THE FUNCTION OF NEUROFIBROMATOSIS TYPE 1 EXON 23A ALTERNATIVE SPlicing IN RAS/ERK SIGNALING AND LEARNING BEHAVIORS IN MICE

BY

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Dedication

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ABBREVIATIONS

• Akt: a serine/threonine-specific protein kinase, also known as Protein kinase B (PKB)
• ANOVA: analysis of variance
• CA1: the first out of four total sub-regions of the hippocampus
• CA3: the third out of four total sub-regions of the hippocampus
• DBC1: Deleted in breast cancer
• CNS: central nervous system
• CS: conditioned stimulus
• ES cells: embryonic stem cells
• G protein: a family of guanine nucleotide-binding proteins which transmits signals from stimuli outside a cell to its interior.
• GAP: GTPase-activating proteins
• GEF: guanine exchange factors
• GRD: GAP-related domain
• GST-RBD: GST-tagged Raf1 Ras binding domain
• ERK: extracellular signal regulated kinase
• LTP: long-term potentiation
• MAPK: mitogen-activated protein kinase
• Nf1: neurofibromatosis type 1
• ISI: inter-stimulus-interval
• Ras-GTP: Ras is bound to guanosine triphosphate, activating Ras.
• Ras-GDP: Ras is bound to guanosine diphosphate, inactivating Ras.
• **RasGRF1**: Ras-specific guanine nucleotide-releasing factor 1, which is one of the GEF proteins

• **Raf/MEK/ERK1/2**: These are target molecules belonging to one of the signaling pathways downstream of Ras.

• **PI3K/Akt/mTOR**: These are target molecules belonging to one of the signaling pathways downstream of Ras.

• **PCR**: Polymerase Chain Reaction

• **PTSD**: Posttraumatic stress disorder

• **RT-PCR**: Reverse Transcription-Polymerase Chain Reaction

• **S6**: a protein component of the 40S ribosomal subunit

• **SEM**: standard error of the mean

• **SynGAP**: a Ras GTPase-activating protein

• **shRNA**: a short hairpin RNA or small hairpin RNA

• **US**: unconditioned stimulus
The Function of Neurofibromatosis Type 1 Exon 23a Alternative Splicing in Ras/ERK signaling and learning behaviors in mice

Abstract

By

HIEU THI NGUYEN

Ras is a key regulator of multiple signaling networks controlling a variety of important biological processes such as cell proliferation, survival, differentiation, and migration. Interestingly, Ras also plays a critical role in the regulation of proper learning and memory.

Neurofibromin is one of the most common and well-studied negative regulator of RasGAPs, which inhibits Ras activity. Neurofibromin is the protein product of neurofibromatosis type 1 (NF1) gene, which inactivates the proto-oncoprotein Ras through its GTPase activating protein domain. Mutations on the NF1 gene will cause neurofibromatosis type 1 disease, which is a common human genetic disorder characterized by phenotypes ranging from increased tumor susceptibility to learning disabilities. NF1 alternative exon 23a lies in the center of the GAP domain and is skipped in brain but included in other tissues.

To study the molecular and biological functions of this regulated splicing event in vivo, we generated mutant mice (Nf1<sup>23aIN/23aIN</sup> mice) with constitutive Nf1 exon 23a inclusion in all tissues, and found increased Ras activation in their brains. In addition, the brain of Nf1<sup>23aIN/23aIN</sup> mice also has elevated levels of phosphorylated ERK1/2 downstream of Ras. Notably, Nf1<sup>23aIN/23aIN</sup> mice show
deficiencies in both short- and long-term spatial learning, as well as in fear associative learning.

Taken together, these results strongly suggest that the regulated inclusion of *Nf1* exon 23a modulates Ras signaling, and is important for proper learning and memory in mice. Therefore, our knock-in mouse is a very helpful tool for future studies on the role of alternative splicing in Ras/ERK signaling and in learning and memory.
CHAPTER 1 - Background and significance

1.1 Introduction

Alternative splicing is a crucial gene expression control mechanism that increases protein diversity by producing different mRNA isoforms from the same pre-mRNA. These mRNA isoforms can subsequently be translated into proteins that carry distinct functions. Alternative splicing has several forms, such as alternative 3’ or 5’ splice site usage, intron retention, mutually exclusive exons, and cassette exons. Exactly how isoforms differ in function is not well understood for most alternative splicing events. In my thesis research, I studied the functions of two distinct neurofibromatosis type 1 (NF1) isoforms generated by alternative splicing of a cassette exon, 23a. Specifically, NF1 alternative exon 23a is predominantly skipped in neurons but included in other cell types.

NF1 is well known as a negative regulator of proto-oncoprotein Ras through its GTPase activating protein (GAP) domain. Mutations on the NF1 gene will cause NF1 disease, which is a common human genetic disorder characterized by phenotypes ranging from increased tumor susceptibility to learning disabilities. Notably, patients carrying a missense mutation that specifically eliminate the NF1 Ras-GAP activity display learning impairments. Ras was shown to play important roles in cognitive behaviors as pharmacological intervention of Ras expression and its downstream signaling targets disrupts learning and memory in mice. NF1 alternative exon 23a lies in the middle of its Ras-regulatory domain (GAP) domain, suggesting that differential expression of the two isoforms modulates Ras activity and is involved in learning functions.
The inclusion of exon 23a is tightly regulated in a tissue- and development stage-specific manner, which is indicative of functional importance of the expression of this exon. Indeed, we showed that the alternative splicing of Nf1 exon 23a is a key regulator of Ras activity in cell models of ES cells and ES-derived neurons.

Using mutant ES cells from our previous study, we generated knock-in mouse model in which exon 23a is constantly included in all tissue types. The goal of my thesis research was to investigate the role of alternative splicing of Nf1 exon 23a in Ras/ERK activity in vivo and in the cognitive behaviors using our mutant mouse model.

1.2 Ras and its signaling pathways

1.2.1 Ras

Ras proteins are a family of small GTPases that bind to and catalyze guanosine nucleotide hydrolysis. They are signaling molecules that play essential roles in multiple signal transduction cascades to regulate many biological processes such as cell proliferation, survival, differentiation, and migration.

The Ras protein family has three major members, including Ras (H-Ras, N-Ras, and K-Ras), Rap1 (also known as Krev-1), and Rap2. They are monomeric proteins, and about 20 kDa in size. They were first identified as oncogenes. H-Ras, N-Ras, and K-Ras are 3 canonical members of the Ras protein family, which were identified over 30 years ago due to their frequent oncogenic activation in human cancer. H-ras, N-ras, and K-ras genes are
expressed in most tissues and widely conserved across species (Barbacid 1987) (Konstantinopoulos et al. 2007).

The activity of Ras is regulated by its guanine nucleotide-binding state. Ras constantly cycles between its active conformation (GTP-bound state) and its inactive state (GDP binding). The cycling of Ras is regulated by guanine exchange factors (GEFs) and GTPase-activating proteins (GAPs) that antagonize the functions of one another (Bernards et al. 2004) (Bos et al. 2007) (Overbeck et al. 1995). GEFs activate Ras by removing GDP so that GTP can bind. In a reverse mode, GAPs increase the rate of GTP hydrolysis of Ras, resulting in the reduction of its activity (Figure 1.1).
Figure 1.1 The cycling of Ras family proteins between inactive and active states. GAP, GTPase activating protein, down-regulates Ras signaling by enhancing the rate at which Ras-GTP is converted into Ras-GDP. In contrast, GEF, guanosine nucleotide exchange factor, activates Ras by promoting the dissociation of GDP from Ras, which facilitates the exchange of GDP for GTP. Active Ras-GTP signals through several molecular pathways to regulate multiple cellular processes.
1.2.2 Ras signaling pathways

There are two main Ras signaling cascades: the Raf/MEK/ERK1/2 pathway (also known as the MAP kinase pathway) and the phosphatidylinositol 3’-kinase (PI3K)/Akt/mTOR pathway (Figure 1.2) (Le et al. 2007, Gutmann et al. 2012, Diggs-Andrews et al. 2013). In each pathway, Ras-GTP binds to and activates a kinase (Raf or PI3K), which then triggers a cascade of kinases, eventually leading to changes in gene expression in the nucleus.

The well-studied downstream signaling pathway of Ras family proteins is the Mitogen-Activated Protein Kinase (MAPK) cascade, mainly Extracellular signal-Regulated Kinase 1/2 (ERK) of the MAPK family (Figure 1.2). Once stimulated, Ras directly binds to the regulatory domain of Raf, leading to its catalytic domain being exposed for subsequent phosphorylation modifications at multiple amino acids for complete activation (Langecarter et al. 1994) (Wood et al. 1992). Thus, Ras family proteins are required for Raf activation by either tethering Raf to the membrane compartment where other cofactors are present or unfolding Raf for further modifications (Langecarter et al. 1994) (Wood et al. 1992).

Another well-characterized effector of Ras is Phospho-inositide 3-kinase (PI3K), which regulates mTOR-dependent translation and cytoskeleton rearrangement during memory processing (Rodriguezviciana et al. 1994, Udo et al. 2005, Horwood et al. 2006, Orme et al. 2006). Ras has been shown to directly interact with the catalytic subunit of PI3K. This interaction consequently promotes the activity of PI3K and triggers its downstream signaling pathways (Sjolander et
al. 1991, Rodriguezviciana et al. 1994, RodriguezViciana et al. 1996, RodriguezViciana et al. 1996) (Figure 1.2).
Figure 1.2 Major downstream signaling cascades of Ras family proteins and their biological roles. The Raf/MEK/ERK1/2 and PI3K/Akt/mTOR cascades are the two most common Ras signaling pathways, regulating multiple biological processes. Adapted from Le and Parada, *Oncogene*, 2007 (Le et al. 2007).
1.3 Regulation of Ras and its signaling

1.3.1 GEF and GAP

As discussed in section 1.1.1, Ras, like a molecular switch, cycles between an active form with GTP bound and an inactive form with GDP bound. These two states are controlled by two slow reactions, GDP/GTP exchange and GTP hydrolysis. To become activated, GDP must be exchanged for GTP. Guanine nucleotide exchange factors (GEFs) catalyze this reaction to release tightly bound GDP, which is subsequently replaced by abundant cellular GTP. Only in the activated form does Ras interact with activate downstream targets to trigger cellular responses. GTP hydrolysis returns active Ras to their inactive state, thereby terminating downstream cascades. This intrinsic reaction is slow and can be accelerated by up to five orders of magnitude through interaction with GTPase activating proteins (GAPs) (Boguski et al. 1993) (Bernards et al. 2003). Therefore, GEFs and GAPs are crucial regulator of Ras activity.

GEFs and GAPs are large and multi-domain proteins. Many of these domains are protein or lipid interaction domains, which are important for their interactions with membrane lipids, or other proteins to target them to specific intracellular compartments (Ye et al. 2010). Therefore, activation of a specific GEF or GAP triggers a particular downstream signaling pathway in a specific cellular location. Each GEF or GAP is activated under the distinct upstream signaling substrates such as cAMP, Ca^{2+}, and tyrosine kinase, thus connecting a specific upstream signaling element to a particular Ras protein (Fan et al. 1998)
(Innocenti et al. 1999) (Krapivinsky et al. 2004). There are a variety of GEFs and GAPs, which target different members of the Ras family.

