.2	Slc22a5	-1.51	3.82E-03	ion transport; transport; sodium ion transport
XM_355366.1	5730407K14Rik	-1.51	2.02E-02	
NM_008702.2	Nlk	-1.51	1.42E-02	protein amino acid phosphorylation; transcription; negative regulation of Wnt receptor signaling pathway; protein kinase cascade; regulation of transcription, DNA-dependent
NM_027564.3	4921507P07Rik	-1.51	2.75E-03	
	Slc30a3	-1.50	9.64E-03	
	Cyp11a1	-1.50	1.48E-02	
NM_013749.1	Tnfrsf12a	-1.50	1.31E-02	angiogenesis; apoptosis; cell adhesion; cell death; cell differentiation; positive regulation of axon extension; development
NM_010807.3	Marcksl1	-1.50	4.00E-03	

Appendix II Domain mapping of Sp4

Sp4 activates the transcription of some target genes, such as TrkC. At the same time, it represses other target genes, such as NT3. We hypothesize there are activation domains and inhibitory domains existing in Sp4 protein. Sp4 might have different effects at different target genes because these functional domains could recruit distinct transcription machineries within the context of specific promoter sequences. The dual functions of Sp4 might also be mediated by post-translational modifications of these functional domains.

In the Sp family, Sp4 is closely related to Sp1 and Sp3 by high degree of primary sequence. In addition to the highly conserved C2H2-type zinc finger DNA binding domain close to the C terminus, all three proteins contain two glutamine-rich regions and two serine/threonine-rich regions in the N-terminal part of the molecule.

For Sp1, the glutamine-rich domains have been identified as activation domains [144]. A domain located adjacent to C-terminal side of Zinc finger region plays a key role in mediating the ability of Sp1 to activate transcription synergistically [145]. The N-terminal inhibitory domain of Sp1 is involved in the protein-protein interactions with corepressors SMRT, NCoR and BCoR [71]. So we want to check whether the homologous domains in Sp4 have similar functions.

We used the Dual-Glo™ Luciferase Assay System (Promega) to determine functional domains in a Neuro-2a cell line. Neuro-2a is a neuroblastoma cell line from mouse, which has endogenous Sp4 expression. Sp4 can also be overexpressed in this cell line.

According to the alignment with Sp1, we constructed 6 N-terminal deletion mutants without the zinc finger DNA binding domain, 4 C-terminal deletion mutants and a middle

fragment deletion mutant, using human Sp4 cDNA as template and pSG424 as vector. The Gal4 DNA binding domain was fused to deletion mutants. The expression level and subcellular localization of deletion mutants were tested by subcellular fractionation and Western Blotting with anti-Gal4 DBD antibody (Figure AII-1). The expression levels of Sp4 deletion mutants were comparable and they had the right subcellular localization in nuclei.

pG5-luc vector have 5 Gal4 DNA binding sites upstream of firefly luciferase reporter. pG5-luc has low basal luciferase activity, and it was used to determine the transcriptional activation potential of each deletion mutant. Experimental results are showed in Figure AII-2. We concluded that the Sp4 494-610 region contain a transactivation domain

In order to test the activation domain in endogenous condition, we chose p35-luc as a reporter. p35 is known be to an activation target of Sp4 [15]. p35-luc reporter contains p35 promoter sequence upstream of the firefly luciferase gene.We constructed 8 Sp4 deletion mutants with the DNA binding zinc finger domains that were N-terminally tagged with 3xFlag. The expression level and subcellular localization was determined by immunoblotting with anti-Flag antibody (data not shown). Experimental results are shown in FigureAII-3. 3xFlag-Sp4 (494-784) has higher activity than other N-terminal deletion mutants, which may also confirm that the 494-610 region contains an important activation domain.

In summary, Sp4 (494-610) may contain an important activation domain.

Similarily, we tried to determine the inhibitory domain of Sp4 by Gal4DBD-Sp4 deletion mutants on TK-G4-luc reporter (with high basal luciferase activity) and 3xFlag-

Sp4 deletion mutants on NT3-luc reporter (NT3 is a repression target of Sp4 [79].). There was no specific region defined for inhibitory domain.