1.3.2 GTP hydrolysis

The GTP hydrolysis is a key reaction in intracellular signal transduction as it terminates Ras-effector interactions. A number of important processes such as protein biosynthesis, cell growth and differentiation, and cytoskeletal reorganization are enzymatically controlled by the conversion of GTP to GDP and inorganic phosphate (P$_i$). GTPase mechanism has attracted considerate attention due to the notion that oncogenic mutants of Ras are unable to hydrolyze GTP, contributing to a growing list of life-threatening diseases.

Ras provides the catalytic machinery to perform the chemical reaction converting GTP to GDP and inorganic phosphate (P$_i$). The primary sequence of Ras folds into an $\alpha/\beta$ structure with conserved residues forming a shallow surface pocket that accommodates the guanine nucleotides GTP or GDP (Bourne et al., 1991). Active and inactive forms differ in the presence or absence of the $\gamma$-phosphate on the nucleotide, leading to significantly conformational differences in regions that contact this terminal phosphate in the GTP-bound form (Milburn et al. 1990) (Pai et al. 1990) Wittinghofer et al 1991). These regions have been consistently termed switch I and switch II and are frequently involved in the interactions between Ras and its effectors or/and regulatory proteins (Vetter et al. 2001) (Dvorsky et al. 2004).

The molecular mechanism underlying the GTPase stimulation relies on stabilizing
residues in the switch I and II regions of Ras such as the essential catalytic residue Q61 (Ahmadian et al. 2003) (Figure 1.3).
Figure 1.3 Schematic view of the intrinsic GTPase reaction mechanism. In the GTP-bound state, the switch regions (Sw I and Sw II) provide critical residues that fix the γ-phosphate directly via the Mg$^{2+}$ ion and the catalytic water molecule. Release of the γ-phosphate after GTP hydrolysis induces a conformational change of switch regions that relax into the GDP-bound state (Scheffzek et al. 2005).
1.3.3. GAP-stimulated GTP hydrolysis

To date, two models of how GAPs could accelerate the hydrolysis of GTP-bound Ras have been proposed. In the first model, Ras would provide the whole catalytic machinery, and the role of GAPs would be to stabilize the catalytically competent conformation (Neal et al. 1988) (Moore et al. 1993) (Nixon et al. 1995). In the second model, GAPs provide a catalytic residue into the active site of Ras to neutralize the developing negative charge on the GTP phosphate oxygen atoms during phosphoryl transfer (Mittal et al. 1996) (Ahmadian et al. 1997) (Rensland et al. 1991). Arginine of GAPs would fit for this role due to two reasons. First, its long side chain could bridge larger inter-protein distances. Second, the positively charged guanidinium group could stabilize the transition state, as has been derived from structures of adenylate kinase-like enzymes (Muller et al. 1992).

A breakthrough in the biochemical investigation of the GAP mechanism came from studies utilizing aluminium fluoride (AlFx). These compounds have been identified as transition state similar with heterotrimeric G-proteins (Chabre et al. 1990). GDP-bound Ras alone does not bind AlFx. Addition of RasGAP enhances the formation of a stable ternary complex between the GDP-bound Ras, AlFx and the GAP component. Notably, oncogenic Ras mutants does not form such a complex nor does inactive GAP mutants, leading to the impairments in GTPase and GAP activities (Mittal et al. 1996). Formation of a GDP-AlFx bound ternary complex is an indicator of a transition state stabilizing mechanism. The residues important for transition stabilization can be identified by mutation.
studies in which formation of an AlFx bound complex should be impaired if the functional group is mutated.

1.4 Roles of Ras family proteins in learning and memory

Recently, a growing body of evidence has demonstrated that Ras and its signaling pathways are critically involved in learning and memory formation, consequently influencing cognitive behaviors. These studies examine the role of Ras in learning behaviors by altering the level of Ras activity in whole animals. Ras activity is altered by the use of inhibitors, genetic approaches that change the expression/activity of Ras, or both.

1.4.1 Studies using Ras inhibitors

Membrane association is critical for Ras signaling. Therefore, disruption of the anchoring of Ras to membranes during different stages of learning and memory processing using Ras inhibitors is one of the most popular approaches to study how Ras regulates cognitive behaviors. A number of studies have locally infused Ras inhibitor, FTase, into specific brain regions. Utilizing this approach, Merino et al. specifically infused FTase into basolateral amygdala (BLA), but not other amygdala subregions prior to training rats (Merino et al. 2006). They found that long-term memory for contextual and cued fear conditioning was disrupted. Intra-BLA infusion of the FTase after training or before testing did not impact the long-term fear conditioning, indicating that localized Ras signaling in BLA is necessary for fear memory formation (Merino et al. 2006).
Long-lasting changes in the strength of synaptic transmission in the brain, such as long-term potentiation (LTP) and long-term depression (LTD), have been shown to be critical for learning and memory (Martin et al. 2000, Neves et al. 2008). Specifically, LTP is an activity-dependent strengthening in the efficacy of neuronal synapses following high-frequency stimulation. In contrast, LTD is an activity-dependent reduction of synaptic efficacy following a long patterned stimulus. Studies of LTP are often undertaken in slices of the hippocampus, which is an important organ for learning and memory. Therefore, in parallel, a number of studies have also investigated the involvement of Ras in synaptic plasticity in terms of LTP. Using the similar approach of Ras inhibitors, FTase was shown to attenuate LTD in the hippocampal dentate gyrus (Murray et al. 2004) and disrupt LTP in the hippocampal CA1 region (O'Kane et al. 2004).

However, there are caveats when using inhibitors. First of all, FTase inhibitors target a number of G proteins. Therefore, their phenotypic effects might not be due to the specific inhibition of Ras signaling. Moreover, FTase inhibitors target nascent Ras without influencing preexisting functional Ras. Thus, the incomplete effect of FTase inhibitors cannot exclude the contribution of Ras signaling to the learning and memory formation process.

1.4.2 Studies using both pharmacological and genetic approaches

To overcome the caveats of the pharmacological approach, genetic manipulations have been widely used to investigate the roles of Ras in memory formation.

Ohno et al. (Ohno et al. 2001) combined pharmacological and genetic
manipulations to study the role of K-Ras in neuronal plasticity. They found that K-Ras heterozygous knockout mice \((K-Ras^{+/--})\) showed normal memory for contextual fear conditioning, and normal LTP and ERK activation in the hippocampus. However, a subthreshold dose of MEK inhibitor, which had no effect on WT control mice, blocked memory formation, LTP, and ERK activation in \(K-Ras^{+/--}\) mice, indicating that K-Ras-ERK signaling is involved in memory processing.

In another study, Manabe et al. showed that \(H-Ras^{-/-}\) mice demonstrated an increase in tyrosine phosphorylation of NMDAR receptors and pronouncedly enhanced hippocampal LTP under high-frequency stimulation. This indicates that H-Ras is engaged in regulating postsynaptic function at glutamatergic synapses (Manabe et al. 2000). Notably, Komiyama et al. demonstrated that LTP induced by pairing low-frequency stimulation with postsynaptic depolarization was normal in \(H-Ras^{-/-}\) mice, indicating that the involvement of H-Ras in LTP is stimulation pattern-specific (Komiyama et al. 2002). Collectively, these studies indicate that the two Ras isoforms, K-Ras and H-Ras, regulate distinct aspects of neuronal plasticity during learning memory formation.

In short, studies at the level of the expression of Ras provide direct and strong evidence to indicate that the signaling of Ras family proteins is crucial for regulating a variety of forms of neuronal plasticity in different brain regions, and that dynamic changes in their activity are essential for many types of memory formation within these same brain regions.
1.4.3 Genetic approaches targeting Ras regulators

Given the fact that Ras can interact with many upstream and downstream targets to engage in many types of neuronal plasticity in various brain regions, changes in the expression of a specific upstream or downstream regulator or scaffold protein can significantly affect the learning and memory process. The majority of these studies focus on manipulating the expression of Ras regulators, mostly GEFs or GAPs (Ye et al., 2010). For example, when the GEF protein RasGRF1 was deleted, long-term memory for cued fear conditioning is significantly compromised in RasGRF1−/− mice (Brambilla et al. 1997). Similarly, heterozygous SynGAP+/− mice performed poorly on Morris water maze, indicating that SynGAP, a neuron-specific Ras-GAP, is important for spatial learning memory formation (Komiyama et al. 2002).

Taken together, the studies at the level of Ras regulators show that either hyperactivation, by inhibiting negative regulators, or inactivation, by inhibiting positive regulators, of Ras family proteins can cause impairments in neuronal plasticity and memory formation.

1.4.4. Dynamic regulation of Ras activity by plasticity-related stimuli

To gain a deeper understanding of how Ras family proteins regulate memory formation and neuronal plasticity, it is crucial to elucidate how stimuli that give rise to neuronal and behavioral plasticity can induce correlated changes in the Ras level. To date, a few recent studies have examined the correlation between the dynamic change in the amount or the activity of Ras and the formation of synaptic and behavioral plasticity.
Eckel-Mahan et al. found a circadian oscillation in Ras activity and downstream ERK activity in mouse hippocampus, which peaked during the day and decreased at night (Eckel-Mahan et al. 2008). In parallel with the molecular oscillation, mice trained in a contextual fear-conditioning task during the day showed robust long-term memory, whereas mice trained or tested at night displayed only short-term memory. Moreover, infusion of ERK activation inhibitors into the hippocampus during the circadian peak impaired long-term memory for contextual fear conditioning. These data established a temporal correlation between Ras-ERK activity in the hippocampus and long-term memory formation (Figure 1.4A) (Eckel-Mahan et al. 2008).

In another study, Harvey et al. investigated the dynamics of Ras activity in single spines of pyramidal neurons in hippocampal slices (Harvey et al. 2008). They used a two-photon laser to locally uncage glutamate at a target spine while imaging Ras activity utilizing a FRET-based indicator. A series of excitatory laser pulses that uncage glutamate were shown to induce robust Ras activation in the stimulated spine, but not nearby spines. Interestingly, there was a correlation between the amplitudes of Ras activation and sustained spine enlargement. The activation of Ras was transient,
Figure 1.4 The dynamics of Ras Family Proteins during memory formation and neuronal plasticity (Ye et al. 2010). (A) The circadian oscillation of hippocampal Ras-ERK activity restricts the temporal window for the induction and expression of long-term memory. Area shaded in green and red indicates permissive and nonpermissive window, respectively, for the induction and expression of long-term memory. (B) Ras activated by a strong glutamate uncaging stimuli at a single spine can spread to and invade neighboring spines and allow the induction of sustained spine enlargement by subthreshold stimuli. Distal spines that do not receive the activated Ras fail to express LTP. (C) The interaction between Ras and Rap1 activity controls ERK activation induced by different patterns of training, and differential routes.
peaking by 1 min and returning to baseline by 15 min. Notably, the activated Ras spread over several micrometers and invaded 10–20 neighboring spines. This spread of Ras activity allowed subthreshold stimuli applied temporally at neighboring spines to also induce sustained spine enlargement, indicating an important function of the spatial distribution of Ras activity in mediating synaptic sharing of LTP (Figure 1.4B) (Harvey et al. 2008).

Finally, Ye et al. (2008) recently analyzed Ras, Rap1, and ERK activation simultaneously in response to various patterns of stimulation, which induces different forms of synaptic facilitation in Aplysia. The authors showed that Ras and Rap1 could serve either as activators or as inhibitors of ERK, depending on the specific stimulation patterns (Ye et al. 2008). Moreover, Ras and Rap1 were functionally interactive during the synaptic plasticity formation. In response to stimuli that induce long-lasting synaptic facilitation, the magnitude of ERK activation was controlled by the specific balance between Ras and Rap1 activation, rather than by the individual activation of either single one. This functional interaction between Ras and Rap1 was absent in response to the pattern that does not induce lasting synaptic plasticity or long-term memory (Figure 1.4C) (Mauelshagen et al. 1998, Sutton et al. 2002, Ye et al. 2008).

Taken together, these findings suggest that the dynamic engagement of Ras and its signaling components critically regulates synaptic plasticity and memory formation.
1.5 Role of *NF1* as a Ras-GAP in learning and memory

1.5.1 Neurofibromatosis type 1 disease

Neurofibromatosis type 1 (NF1) is an autosomal dominant genetic disorder that was first identified by Friedrich von Recklinghausen in 1882 (Crump 1981, Reynolds *et al.* 2003). NF1, the most common human genetic disorder affecting the nervous system, has an incidence of around 1 to every 3000, and is caused by mutations on the *NF1* gene (Huson *et al.* 1988, Gutmann *et al.* 1997). About half the cases of NF1 disease are hereditary and the other half are caused by de novo mutations (Littler *et al.* 1990).
<table>
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<tr>
<th>NF1-defining diagnostic criteria</th>
<th>Other NF1-associated features</th>
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<tr>
<td>Patients must have two or more of the following features:</td>
<td>- Learning disabilities, memory loss, attention deficits, and social difficulties</td>
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<tr>
<td>- Two or more neurofibromas, or one plexiform neurofibromas (benign tumors of the peripheral nerve sheath) are considered as hallmarks of NF1 disease</td>
<td>- Cardiovascular abnormalities</td>
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<tr>
<td>- Six or more café-au-lait macules: brown birthmarks</td>
<td>- Increased risk for tumors and cancers including:</td>
</tr>
<tr>
<td>- Skin fold freckling</td>
<td>- Malignant peripheral nerve sheath tumor</td>
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<td>- Specific skeletal defects</td>
<td>- Juvenile myelomonocytic leukemia</td>
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<td>- Optic pathway gliomas</td>
<td>- Gastrointestinal stromal tumors</td>
</tr>
<tr>
<td>- Two or more Lisch nodules</td>
<td>- Pheochromocytomas</td>
</tr>
<tr>
<td>- First degree relative with NF1 patients</td>
<td><strong>Table 1.1.</strong> Summary of human NF1 disease phenotypes</td>
</tr>
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</table>
The disease manifestation of NF1 varies greatly between individuals (Figure 1.5) (Williams et al. 2009). To be diagnosed with NF1, one must have at least two of the following characteristics: neurofibromas, Lisch nodules, skin fold freckling, specific skeletal defects, café-au-lait macules, optic pathway gliomas (OPGs), or a first-generation relative with NF1 patient (1988, Szudek et al. 2003). In addition to the defining features of NF1, patients also usually demonstrate other characteristics including learning and memory deficits, cardiovascular abnormalities, and increased susceptibility to various tumors in addition to neurofibromas.

The NF1 genetic disease includes a wide variety of clinical features that involve several different organ systems within the body. This suggests that NF1 has important functions in many biological processes, although not all of them are well studied. Analyses of the phenotypes of families of NF1 patients suggest that modifying genes and the differences in the normal allele of NF1 may affect disease phenotypes (Easton et al. 1993, Szudek et al. 2002).

1.5.2 History of the discovery of the NF1 gene

Two decades ago, scientists invested extensive effort towards identifying the gene that is mutated in NF1 disease. Using conventional positional cloning techniques, the human NF1 gene was mapped to chromosome 17q11.2 (Barker et al. 1987, Fain et al. 1989, Ledbetter et al. 1989, Wallace et al. 1990, Xu et al. 1990, Yagle et al. 1990). NF1 is a large gene, spanning over 300 kb with over 60 exons encoding 2818 amino acids (Marchuk et al. 1991). A mouse homolog, Nf1, is mapped on chromosome 11, and the mouse coding sequence is about 98%
conserved with the human NF1 sequence (Bernards et al. 1993).

1.5.3 Domain structure and function of NF1 protein

1.5.3.1 Domain structure of NF1 protein

Neurofibromin is the protein product of the NF1 gene. It is a large protein with a molecular weight of 250 kDa (Figure 1.4). Neurofibromin contains several proposed functional domains, of which, the GTPase activating protein (Krapivinsky et al.) domain is the best characterized domain (Barron et al. 2012). The remaining sequence contains several domains that are less well characterized (D’Angelo et al., 2006). Missense mutations detected in NF1 patients are located throughout the entire gene, indicating that the additional domains contribute to the overall neurofibromin functions (Bernards and Settleman, 2004).

The GAP domain, sometimes referred to as the GAP-related domain (GRD) or the Ras-GAP domain, consists of ~360 amino acids in the middle of the NF1 gene. Fragments in the residue range between 1095 and 1577 have commonly been studied as “GAP” but the catalytic activity can be narrowed down to a currently minimal segment of 229 amino acids (residues 1248–1477) (Ahmadian et al. 1996).

An x-ray crystallographic analysis of the NF1 GAP domain demonstrated that it is composed of several α-helices. In addition, its structure is analogous to that of the p120GAP protein, even in areas where the sequences do not show much similarity (Scheffzek et al. 1998) (Figure 1.5). The structure of the GRD
consists of a central portion consistent with the minimal central catalytic domain (GAPc) (Ahmadian et al. 1996) and an extra domain (GAPex) formed by a coiled helical arrangement of about 50 residues from the N- and C-terminus, respectively. A shallow pocket in the surface of
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Figure 1.5  
A. NF1-GRD crystal structure (Scheffzek et al. 1998)
B. p120-GAP crystal structure (Scheffzek et al. 1996)
Figure 1.6 Model of the GRD–Ras complex and the GAP mechanism. (a) Ribbon representation of GRD and Ras. GRD (PDB: 1NF1) was superimposed with the p120GAP component from the Ras: RasGAP complex (PDB: 1WQ1). GDP, AlF$_3$, and Mg$^{2+}$ are depicted as yellow stick models. The positions of non-truncating, patient-derived mutations are visualized as yellow spheres. (b) GRD strongly accelerates the hydrolysis of GTP to GDP + Pi by Ras. The P-Loop, Mg$^{2+}$ ion, and NKxD and DxxG motifs stabilize bound GTP in a favorable orientation for the hydrolysis reaction. The reaction itself is initiated by a water molecule, positioned by Ras-Gln61. Only after binding of GRD can the reaction proceed efficiently. GRD stabilizes the switch I and II regions of Ras and complements the Ras active site with Arg1276. Arg1276 stabilizes the transition state of the reaction by neutralization of developing negative charges and helps positioning of Ras-Gln61 (Bos et al. 2007; Scheffzek and Ahmadian 2005).
GAPc forms the Ras-binding region (Scheffzek et al. 1998).

The respective Ras–GRD complex shows Ras bound to the abovementioned pocket in GAPc (Figure 1.6). As expected, the GTP/GDP sensing switch I and II regions along with regions of the nucleotide binding pocket form major components of the Ras–GRD interface, that is dominated by largely polar interactions (Scheffzek et al. 1997). The active site is bound to GDP–AlF₃, a presumed transition state analog of the phosphotransfer reaction in which the planar AlF₃ moiety recapitulates the terminal γ-phosphate group during its transfer to the hydrolytic water molecule or the nucleophile derived from it (Wittinghofer 1997). GDP–AlFx complex with Ras is only formed in the presence of the GRD, indicating that NF1 GRD acts to stabilize the active site of GTP-bound Ras (Mittal et al. 1996) in a heterodimeric protein complex (Scheffzek et al. 1998b). In the Ras–GRD complex, the chemical arrangement is stabilized by the interaction with the GAP domain characterized by a highly conserved arginine residue (Arg1276 in neurofibromin). This residue is also called the arginine “finger”, that interacts with the presumed GDP–AlFx transition state. Additional residues partly derived from the FLR finger print motif stabilize the catalytically important switch I/II regions (Scheffzek et al. 1997). A key component in the catalytic process is the highly conserved Gln61 that stabilizes the hydrolytic water molecule. Its conformation is stabilized by Arg1391 along with switch II contributions (Figure 1.6). Mutational analysis has confirmed the importance of Arg1276 and Arg1391 for GAP catalysis, leading to a 2,000- and 50-fold reduction of GAP activity, respectively (Ahmadian et al. 1997b; Sermon et
al. 1998). Importantly, both arginines have been identified to be mutated in NF1 patients with severe disease phenotypes (Upadhyaya et al. 1997b; Klose et al. 1998)

Insertion of a 21 amino acid (exon 23a) stretch Gln370 (Figure 1.7) has no counterpart in p120GAP, IRA1, and IRA2. In the structural model, exon 23a insertion may disrupt the C-terminus of helix $\alpha_{5c}$, which is an exposed part of the NF1-GRD domain. Exon 23a carrying many charged amino acids was speculated to be involved in protein-protein interactions. Based on the structure of NF1-GRD, the insertion may not directly interfere with Ras binding, but rather alter the GRD-Ras interaction by long-range electrostatic bonds.
Figure 1.7 Sites of NF1-GRD mutations. The position where 21 amino acids are inserted (type II transcript) is indicated at the bottom (Scheffzek et al. 1998).
Immediately adjacent to the NF1 GAP domain is a bipartite module, which consists of a domain with sequence similarity to the yeast Sec14p protein. Just downstream of the GAP domain is a pleckstrin homology (PH)-like domain (Figure 1.8). The Sec14-PH domain interacts with phospholipids (D'Angelo et al. 2006, Welti et al. 2007). In other contexts, Sec14 domains allow proteins to shuttle lipids between different cellular compartments, so it is hypothesized that NF1 could also have a similar function (Bankaitis et al. 1990, Cleves et al. 1991, Kostenko et al. 2005). Farther downstream of the GAP domain are a cluster of Caveolin binding domains which allow NF1 to interact with the protein Caveolin-1 (Figure 1.4) (Boyanapalli et al. 2006). Caveolin-1 localizes to caveolae, which are forms of lipid rafts in the plasma membrane that consist many signaling molecules. It is believed that the interaction of NF1 with Caveolin-1 might be important for the regulation of NF1 signaling.