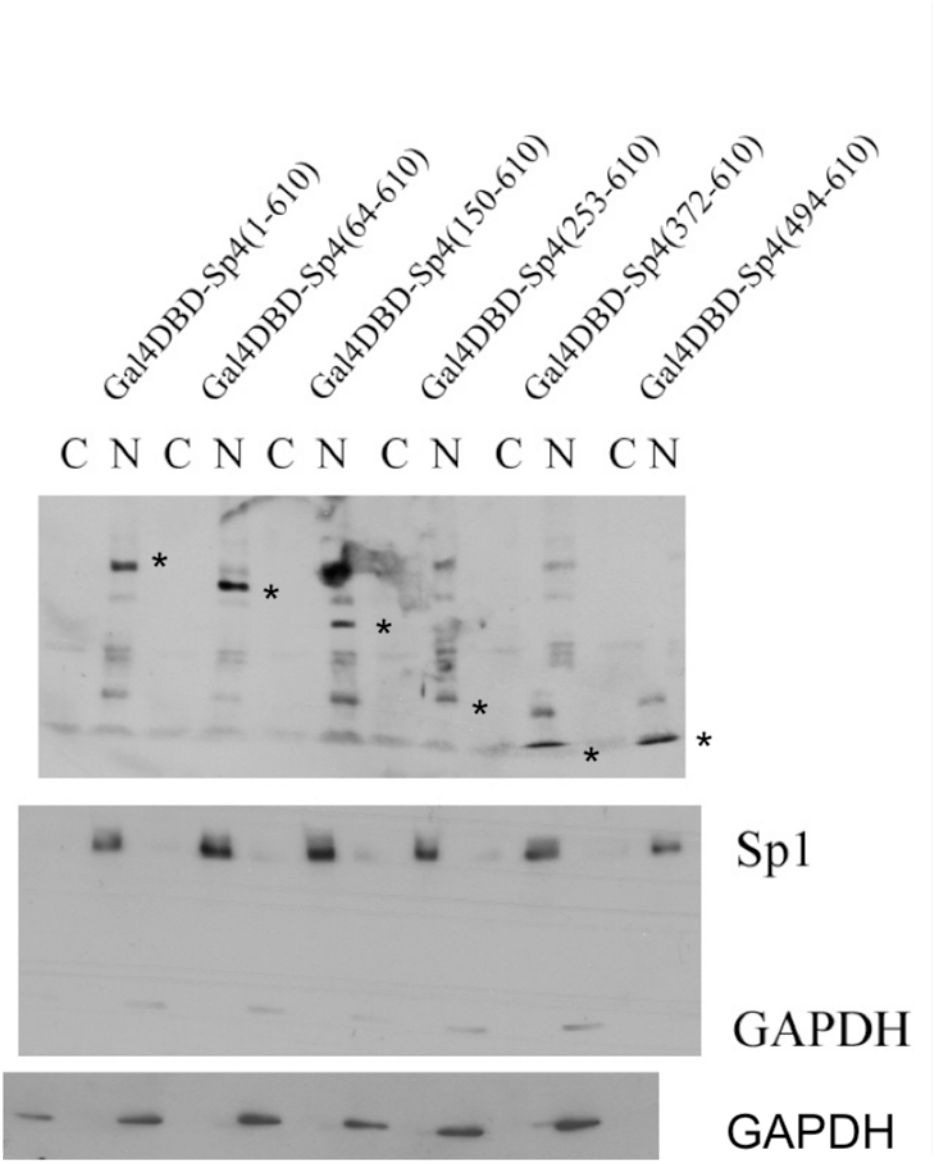

Figure AII-1 Subcellular localization of Gal4DBD-fused Sp4 deletions in N2A cells.

Neuro2a cells were transfected with expression plasmids of Gal4DBD-Sp4 deletion mutants. Protein lysates from cells were immunoblotted for Gal4DBD (mouse monoclonal from Santa Cruz), Sp1(Rabbit polyconal from Upstate) and GAPDH 48 hours after transfection. The upper panel is the blotting for Gal4DBD. The lower panel is the blotting for Sp1 and GAPDH. Sp1 works as a nucleus marker, and GAPDH works as a cytoplasm marker (c: cytoplasm; N: nucleus). Asterisks (*) are next to the specific bands.