Upstream of the NF1 GAP domain is a cysteine-serine-rich domain (CSRD) (Figure 1.8) (Fahsold et al. 2000). Residues within this domain are believed to be phosphorylated by cAMP-dependent protein kinase A (PKA) (Izawa et al. 1996). Moreover, serines located in the CSRD are phosphorylated by PKCα in response to epidermal growth factor (EGF). This phosphorylation promotes the NF1-actin association. Once PKCα is downregulated, it decreases the ability of a combined CSRD-GAP domain in inhibiting Ras (Mangoura et al. 2006). Within the CSRD is a microtubule-associated protein (MAP) domain, which is believed to be important for NF1-microtubule interactions (Gregory et al. 1993).
Figure 1.8 Neurofibromin and its roles (Ratner et al. 2015). Neurofibromin contains multiple domains (light blue). These include a cysteine–serine-rich domain (CSRD), a tubulin-binding domain (TBD), a central GTPase-activating protein-related domain (GRD), a SEC14 domain, a pleckstrin homology (PH) domain, a carboxy-terminal domain (CTD), and a syndecan-binding domain (SBD). The SEC14 and PH domains bind to phospholipids.
1.5.3.2 NF1 protein expression and its functions

Neurofibromin is ubiquitously expressed in almost all human tissues but is particularly enriched in neurons, oligodendrocytes, and Schwann cells (Daston et al. 1992). Neurofibromin is present in early rodent embryos, with changes in relative expression in different tissues throughout development. For instance, neurofibromin is barely detectable in mouse heart at embryonic day 11 (E11), but then increases dramatically in expression at E12, and returns to baseline expression levels by E13. These dynamic changes in expression indicate a possible function of neurofibromin in development. During late fetal and early postnatal development, neurofibromin becomes enriched in tissues of the nervous system and expression decreases in other tissues (Daston et al. 1992, Huynh et al. 1994).

On a subcellular level, neurofibromin is localized mainly in the cytoplasm. Nevertheless, there were several studies reported that neurofibromin contains a nuclear localization signal, enable it to shuttle back and forth from the nucleus to cytoplasm (Vandenbroucke et al. 2004). In one study of rat neurons, neurofibromin was demonstrated to be present throughout the cell body and axons, and particularly enriched in dendrites and near the endoplasmic reticulum (Nordlund et al. 1993). Another study indicated that neurofibromin can associate with microtubules in the cytoplasm, and that sequences within the GAP domain are necessary for this interaction (Gregory et al. 1993, Xu et al. 1997).

Inactivation of Ras is the most well-known and well-studied function of neurofibromin. As a Ras-GAP, neurofibromin enhances the rate at which Ras-
GTP (active Ras) is hydrolyzed into Ras-GDP (inactive Ras) (Le et al. 2007, Gutmann et al. 2012, Diggs-Andrews et al. 2013). Since neurofibromin inhibits Ras, which is up-regulated in many cancers, it is considered to be a tumor suppressor protein. By inactivating Ras, NF1 serves to attenuate signaling through both the MAPK and PI3K cascades.

The first evidence that neurofibromin functions as a Ras-GAP came shortly after the NF1 gene was cloned due to its sequence homology with the mammalian GAP gene and the yeast IRA1 and IRA2 GAP genes (Ballester et al. 1990, Xu et al. 1990). Later, the expression of the NF1 GAP domain was shown to rescue the heat shock sensitivity of yeast IRA1 and IRA2 mutants, indicating that they were functionally conserved (Ballester et al. 1990). In addition, the NF1 GAP domain was reported to stimulate the GTPase activity of Ras proteins both in vitro and in vivo (Ballester et al. 1990, Martin et al. 1990, Xu et al. 1990, DeClue et al. 1992). These studies strongly support that neurofibromin is a GAP protein.

Further evidence that NF1 is a Ras-GAP came from the discovery that active Ras levels are significantly upregulated in tumors and cells of NF1 patients (Guha et al. 1996, Feldkamp et al. 1999, Thomas et al. 2006). The Ras signaling pathways are also upregulated in many NF1 mutant tissues and tumors, and different branches of the cascade appear to be essential for distinct NF1-associated phenotypes (Le et al. 2007, Diggs-Andrews et al. 2013).
1.5.4 Studies of NF1-associated cognitive deficits

1.5.4.1 Learning deficits of NF1 patients

Although tumors are the hallmarks of NF1, most patients show neurocognitive deficits. Learning disabilities appear to be in about 30-60% of children with NF1 (Ferner et al. 2007, Shilyansky et al. 2010). NF1 patients often have IQs lower than the average range, and have an increased risk of intellectual disability relative to the general population (Ferner et al. 1996, Ozonoff 1999). They usually have specific learning disabilities, including spatial deficits, nonverbal and verbal language deficits, and difficulties with motor skills (Hofman et al. 1994, Hyman et al. 2005). Difficulties in reading, spelling, and math are also often associated with NF1 (Hofman et al. 1994, Hyman et al. 2005). Patients also often show behavioral issues, particularly prevalent with attention deficit and hyperactivity disorder (Mautner et al. 2002, Hyman et al. 2005).

1.5.4.2 Animal models of NF1-associated cognitive deficits

1.5.4.2.1 Drosophila studies

The Drosophila NF1 gene is quite well conserved with the human gene, sharing about 60% similarity in amino acid sequence (The et al. 1997). Therefore, it has been used as a model system for studying NF1 functions in vivo. Unlike mice, Drosophila NF1−/− mutants are viable, but they are smaller than WT controls (The et al. 1997). Drosophila NF1 has been shown to increase cAMP by regulating the rutabaga-encoded adenylyl cyclase (Guo et al. 1997). Manipulation of Ras signaling does not rescue the NF1 mutant size phenotype, but manipulation to upregulate the cAMP pathway does (The et al. 1997). A
genetic modifier screen further provided evidence, indicating that reduced cAMP signaling in part of the neuroendocrine ring gland contributes to the growth defects in \textit{NF1}\textsuperscript{-/-} Drosophila (Walker \textit{et al.} 2013). This suggests that the regulation of cAMP by NF1 is important for Drosophila development. \textit{NF1}\textsuperscript{-/-} Drosophila has deficits in both olfactory associative learning and short and long-term memory. Notably, studies indicate that the associative learning and short-term memory phenotypes of \textit{NF1}\textsuperscript{-/-} Drosophila are mediated by the cAMP pathway, while the long-term memory phenotypes are mediated by Ras signaling (Guo \textit{et al.} 2000, Ho \textit{et al.} 2007). Hence, in Drosophila, both major arms of \textit{NF1}-related signaling pathways appear to be important for learning and memory.

1.5.4.2.2 Mouse studies

Just like humans and Drosophila, \textit{Nf1} mutant mice demonstrated specific learning deficits. \textit{Nf1}\textsuperscript{+/--} mice performed poorly on tests of hippocampus-based spatial learning and memory including the Morris water maze (Silva \textit{et al.} 1997). These learning deficits can be overcome with extended training, and are specific because the \textit{Nf1}\textsuperscript{+/--} mice performed normally on amygdala-dependent and hippocampal-independent memory tasks such as the cued fear-conditioning test of associative learning. This suggests that neurofibromin have no effect on amygdala processing of fear memory. The \textit{Nf1}\textsuperscript{+/--} mice also show deficits in long-term potentiation (LTP), which appear to be mediated by increased GABA-mediated inhibition (Costa \textit{et al.} 2002).

Follow-up studies were carried out to explore the cellular and molecular mechanisms underlying memory impairment in \textit{Nf1} mutant mice. Up-regulating
Ras signaling has been shown to be partially responsible for the learning deficits in $Nf1^{+/}$ mice, as manipulations that decrease the levels of Ras, both genetically and pharmacologically, reverse the spatial learning deficits as well as the impairments in long-term potentiation in $Nf1^{+/}$ mice (Costa et al. 2002). Costa et al. (2002) found that the spatial memory deficits in $Nf1^{+/}$ mice were rescued by a further heterozygous knockout in K-Ras or N-Ras. Prior to training, injection of an FTase inhibitor or lovastatin, both of which inhibit membrane anchoring of Ras and thus disrupt Ras signaling, also rescued the memory impairment (Costa et al., 2002; Li et al., 2005).

Lovastatin, the FDA-approved drug, can reverse the GABA release, LTP, and learning impairments in adult mice, and was shown to improve impairments in synaptic plasticity in human NF1 patients (Li et al. 2005, Mainberger et al. 2013). Extensive studies in complex neuron-specific NF1-deficient mouse models indicates that the Ras signaling is responsible for hippocampal learning deficits (Kushner et al. 2005, Guilding et al. 2007, Cui et al. 2008, Staley et al. 2009, Shilyansky et al. 2010). According to their model, in interneurons, the mutation of $Nf1$ leads to activation of Ras, which subsequently triggers a phosphorylation cascade causing activation of ERK1/2. This then results in the phosphorylation and activation of Synapsin1, a neurotransmitter vesicle protein, which leads to elevated GABA neurotransmitter release in the hippocampus. The increased level of GABA then inhibits LTP and learning. Although, Guilding et al. (2007) found that basal ERK phosphorylation and downstream CREB phosphorylation was elevated in the hippocampus of $Nf1^{+/}$ mice, there was no
change in the PI3K cascade (Guilding et al. 2007). This suggests that neurofibromin exclusively suppresses the ability of Ras to activate the Raf-MEK-ERK-CREB cascade. Taken together, these findings indicate that the abnormal increase in Ras activity may account for memory impairments observed in Nf1 mutant mice.

In summary, these in vivo studies clearly demonstrate that hyperactive Ras induced by loss-of-function mutations in neurofibromin, a specific RasGAP, can result in memory impairments. Despite the ubiquitous distributions of neurofibromin, surprisingly, its engagement in cognitive behaviors is specific to certain types of memory tasks and certain neuronal cell types (GABAergic neurons and not excitatory neurons or astrocytes), and a particular Ras signaling pathway (Ras-ERK cascade but not Ras-PI3K cascade). In addition, despite the importance of neurofibromin in cognitive function, little is known about how the activity of neurofibromin is regulated, especially by stimuli that give rise to memory formation and synaptic plasticity. The exact
<table>
<thead>
<tr>
<th>Nf1 mutation</th>
<th>Cognitive abnormality</th>
<th>Refs</th>
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<tbody>
<tr>
<td>Flies</td>
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<tr>
<td>Mice</td>
<td></td>
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<tr>
<td>Nf1^+/−  (129/C57 hybrid)</td>
<td>Heterozygote</td>
<td>Spatial learning memory attention</td>
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<tr>
<td>Nf1^floxi− GFAP−Cre</td>
<td>Heterozygosity in neurons</td>
<td>Spatial learning memory attention</td>
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<tr>
<td>Nf1^floxi− Syn1−Cre</td>
<td>Heterozygosity in neuronal subset</td>
<td>Spatial learning memory</td>
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**Table 1.2** Summary of animal models of NF1-associated cognitive deficits (Diggs-Andrews, 2013).
molecular mechanisms at play in these studies remain to be elucidated.