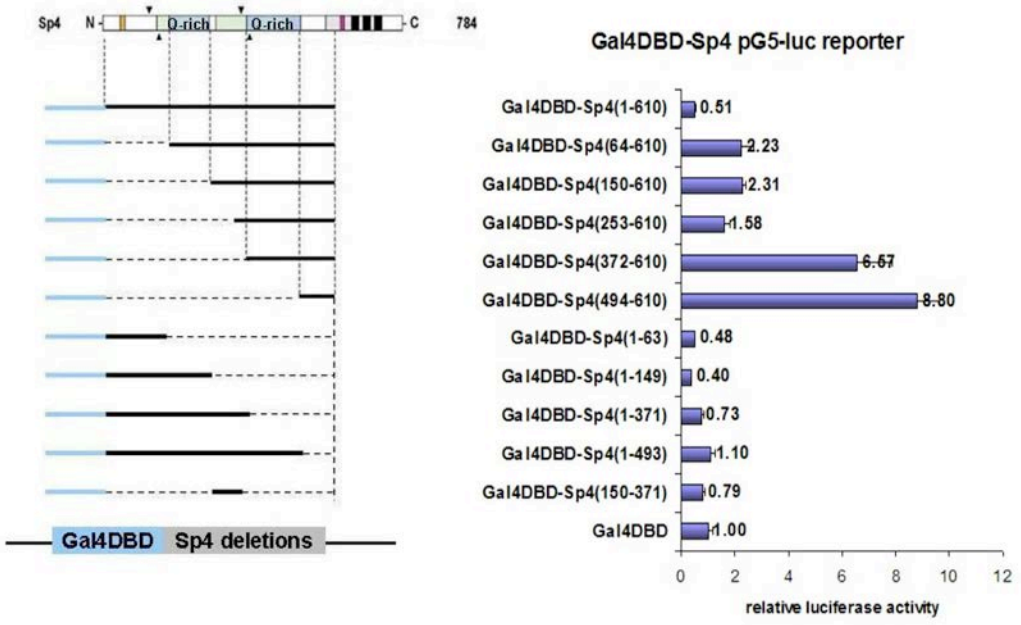

Figure AII-2 Transcription activity of Gal4DBD-Sp4 deletion mutants on pG5-luc reporter.

11 deletion mutants of Sp4 without DNA binding domain were fused with Gal4 DBD. Neuro2A cells were transiently cotransfected with expression plasmids of Gal4DBD-Sp4 deletion mutants, reporter pG5-luc plasmid and renilla luciferase plasmid. 48 hours after transfection, cell extracts were analyzed for reporter firefly luciferase activities which were normalized with renilla luciferase acitivity. Relative luciferase activity of only Gal4DBD was set as 1. Each sample was done in triplicate, and the experiments have been repeated twice.

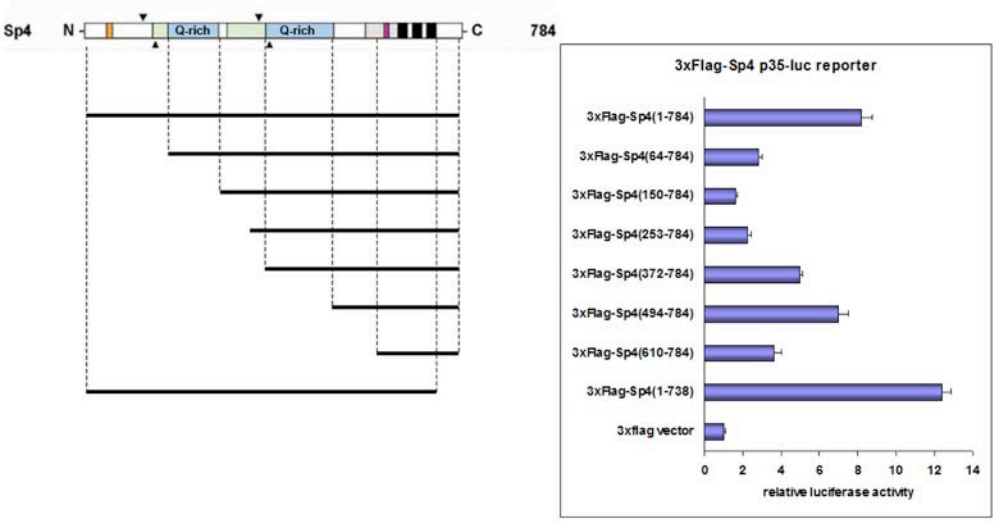

Figure AII-3 Transcription activity of 3xFlag-Sp4 deletion mutants on p35-luc reporter.

8 deletion mutants of Sp4 with zinc-finger DNA binding domain were tagged with N-terminal 3xFlag. Neuro2A cells were transiently cotransfected with expression plasmids of 3xFlag tagged Sp4 deletion mutants, reporter p35-luc plasmid and renilla luciferase plasmid. 48 hours after transfection, cell extracts were analyzed for reporter firefly luciferase activities, which were normalized with renilla luciferase acitivity. Relative luciferase activity of 3xFlag vector was set as 1. Each sample was done in triplicate, and the experiments have been repeated twice.

Appendix III Gross anatomy and histological analysis of Sp4 hypomorphic mice.

We observed the gross cerebellum structure by H&E staining at postnatal Day7, 11,21. We stained the Purkinje cells and astrocytes in postnatal Day7,11,21. There were no big difference between WT and Sp4 hypomorphic mice.

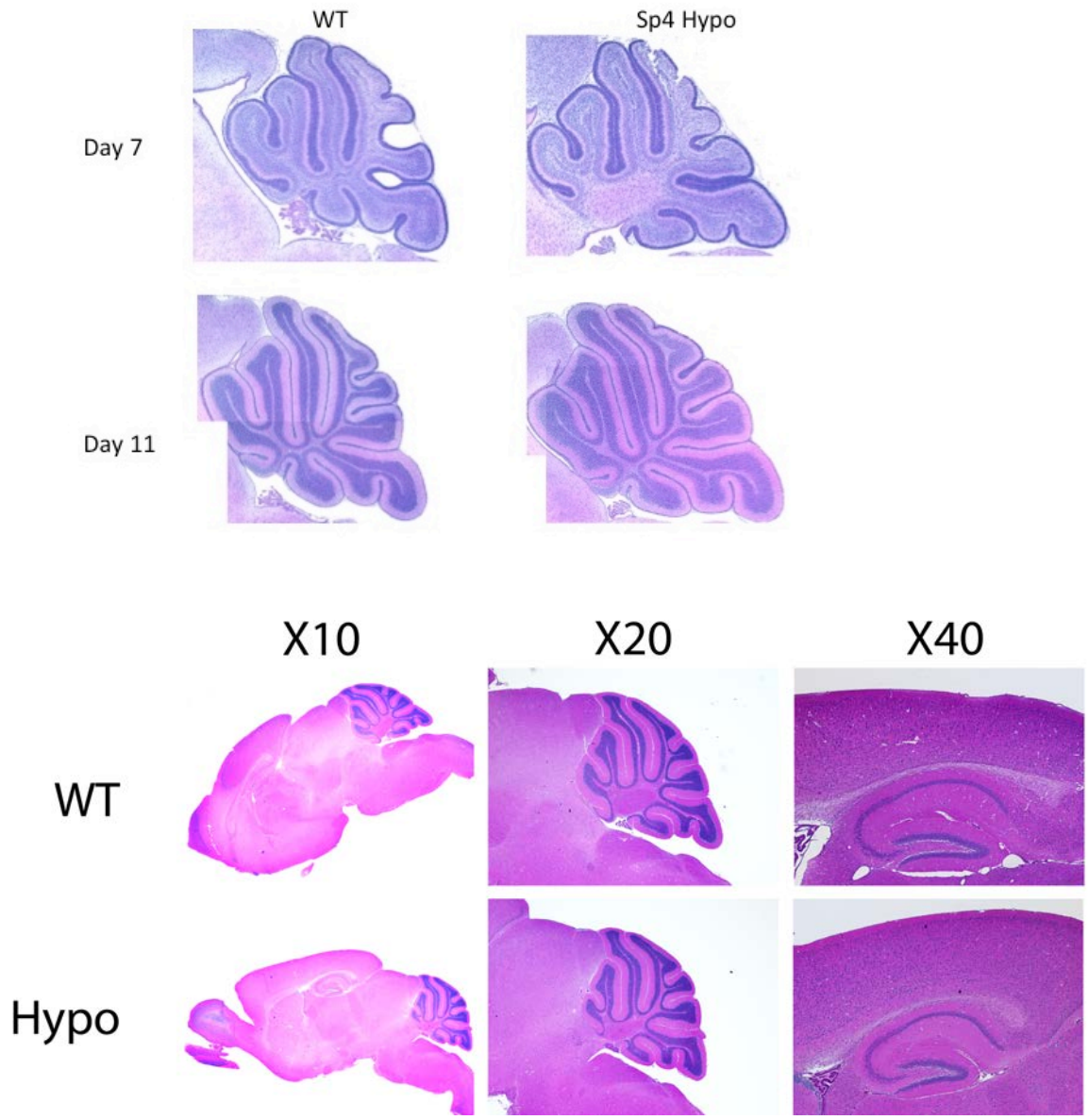

Figure AIII-1 Brain structure of Sp4 WT and hypomorphic mice at Day 7, Day11, and Day 21.