1.6 Alternative splicing of **NF1**

1.6.1 Alternative exons of **NF1**

Our previous study showed that the functions of **NF1** are modulated by alternative splicing in cell models (Hinman et al, 2014). The human **NF1** gene contains three in-frame alternative cassette exons: 9br/9a, 23a, and 48a. These alternative exons are conserved between humans and rodents. Exon 9br is located in the N-terminal portion of **NF1**, and its inclusion leads to the addition of 10 amino acids to neurofibromin. Exon 9br is included in neurons of the central nervous system, but excluded from all other tissues, including the peripheral nervous system (Danglot et al. 1995, Geist et al. 1996, Gutmann et al. 1999). Exon 48a is located near the extreme 3’ end of **NF1**, and its inclusion adds 18 amino acids to neurofibromin. It is expressed specifically in cardiac, skeletal, and smooth muscle (Gutman et al. 1993, Danglot et al. 1994, Gutmann et al. 1995). The function of **NF1** exon 48a expression in muscles is unknown.

**NF1** exon 23a, which is conserved in humans and rodents but not in Drosophila (The et al. 1997), is located between constitutive exons 23.2 and 24, is 63 nucleotides in length, and its inclusion adds 21 amino acids to the NF1 protein. The type 2 **NF1** transcript includes this exon while the type 1 transcript, which predominates in the brain, excludes it. No NF1 disease-causing mutations have been found within exon 23a, but the location of this alternative exon within the Ras-GAP domain of **NF1** has made it the focus of several studies to
understand its function, including my thesis research (Andersen et al. 1993, Ars et al. 2003, Pros et al. 2008).

An additional 41 bp alternative exon, 23b, is detectable only in rodents. Its inclusion causes a frame shift leading to a premature termination codon, and possibly to nonsense-mediated decay of the Nf1 mRNA (Andersen et al. 1993, Mantani et al. 1994). Supporting this idea is the finding that exon 23b inclusion increases with cycloheximide treatment, which inhibits translation and thus nonsense-mediated decay (Metheny et al. 1996). The function of this exon is currently unknown.

1.6.2 Expression pattern of NF1 exon 23a

NF1 exon 23a inclusion is developmental stage-specific and tissue-specific. A number of molecular mechanisms are in place to regulate NF1 exon 23a inclusion during development and in specific tissues. During development in whole mouse embryos, the type 2 mRNA containing Nf1 exon 23a predominates from E8-E10, and the type 1 mRNA predominates since day 11 toward adulthood (Huynh et al. 1994, Gutmann et al. 1995). This is consistent with immunostaining using an antibody specific to the peptide encoded by exon 23a, which shows low expression of the type 2 neurofibromin isoform by E12 (Huynh et al. 1994). In the rat, Nf1 exon 23a is mainly skipped in the brain throughout development from E14 to P14, whereas other tissues, such as kidney and lung, mainly have exon 23a included throughout development. Some tissues exhibit changes in exon 23a inclusion throughout development. For example, in testes, exon 23a inclusion switches from nearly 100% at P1 to mainly skipping at P14 (Gutmann et al.
1995). Cultured cells from E16 mouse cerebral cortex was prepared to determine the expression of \textit{Nf1} isoforms in individual cell types. Cultures enriched for neurons express mainly the type 1 isoform, while cultures enriched for glia express mostly the isoform with exon 23a included (Gutmann \textit{et al.} 1995).

There is a substantial body of evidence to indicate that there is a developmental switch from exon 23a inclusion to exon 23a skipping during neuronal differentiation. In one study, rat PC12 cells, which are derived from a pheochromocytoma of the adrenal medulla, were induced to differentiate into neuron-like cells by treatment with nerve growth factor, and exon 23a inclusion switched from predominantly inclusion to mainly skipping (Metheny \textit{et al.} 1996). Similarly, there is a switch from exon 23a inclusion to exon 23a skipping upon neuronal differentiation of mouse P19 teratocarcinoma cells by retinoic acid treatment (Mantani \textit{et al.} 1994). This switch to the type 1 isoform upon differentiation appears to be specific to neurons. For instances, in the MT4H1 Schwann cell line, the abundance of the type 2 isoform increases upon treatment to induce Schwann cell differentiation (Gutmann \textit{et al.} 1993). It seems likely from these data that skipping of \textit{Nf1} exon 23a might play a role in some aspect of neuronal differentiation.

In adult tissues, the general trend is toward skipping of \textit{NF1} exon 23a in neural tissues. One study showed that both type 1 and type 2 isoforms are present in various adult human tissues, but the type 1 isoform is most abundant in brain, while the type 2 isoform predominates in other tissues (Figure 1.7) (Andersen \textit{et al.} 1993). Another study showed similar results in human tissues,
with NF1 exon 23a predominantly included in most tissues including peripheral nerves, but skipped in brain tissue and equally included and skipped in muscle and hematopoietic cells (Danglot et al. 1994). Melanocytes from café-au-lait spots in NF1 patients as well as melanocytes from healthy donors show a predominance of the type 2 isoform (Eisenbarth et al. 1995). Just as in human tissues, the isoform lacking Nf1 exon 23a predominates in the neural tissues of adult mice, including the brain and spinal cord (Mantani et al. 1994). Adult rats show predominantly skipping of Nf1 exon 23a in the brain, and approximately 50% inclusion in the spinal cord (Gutmann et al. 1995).

While NF1 exon 23a is mainly skipped in adult brain, the general trend is toward inclusion of the exon in brain tumors. In one study of 16 sporadic human brain tumors including glioblastomas, astrocytomas, and meningiomas, the type 2 isoform predominated in the tumors, whereas the type 1 isoform predominated in surrounding normal brain tissue (Suzuki et al. 1991). Likewise, in another study of 16 sporadic human astrocytomas, NF1 exon 23a was mostly included in tumor samples but skipped in normal brain samples (Tokuyama et al. 1995). In a third study, the NF1 type 2 transcript was most abundant in 8 of 10 human brain tumors from non-NF1 patients, and the type 1 transcript was most abundant in all parts of the normal brain (Takahashi et al. 1994). A fourth study reported mainly skipping of exon 23a in tumors but more inclusion in the adjacent normal brain tissue, which contradicts numerous other studies showing skipping of exon 23a in brain (Mochizuki et al. 1992). Collectively, these results enable us to hypothesize that the NF1 isoform containing exon 23a may promote the
formation or maintenance of some brain tumors. This hypothesis remains to be tested in future studies.

In summary, *NF1* exon 23a inclusion changes dynamically throughout development, and the exon is preferentially skipped in brain tissue and neurons. The tight regulation of exon 23a inclusion leads to the hypothesis that exon 23a skipping
Figure 1.9 The alternative splicing of NF1 exon 23a. The type I isoform of NF1, in which exon 23a is excluded, predominates in neurons. The type II isoform of NF1, which contains exon 23a, is more abundant in other cell types. Boxes represent exons and lines represent introns.
may be important for the function of NF1 in the brain, and that this function may be disrupted in some brain tumors.

1.6.3 Regulation of NF1 exon 23a alternative splicing

Complex molecular mechanisms are in place for the regulation of NF1 exon 23a inclusion (Zhu et al. 2008, Barron et al. 2010, Zhou et al. 2011, Barron et al. 2012, Fleming et al. 2012). Several RNA binding proteins have been shown to contribute to this regulation. These include the CELF (CUG-BP [CUG-binding protein] and ETR-3 [ELAV (embryonic lethal abnormal vision)-type RNA binding protein]) family, the MBNL (Muscleblind-like) family, TIA-1 (T-cell intracellular antigen 1-related protein), TIAR (TIA-1 related protein), and the Hu/ELAVL (ELAV-like) family (Zhu et al. 2008, Barron et al. 2010, Zhou et al. 2011, Fleming et al. 2012). These RNA-binding-proteins act by directly affecting the interaction of the spliceosome with the NF1 pre-mRNA.

Besides RNA binding proteins, studies in our laboratory showed that the inclusion of NF1 exon 23a is also under the epigenetic regulation of chromatin structure, histone modifications, which indirectly impact transcriptional elongation rate.

We demonstrated that Hu proteins regulate NF1 exon 23a inclusion at the epigenetic level by influencing the transcriptional elongation rate across the NF1 gene (Zhou et al. 2011). Hu proteins interact with histone deacetylase 2 (HDAC2) and inhibit its activity. The AU-rich sequences around exon 23a region are canonical binding sites for Hu proteins. The elevated concentration of Hu proteins surrounding exon 23a in the NF1 gene induces local histone
hyperacetylation in this region, leading to an increase in the local transcriptional elongation rate. Based on the kinetic model of splicing regulation, a faster transcriptional elongation rate allows the spliceosome less time to recognize $NF1$ exon 23a before the downstream constitutive exon is transcribed, thus favoring skipping of the exon.

The current model is that Hu proteins bind to the $NF1$ nascent mRNA while it is still attached to the $NF1$ gene during transcription. The Hu proteins bind to AU-rich sequences surrounding exon 23a and interact with HDAC2 around this region. These Hu-HDAC2 interactions inhibit the activity of HDAC2, preventing deacetylation of the histones in the nearby region around $NF1$ exon 23a. During the next round of transcription, the region surrounding exon 23a in the $NF1$ gene is already hyperacetylated, consequently resulting in faster transcriptional elongation and skipping of the exon.

In addition, the exon 23a splicing patterns can also be regulated under the stimulation of external signaling elements. We previously showed increased intracellular $\text{Ca}^{2+}$ under KCl treatment can change the $Nf1$ exon 23a splicing patterns in cardiomyocytes by inducing the global histone hyperacetylation along the gene, leading to a faster transcriptional elongation rate over the $Nf1$ gene body and skipping of this exon (Sharma et al. 2014).

1.6.4. Role of exon 23a in $NF1$ GAP domain

A hint about the possible function of $NF1$ exon 23a comes from the fact that it falls within the GRD domain, which is the key functional domain of NF1 that inactivates the oncogene Ras. A structural analysis of the NF1 GRD domain
lacking exon 23a indicated that the GAP domain is composed of a series of α-helices. The 21 amino acid exon 23a is predicted to disrupt the C-terminal portion of one of these helices, α5c, which is an exposed part of the GAP domain. The authors of this study speculated that since exon 23a is in a relatively exposed portion of the GAP domain and contains many charged residues (six lysines and two glutamates), it might function in interactions with other proteins or with other portions of the large NF1 protein. They also hypothesized that exon 23a is unlikely to directly disrupt the binding of NF1 to Ras, but that it could modify the interaction by long-range electrostatic contributions (Scheffzek et al. 1998).