Paraffin sections from Day7, Day11 (upper panel) and Day21 (lower panel) mouse brain were stained with H&E.

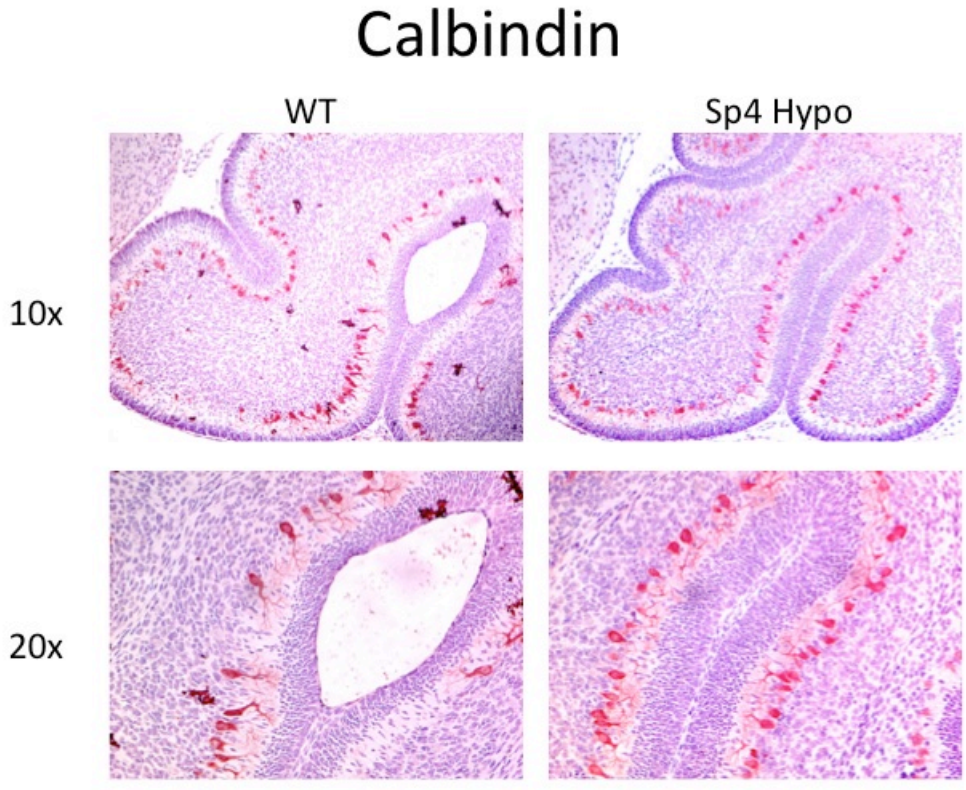

Figure AIII-2 Purkinje cells in Day 7 mouse cerebellum.

Paraffin sections from Day 7 mouse cerebellum were stained with anti-Calbindin antibody (mouse monoclonal from Sigma)(red signal), a Purkinje cell marker. The slides were conterstained with hematoxylin.

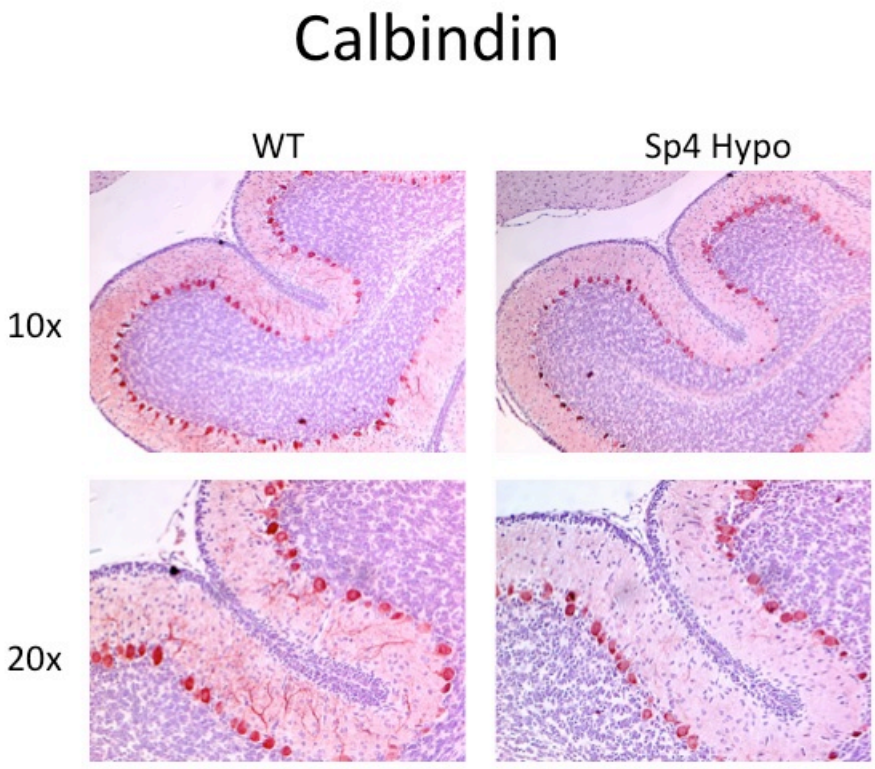

Figure AIII-3 Purkinje cells in Day 11 mouse cerebellum.

Paraffin sections from Day 11 mouse cerebellum were stained with anti-Calbindin antibody (red signal), a Purkinje cell marker. The slides were conterstained with hematoxylin.

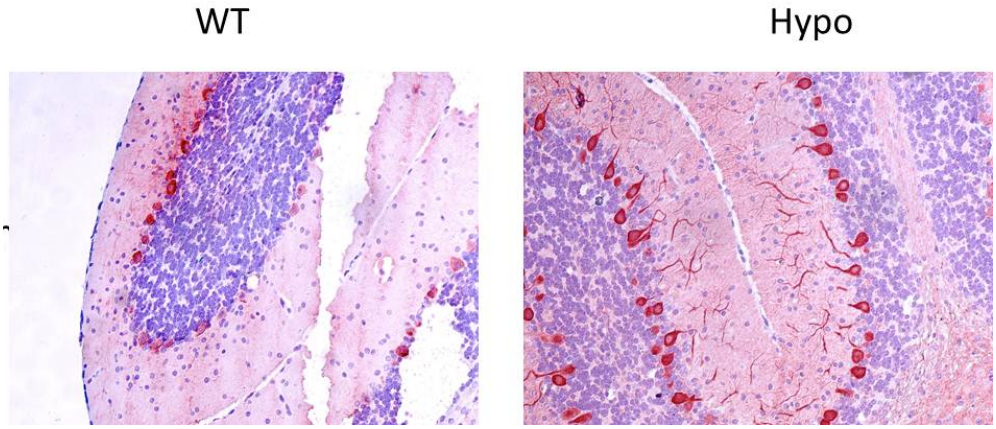

Figure AIII-4 Purkinje cells in Day 21 mouse cerellum.

Paraffin sections from Day 21 mouse cerebellum were stained with anti-Calbindin antibody (red signal), a Purkinje cell marker. The slides were conterstained with hematoxylin. The objective len of microscope is 20X.