Costa et al. partially characterized the biological function of Nf1 exon 23a by creating a homozygous deletion of Nf1 exon 23a in mice (Nf1^{23a/-} mice). In these mutant mice, exon 23a was deleted but the remainder of the gene was intact (Costa et al. 2001). These mice were born at Mendelian ratios and did not have an increased predisposition for tumor formation, and also survived normally when aged. Nf1^{23a/-} mice have deficiency in spatial learning, as demonstrated by poor performance on the hidden version of the Morris water maze and on a contextual discrimination test. However, when the Nf1^{23a/-} mice were given extended training, they were capable of performing as well as WT mice. Similar deficits in spatial learning were observed in Nf1^{+/} mice (Silva et al. 1997). The Nf1^{23a/-} mice also demonstrated delayed acquisition of motor skills, as measured by the accelerating rota-rod test. The learning deficits in these mice were specific, as the mutant mice performed normally on other tests of learning, such as the
social transmission of food preferences. Collectively, the results of this study indicate that the type 2 isoform of Nf1 plays a role in spatial learning in mice.

1.7 Summary of research

In my thesis research, I sought to understand the biological functions of Nf1 alternative splicing in the context of the endogenous full-length gene in an *in vivo* model system, particularly the behavior phenotypes caused by alterations in Nf1 alternative splicing. Given the fact that Nf1 exon 23a lies in the middle of the Ras-GAP domain of Nf1 and is specifically skipped in neurons, I hypothesized that exon 23a inclusion is tightly regulated to provide appropriate Ras signaling *in vivo*, and is important for proper learning and memory in mice.

The biological function of Nf1 exon 23a in vivo was initially characterized in Nf1\(^{23a/-}\) mice in which the exon was deleted (Costa *et al.* 2001). Nf1\(^{23a/-}\) mice are viable, grow normally, and are not susceptible to tumor formation. However, they demonstrate learning impairments similar to those of NF1 patients and Nf1\(^{+/}\) mice. Several questions arose from this study. Given that Nf1 exon 23a is predominantly skipped in mouse brain (Mantani *et al.* 1994), is the small amount of Nf1 exon 23a inclusion in the brain critical for function, and how do learning phenotypes arise when the exon is deleted? In addition, the authors did not investigate how deletion of exon 23a impacts the downstream molecular cascades in mice. Were the levels of Ras-GTP and signaling downstream of Ras affected in tissues of Nf1\(^{23a/-}\) mice? Is there a narrow window of appropriate Ras signaling for learning? Are the learning deficits in Nf1\(^{23a/-}\) mice due to alterations
in a Ras signaling pathway? Most importantly, what would happen if \textit{Nf1} exon 23a were included in the brain, where it is normally skipped?

Previously, we showed that the alternative splicing of \textit{Nf1} exon 23a is a key regulator of Ras activity in cell models (Hinman \textit{et al.} 2014). Specifically, we engineered mutations into the splicing signals surrounding endogenous alternative \textit{Nf1} exon 23a in mouse embryonic stem (ES) cells, creating \textit{Nf1}^{23a\text{IN}/23a\text{IN}} cells in which the exon is constitutively included, and \textit{Nf1}^{23a\text{IN}/23a\text{IN}} cells in which the exon is deleted. We showed both in ES cells and in ES cell-derived neurons that increased levels of active Ras correlates with increased \textit{Nf1} exon 23a inclusion. We further showed that ERK1/2 activation downstream of Ras is elevated in \textit{Nf1}^{23a\text{IN}/23a\text{IN}} cells. These data suggest that the function of the alternative splicing of \textit{Nf1} exon 23a in cells is to modulate Ras regulatory activity of \textit{Nf1} GRD.

Using mutant ES cells from our previous study, we generated knock-in mouse model in which exon 23a is constantly included in all tissue types, including the brain, where \textit{Nf1} exon 23a is normally skipped. The goal of my thesis research was to investigate the role of alternative splicing of \textit{Nf1} exon 23a in Ras/ERK activity \textit{in vivo} and in the learning and memory processes using our mutant mice.
CHAPTER 2 - Neurofibromatosis type 1 alternative splicing is a key regulator of Ras/ERK signaling and learning behaviors in mice

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2.1 Abstract

Appropriate activation of the Ras/ERK protein signaling cascade within the brain is crucial for optimal learning and memory. One key regulator of this cascade is the Nf1 Ras GTPase activating protein (RasGAP), which attenuates Ras/ERK signaling by converting active Ras-GTP into inactive Ras-GDP. A previous study using ES cells and ES cell-derived neurons indicated that Nf1 RasGAP activity is modulated by the highly regulated alternative splicing of Nf1 exon 23a. In this study, we generated Nf1^{23aIN/23aIN} mice, in which the splicing signals surrounding Nf1 exon 23a were manipulated to increase exon inclusion. Nf1^{23aIN/23aIN} mice are viable and exon 23a inclusion approaches 100% in all tissues, including the brain, where the exon is normally almost completely skipped. Ras activation and phosphorylation of ERK1/2 downstream of Ras are both greatly increased in Nf1^{23aIN/23aIN} mouse brain lysates, confirming that exon 23a inclusion inhibits Nf1 RasGAP activity in vivo as it does in cultured cells. Consistent with the finding of altered Ras/ERK signaling in the brain, Nf1^{23aIN/23aIN} mice showed specific deficits in learning and memory compared to Nf1^{+/+} mice. Nf1^{23aIN/23aIN} mice performed poorly on the T-maze and Morris water maze tests, which measure short- and long-term spatial memory, respectively. In addition, Nf1^{23aIN/23aIN} mice showed abnormally elevated context-dependent fear and a diminished ability to extinguish a cued fear response, indicating defective associative fear learning. Therefore, the regulated alternative splicing of Nf1 is an important mechanism for fine-tuning Ras/ERK signaling as well as learning and memory in mice.
2.2 Introduction

Members of the Ras family of small G proteins play essential roles in many cellular processes, including cell proliferation, survival, differentiation, and migration. In the central nervous system (CNS), maintaining an optimal level of Ras activity is crucial for brain development and cognitive functions such as learning and memory (Ye et al. 2010). The Ras/extra-cellular signal regulated kinase (ERK) subfamily of the mitogen-activated protein kinase (MAPK) cascade is particularly important in cognition (Samuels et al. 2009, Zhong 2016).

Ras proteins exist in two forms: the active GTP-bound and the inactive GDP-bound forms. The conversion between the two forms is regulated by guanine exchange factors (GEFs), which are Ras activators, and GTPase-activating proteins (GAPs), which are Ras inactivators. Interestingly, genetic inactivation of either a GEF or a GAP in mouse, resulting in hypo- or hyper-activation of Ras, respectively, leads to abnormal cognitive behaviors such as learning disabilities, memory deficits, and impaired synaptic plasticity. For example, when the GEF protein RasGRF1 was deleted in RasGRF1\(^{-/-}\) mice, the mutant mice exhibited deficits in long-term memory formation and in fear conditioning tests (Brambilla et al. 1997). Similarly, when one copy of SynGAP, a neuron-specific Ras-GAP, was deleted in the SynGAP\(^{+/}\) mice, the animals also exhibited deficiencies in long-term memory formation, as examined in tests of spatial learning memory (Komiyama et al. 2002). These results suggest that balanced cellular Ras activity must be achieved to support proper learning and memory.
Interestingly, accumulating evidence indicates that Ras/ERK activity during memory formation is highly dynamic (Impey et al. 1999). For example, it has been demonstrated that Ras is activated by spontaneous neuronal activity, which is required for long-term potentiation (LTP) induction and associated dendritic spine enlargement (Zhu et al. 2002, Harvey et al. 2008). Furthermore, timely inactivation of Ras following its induction also appears to play a crucial role in the maintenance of spine structure, as continued hyperactivation of stimulus-evoked Ras activity leads to impaired synaptic plasticity and dendritic spine loss (Oliveira et al. 2014).

Although it is well recognized that a dynamic balance of Ras activation is important for proper learning and memory, it is not well understood how this process is regulated. As many studies have utilized pharmacological inhibitors to investigate the role of Ras/ERK signaling in learning and memory, the contributions of specific GEFs and GAPs in this process are not well understood.

Neurofibromin, the protein product of the neurofibromatosis type 1 (NF1) gene, is an important RasGAP in the nervous system (King et al. 2013). Neurofibromin contains a GAP-related domain (GRD) that is responsible for converting active Ras-GTP to inactive Ras-GDP (Tanaka et al. 1990). Inactivation of the Nf1 gene in mice, either in heterozygous Nf1\(^{+/}\) or in tissue-specific Nf1\(^{-/-}\) mutants, leads to spatial learning deficits and/or social interaction impairments (Silva et al. 1997, Cui et al. 2008, Molosh et al. 2014).

Notably, the mammalian Nf1 RasGAPs have a unique feature: they can be regulated by alternative splicing of exon 23a. This exon, encoding 21 amino
acids, is located in the Nf1-GRD (Andersen et al. 1993). Two protein isoforms are generated by alternative splicing of exon 23a, one containing the exon and the other one lacking it. Alternative splicing of this exon is tightly regulated, exhibiting evolutionarily conserved cell type- and developmental stage-specific splicing patterns, which is indicative of functional importance of the expression of this exon (Baizer et al. 1993, Huynh et al. 1994, Mantani et al. 1994, Gutmann et al. 1995, Gutmann et al. 1995). In adult mammals, exon 23a is predominantly skipped in CNS neurons, while in some PNS neurons, as well as in non-neuronal cells, including glia and many other cell types, exon 23a is included at varying levels (Gutmann et al. 1995). Furthermore, a switch from inclusion to skipping occurs during early embryonic development between day E10 and E11 in mouse brain, and between day E7 and E13 in chicken brain (Baizer et al. 1993, Huynh et al. 1994, Gutmann et al. 1995).

Studies using yeast and mammalian cell lines indicate that although both isoforms have RasGAP activity, the isoform that contains the exon shows reduced RasGAP activity by up to ten-fold compared with the isoform that lacks the exon (Andersen et al. 1993, Yunoue et al. 2003). To understand the biological role of exon 23a expression, we generated mutant Nf1 alleles, Nf1^{23aIN} and Nf1^{23aΔ}, in mouse embryonic stem (ES) cells using a gene targeting approach (Hinman et al. 2014). The Nf1^{23aIN} allele leads to production of the neurofibromin isoform that contains exon 23a, whereas the Nf1^{23aΔ} allele leads to production of the isoform that does not contain the exon (Hinman et al. 2014). Our studies using the mutant ES cells and ES cell-derived neurons demonstrate
that inclusion of exon 23a significantly increases Ras/ERK signaling (Hinman et al. 2014). Thus, the alternative splicing of \( \textit{Nf1} \) exon 23a is a key regulator of cellular Ras activity in ES and neuronal cells (Hinman et al. 2014).

In the present study, we investigated the role of alternative splicing of \( \textit{Nf1} \) exon 23a in the cognitive behaviors of mice. We used mutant ES cells to generate mutant \( \textit{Nf1}^{23\text{aIN}/23\text{aIN}} \) mice in which exon 23a is constantly included in all tissue types. We found that the mutant mice were viable, fertile, and had no obvious physical abnormalities. In brain tissues, where exon 23a is predominantly skipped in wild type mice, active Ras levels were elevated in the \( \textit{Nf1}^{23\text{aIN}/23\text{aIN}} \) mutant mice, indicating that exon 23a alternative splicing is an important regulator of Ras activity \textit{in vivo}. Furthermore, exon 23a inclusion led to a six-fold increase in phospho-ERK1/2 levels in the mutant brains. Most notably, \( \textit{Nf1}^{23\text{aIN}/23\text{aIN}} \) mice showed significant deficits in specific learning behaviors including spatial and associative fear learning. These results demonstrate an important role of the regulated expression of \( \textit{Nf1} \) exon 23a in Ras/ERK activity and proper learning and memory in mice.

2.3 Results

2.3.1 \( \textit{Nf1}^{23\text{aIN}/23\text{aIN}} \) mice are viable

To determine the biological function of regulated \( \textit{Nf1} \) exon 23a inclusion \textit{in vivo}, we generated mice using ES cells in which one \( \textit{Nf1} \) allele is mutated (Hinman et al. 2014). The mutations are engineered at both ends of exon 23a to better match the consensus splicing signals, so that the exon is constitutively included (Hinman et al. 2014). We obtained both heterozygous and homozygous
mutant mice, termed $Nf1^{23aIN/+}$ and $Nf1^{23aIN/23aIN}$, respectively (Hinman et al. 2014). After ten generations of back-crossing, all mice are in the C57BL/6J genetic background.

Both $Nf1^{23aIN/+}$ and $Nf1^{23aIN/23aIN}$ mice are viable, fertile, and appear physically normal on a gross level. Several crosses were set up between $Nf1^{23aIN/+}$ mice, and the offspring were born and reached adulthood at the expected ratios according to
Figure 2.1 Mutations designed to increase Nf1 exon 23a inclusion do not affect mouse viability. A. Mouse pups were genotyped by PCR. B. The genotypes of weaning-age offspring from crosses between Nf1^{23aIN/+} mice. Total numbers of mice are shown along with percent of total mice in parentheses. Chi-square test: P=.95
Mendelian genetics (Fig. 1A and 1B). Therefore, \(Nf1^{23alN}\) mutations do not affect the viability of mice. This contrasts with \(Nf1^{-/-}\) mice, which lack all functional \(Nf1\) and die at mid-gestation (Brannan et al. 1994, Jacks et al. 1994).

2.3.2 \(Nf1^{23alN}\) mutations increase \(Nf1\) exon 23a inclusion \textit{in vivo}

To analyze alternative splicing, we isolated total RNA from brain, heart, lung, kidney, liver, spleen, skeletal muscle, and testes tissues of adult \(Nf1^{+/+}\), \(Nf1^{23alN/+}\), and \(Nf1^{23alN/23alN}\) mice. \(Nf1\) exon 23a inclusion was measured by radioactive, semi-quantitative RT-PCR using primers in the exons surrounding exon 23a (Hinman et al. 2014). The RT-PCR results indicate that \(Nf1\) exon 23a inclusion is tissue-specific in adult \(Nf1^{+/+}\) mice. Particularly, \(Nf1\) exon 23a is almost exclusively skipped in brain and testes, but its inclusion is higher in other tissues (Fig. 2). Within the brain, exon 23a skipping predominates across various regions, including cerebellum, cortex, hippocampus, and brain stem (Fig. S1A). There is a developmental switch in exon 23a inclusion, with higher inclusion in whole embryonic brain than in newborn or adult brains (Fig. S1B). These results are consistent with previously reported studies (Baizer et al. 1993, Huynh et al. 1994, Mantani et al. 1994, Gutmann et al. 1995, Gutmann et al. 1995). Furthermore, as expected, the mutant \(Nf1^{23alN/23alN}\) mice exhibit nearly 100% exon 23a inclusion in all tissues, including the brain and testes (Fig. 2). Meanwhile, \(Nf1^{23alN/+}\) mice have an \(Nf1\) exon 23a inclusion level that stands between that of \(Nf1^{+/+}\) and \(Nf1^{23alN/23alN}\) mice in all tissues (Fig. 2). Therefore, the
23aIN mutations that improve splicing signals are an effective strategy for preventing alternative exon 23a skipping in vivo.
Figure 2.2 Nf123aIN mutations increase endogenous Nf1 exon 23a inclusion in mouse tissues. RT-PCR showing endogenous Nf1 exon 23a inclusion in mouse tissues. PCR primers are located on surrounding exons. Error bars represent standard error. N≥3. Brain: *P=7.3x10^{-8}, **P=1.6x10^{-8}; Heart: *P=1.0x10^{-2}, **P=1.5x10^{-2}; Lung: *P=1.0x10^{-3}, **P=1.5x10^{-4}; Kidney: *P=2.8x10^{-4}, **P=5.9x10^{-5}; Liver: *P=2.1x10^{-5}, **P=1.0x10^{-5}; Spleen: *P=5.4x10^{-4}, **P=1.2x10^{-5}; Muscle: *P=5.8x10^{-3}, **P=1.1x10^{-4}; Testes: *P=1.3x10^{-2}, **P=4.5x10^{-6}
2.3.3 Nf1 exon 23a inclusion increases active Ras levels in the brain

In brain tissues, where Nf1 expression is highly enriched, exon 23a inclusion changes very dramatically between wild type and mutant mice, from predominant skipping to complete inclusion (Fig. 2). Therefore, we decided to focus on brain tissue for our phenotypic studies. We first compared the brains dissected from Nf1+/+ and Nf1^23aIN/23aIN mice and found no obvious differences in their general gross morphology (Fig. S2).

We then investigated the molecular and biochemical differences between wild type and mutant mouse brains. We asked whether Nf1 exon 23a inclusion affects the level of active Ras in vivo like it does in cells (Hinman et al. 2014). We carried out an active Ras pull-down assay as described previously (Hinman et al. 2014). In this assay, we used GST-tagged Raf1 Ras binding domain (RBD) to pull down Ras-GTP, the active form of Ras, from adult mouse brain lysates. As shown in Fig. 3A, active Ras-GTP levels were highest in Nf1^23aIN/23aIN whole brain lysates, lower in Nf1^23aIN/+ lysates, and were nearly undetectable in Nf1^+/+ lysates. Importantly, neurofibromin, the Nf1 gene product, was expressed at similar levels in mice of the three genotypes, indicating that only the isoforms of Nf1 were changed (Fig. 3B). Therefore, increasing Nf1 exon 23a inclusion correlates with increasing active Ras levels in mouse brain tissues. These data indicate that Nf1 exon 23a alternative splicing is a key regulator of Ras activity in vivo. 2.3.4 Nf1 exon 23a inclusion increases ERK signaling.

We next asked which signaling pathway(s) downstream of Ras are activated by increased Nf1 exon 23a inclusion. Given that the Raf/MEK/ERK1/2
and PI3K/Akt/mTOR pathways are the most prominent pathways activated by Ras, we
Figure 2.3 Increased endogenous Nf1 exon 23a inclusion leads to increased Ras activation in mouse brain. **A.** GST-Raf1RBD was used to pull down active Ras (Ras-GTP) from mouse brain lysates, followed by western blot analysis using anti-Ras antibody. Mock pull-downs were performed using GST. Four percent of total lysate was loaded in the total Ras lanes. U1 70K is a loading control. **B.** Western blot analysis showing Nf1 protein expression in mouse brain lysates. U1 70K is a loading control.
Figure 2.4 Western blot analysis showing the expression of downstream targets of Ras in lysates from whole mouse brains. A. Phospho-ERK1/2 (~42/44 kDa) and total ERK1/2 (~42/44 kDa). Error bars represent standard errors. N=3. *P=1x10^{-2}, **P=5.85x10^{-6}. B. Phospho-Akt (~60 kDa) and total Akt (~60 kDa). Error bars represent standard errors. N=3. C. Phospho-S6 (~32kDa), total S6 (~32 kDa), and γ-tubulin (~48 kDa, a loading control). Error bars represent standard errors. N=3.
measured the levels of phosphorylated ERK1/2 and Akt as indicators of activation of these pathways (Le et al. 2007, Gutmann et al. 2012, Diggs-Andrews et al. 2013). The level of phospho-ERK1/2 is greatly elevated in \( Nf1^{23aIN/23aIN} \) and \( Nf1^{23aIN/+} \) mouse brains compared to wild type (Fig. 4A). Specifically, there is a six-fold increase in the \( Nf1^{23aIN/23aIN} \) brains and a three-fold increase in \( Nf1^{23aIN/+} \) brains (Fig. 4A). No changes in phospho-Akt or phospho-S6 levels were observed (Fig. 4B and 4C). This indicates that \( Nf1 \) exon 23a inclusion specifically elevates Raf/MEK/ERK1/2 signaling downstream of Ras. These results are consistent with previous studies showing activation of the Raf/MEK/ERK1/2 pathway but not the PI3K/Akt/mTOR pathway in \( Nf1 \) mutant mouse brains (Guilding et al. 2007, Lush et al. 2008). They indicate that \( Nf1 \) exon 23a inclusion specifically activates the Ras-ERK signaling pathway in mouse brains.

### 2.3.4 Increased \( Nf1 \) exon 23a inclusion leads to specific learning deficits in mice

Ras signaling plays an important role in learning and memory behaviors. Since we demonstrated significantly elevated Ras-ERK activity in the \( Nf1 \) mutant mice, we hypothesized that they would exhibit deficits in learning and memory. To test this hypothesis, we assessed the sensorimotor performance and overall learning and memory behaviors of the \( Nf1^{+/+} \), \( Nf1^{23aIN/+} \), and \( Nf1^{23aIN/23aIN} \) mice.

We conducted the sticky paper test to assess general tactile sensitivity (Bouet et al. 2009), and the rotarod and beam walking tests to assess overall motor coordination. All three mouse genotypes had normal paw tactile sensation in the sticky paper test and normal body coordination in the beam walking and
rotarod tests (Fig. S3A–C). In the beam walking test, \( Nf1^{23alN/23alN} \) mice exhibited slightly faster crossing latencies than \( Nf1^{+/+} \) mice using a 16-mm square beam, but this was not the case with other beams (Fig. S3B). We assessed the exploratory/anxiety behaviors of these mice using an open field test. There were no differences in anxiety measures among these mice, including the time spent immobile and the percent of time spent in the inner area of the test apparatus (Fig. S3D–S3E). These data indicate that regulation of \( Nf1 \) exon 23a inclusion has little impact on sensorimotor and emotional function in mice.