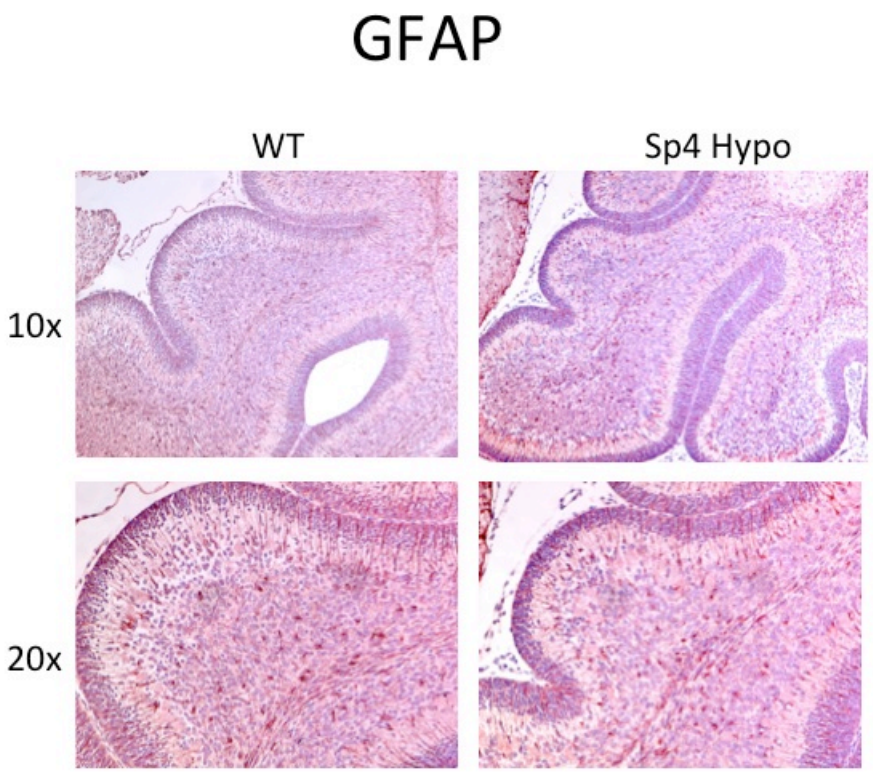

Figure AIII-5 Astrocytes in Day 7 mouse cerebellum.

Paraffin sections from Day 7 mouse cerebellum were stained with anti-GFAP antibody (rabbit polyclonal from DAKO) (red signal), an astrocyte marker. The slides were conterstained with hematoxylin.

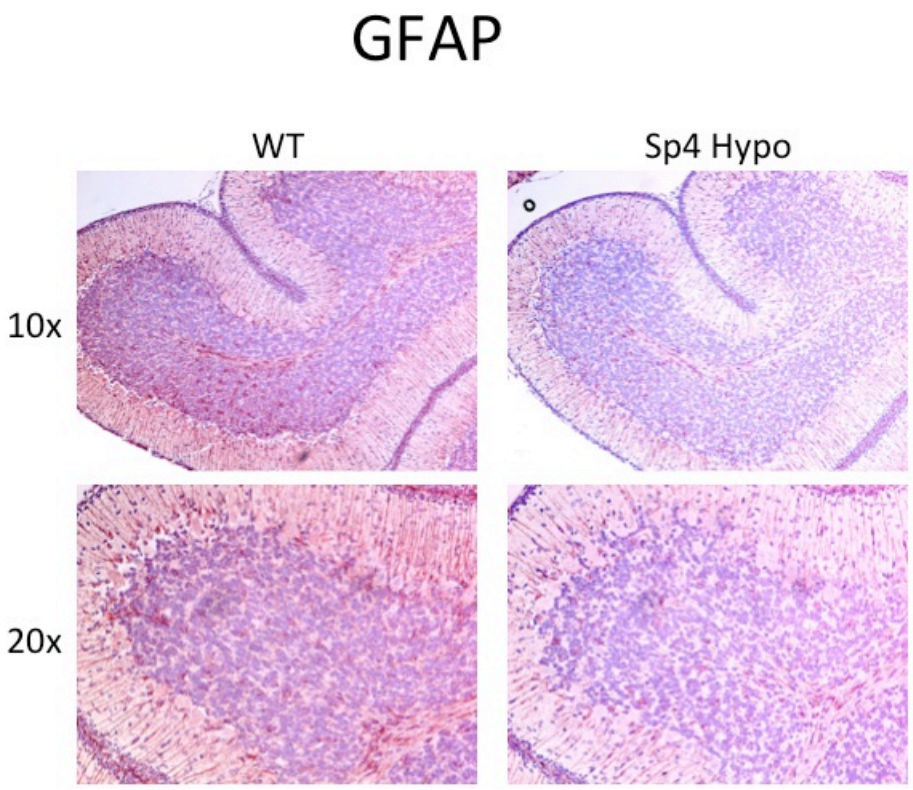

Figure AIII-6 Astrocytes in Day 11 mouse cerebellum.

Paraffin sections from Day 11 mouse cerebellum were stained with anti-GFAP antibody (red signal), an astrocyte marker. The slides were conterstained with hematoxylin.

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