In contrast to the motor and anxiety tests, mice with increased \( Nf1 \) exon 23a inclusion showed robust impairment in learning and memory performance. We performed the T-maze test to assess novelty-based short-term spatial memory and the Morris water maze test to assess long-term spatial memory in these mice. \( Nf1^{23alN/23alN} \) mice exhibited poor learning performance in the two spatial memory tests, indicating that increased \( Nf1 \) exon 23a inclusion leads to impaired hippocampus-dependent spatial memory. In the T-maze, \( Nf1^{+/+} \) mice displayed a clear preference for exploring an unfamiliar arm over a familiar arm (~67%), whereas mutant mice showed no preference (~50%) (Fig. 5A). In the Morris water maze test, \( Nf1^{+/+} \) mice displayed improvement in the time it took to find a hidden platform over successive trials. In contrast, \( Nf1^{23alN/23alN} \) mice showed less improvement over successive trials, and took significantly more time to reach the platform than \( Nf1^{+/+} \) mice on days 3 and 4 (Fig. 5B). Following the final trial in the water maze, a probe test was performed in which the hidden platform was removed. \( Nf1^{23alN/23alN} \) mice spent less time swimming in the
quadrant where the platform had previously been located than $Nf1^{+/+}$ mice, confirming impaired acquisition of spatial memory (Fig. 5C). In addition, $Nf1^{23aIN/23aIN}$ mice showed a consistent impairment in performance on the novel object recognition test, which
Figure 2.5 Spatial learning and memory tests. n=9 for each genotype.

Error bars represent standard error.  

A. T-maze test. Mice were trained for 10 minutes in a T-maze where one of the arms was blocked and were then tested after 2 hours in the same T-maze, except all arms were open. The percent of time spent exploring the previously blocked arm was measured (unfamiliar arm choice %). The dotted line indicates the unfamiliar arm choice percentage expected by chance. *$Nf1^{23aIN/+}$ and $Nf1^{23aIN/23aIN}$ were significantly lower than $Nf1^{+/+}$ ($F_{2,27} = 3.82, P=0.0346$).

B-C. Morris water maze test. Four trials were performed each day for four days. B. Latency to reach the hidden platform was recorded [Gene; $F_{2,27} = 3.302, P=0.0521$, Days: $F_{3,81} = 50.209, P<0.0001$, Gene x Days: $F_{6,81} = 4.35, P=0.0007$]. *Day 3: $Nf1^{23aIN/23aIN}$ was significantly higher than $Nf1^{+/+}$ (P<0.05), Day 4: $Nf1^{23aIN/23aIN}$ was significantly higher than $Nf1^{+/+}$ (P<0.01).

There was no significant difference between genotypes on Day 1 or Day 2. C. Following the final session, the platform was removed and the animals were allowed to swim for 60 seconds. The percent time spent in the quadrant where the platform was previously located was measured. *$Nf1^{23aIN/23aIN}$ was significantly lower than $Nf1^{+/+}$ ($F_{2,27} = 3.945, P=0.0314$).
measures reference memory. In this test, preference for investigating a novel object over a familiar object is used as an indicator of reference memory. There was no difference in performance among genotypes in the 1.5-hour (short-term memory) version of the object recognition test, but \( Nf1^{23aIN/23aIN} \) mice performed poorly in the 24-hour version of the test, indicating impairment in long-term reference memory (Fig. S3F). Taken together, these results suggest that appropriate regulation of \( Nf1 \) exon 23a inclusion is required for functional spatial learning. These impairments in spatial learning are similar to those observed in \( Nf1^{+/} \) mice (Silva et al. 1997), and are consistent with the finding that exon 23a inclusion decreases the molecular activity of Nf1 as a RasGAP (Fig. 3A).

We further investigated memory function in these mutant mice using a fear conditioning paradigm. Conditioned responses and extinction of those responses were assessed in cue- and context-dependent fear conditioning tests. All the mice showed an acquired fear response to repeated foot shock exposures (unconditioned stimuli, US) during a training session, as indicated by increased freezing over time in response to an auditory cue (conditioned stimulus, CS) (Fig. 6A). Twenty-four hours later, the mice were placed back into the same context where they had previously received foot shocks. Both \( Nf1^{23aIN/23aIN} \) and \( Nf1^{23aIN/+} \) mice exhibited increased freezing compared to \( Nf1^{+/+} \) mice, indicating abnormally elevated context-dependent fear (Fig. 6B). Two hours later, cued memory was assessed by placing the mice into an unfamiliar context and measuring freezing in response to the auditory cue in the absence of a foot shock. All three genotypes of mice initially showed similar levels of freezing in response to the
auditory cue (Fig. 6C, Block 1). Over subsequent trials, $Nf1^{+/}$ mice exhibited decreased
Figure 2. 6 Fear learning tests. Error bars represent standard error. A. Mice were trained to associate a conditioned stimulus (CS, a 30-second-long pure tone of 5kHz and 80 dB) with an unconditioned stimulus (US, a one second long electrical shock of 0.5 mA), over four trials [Gene: $F_{2,27} = 0.903$, $P=0.4171$, Trial: $F_{4,108} = 91.114$, $P<0.0001$, Gene x Trial: $F_{8,108} = 0.794$, $P=0.609$]. Freezing was significantly higher than baseline for CS 2, CS 3, and CS 4 ($P<0.01$). There was no significant difference in freezing between genotypes. B. 24 hours later, mice were evaluated for their learned aversion for an environment associated with the shock (context-dependent fear), as measured by the percent time freezing in the environment where they had previously been shocked. *$Nff^{23aIN/+}$ and $Nff^{23aIN/23aIN}$ were significantly higher than $Nff^{+/+}$ ($F_{2,27} = 12.997$, $P=0.0001$). C. Two hours later the animals were reintroduced into a contextually altered box, the same tone (30 sec, 5kHz, 80dB) was delivered 10 times without an electrical shock, and freezing behavior was measured [Gene: $F_{2,27} = 1.30$, $P=0.289$, Block: $F_{4,108} = P<0.0001$, Gene x Block: $F_{8,108} = 2.934$, $P=0.0053$]. *Block 4 and 5: $Nff^{23aIN/+}$ and $Nff^{23aIN/23aIN}$ were significantly higher than $Nff^{+/+}$ ($P<0.05$).
freezing, indicating that they had learned to extinguish the cued fear response, whereas $Nf1^{23a IN/+}$ and $Nf1^{23a IN/23a IN}$ mice showed a significantly reduced ability to extinguish the freezing response (Fig. 6C, Blocks 2-5). Overall, these results indicate that associative fear learning is altered in mutant mice compared to $Nf1^{+/+}$ mice.

2.3.5 $Nf1^{23a IN/23a IN}$ neurons exhibit similar dendritic spine density and morphology as in wild type neurons.

Since $Nf1$ is known to be a major regulator of Ras activity and synaptic plasticity in dendritic spines (Oliveira et al. 2014), we hypothesized that altered dendritic spine formation might contribute to the learning phenotypes in $Nf1^{23a IN/23a IN}$ mice. As an initial experiment to test this hypothesis, we cultured cerebellar neurons from wild type and mutant P8 mice, transfected them with a GFP expression plasmid, and imaged their dendritic spines. We found no obvious differences in dendritic spine morphology and density between wildtype and mutant neurons (Fig. S4), although we cannot rule out the possibility that subtle differences exist, or that dendritic spines are altered in other neuron types. Our future studies will investigate these possibilities.

2.4 Discussion

2.4.1 A new mouse model to study the role of Ras/ERK signaling in learning behaviors

In this study, we demonstrated that mice with constitutive inclusion of exon 23a of the $Nf1$ gene are viable but have significantly increased Ras-ERK signaling in their brains, which leads to deficiencies in both short- and long-term spatial learning, as well as in associative fear learning.
This mouse model is a very useful genetic tool to study the role of Ras/ERK signaling in learning, which has been an intensely investigated subject. The dynamic regulation of Ras/ERK activity during learning has been demonstrated by many studies. However, the vast majority of these studies employed pharmacological approaches with specific Ras or ERK inhibitors to inactivate Ras/ERK signaling (Samuels et al. 2009, Zhong 2016). This is partly due to the potential complications of genetic models, i.e., the difficulties in separating the neurodevelopmental defects from learning behavior deficiencies. As Ras signaling is essential for proper development, any drastic changes in active Ras levels lead to either lethality or major developmental defects. In this regard, the mouse model we generated represents a “gentler” model. In this model, the Nf1 RasGAP activity is not completely abolished but rather is greatly reduced, to a level that still supports viability and generally normal development. Thus, this genetic model will enable us to probe more deeply into the dynamic regulation of Ras/ERK signaling during learning and memory formation.

Furthermore, Pavlovian fear conditioning and extinction has been used to model human anxiety disorders, such as posttraumatic stress disorder (PTSD), and is considered an excellent experimental paradigm to study PTSD (Mahan et al. 2012). The mouse model presented in this report shows robust defects in fear extinction (Fig. 6). In this regard, this mouse model could be used for studies investigating the molecular mechanisms of PTSD.
2.4.2 Role of Nf1 RasGAP in spatial and associative fear learning

Our findings that the RasGAP activity of neurofibromin plays an important role in learning behaviors are consistent with previous studies carried out using heterozygous or tissue-specific Nf1 null mutant mice (Silva et al. 1997, Cui et al. 2008). These models all demonstrated defective spatial learning behaviors in the Morris water maze test. Interestingly, in addition to spatial learning, our model also exhibited significant defects in contextual and cued fear conditioning tests, which were not observed in other mouse models. One possible explanation for this discrepancy is the different levels of Ras/ERK signaling activity in these different models. Specifically, Nf1 RasGAP activity is abolished in cells where both Nf1 alleles are deleted, and is presumably 50% of the wild type level in the Nf1+/- mice. In the Nf1^{23aIN/23aIN} mice, the Nf1 RasGAP activity is expected to be lower than that in the Nf1+/- mice, as several studies indicated that the RasGAP activity of the neurofibromin isoform containing exon 23a is up to 10 times lower than that of the isoform without the exon (Andersen et al. 1993, Yunoue et al. 2003, Hinman et al. 2014). It should be noted that in most CNS neurons of wild type mice, exon 23a is predominantly skipped, leading to production of the neurofibromin isoform without the amino acids encoded by the exon (Huynh et al. 1994, Mantani et al. 1994, Gutmann et al. 1995). As indicated in Fig. 2, in whole brain lysates that include multiple cell types, inclusion of exon 23a switches from 11% in wild type to 99% in homozygous mutant mice. Thus, it is expected that in the Nf1^{23aIN/23aIN} mice the most significant change in Nf1 RasGAP activity occurs in the brain.
What is the role of Nf1 RasGAP in the dynamic regulation of Ras/ERK during learning? It has been demonstrated by numerous studies that proper neuronal activity-induced activation and inactivation of Ras is important for memory formation (Impey et al. 1999). On one hand, it has been demonstrated that Ras is activated by spontaneous neuronal activity, and that this activation is required for long-term potentiation (LTP) induction and is associated with dendritic spine enlargement (Zhu et al. 2002). Infusion of an ERK inhibitor in the amygdala leads to impaired long-term memory of fear conditioning as well as LTP in the amygdala in vitro (Schafe et al. 2000). On the other hand, timely inactivation of Ras following its induction is also important for the maintenance of spine structure. Continued hyper-activation of stimulus-evoked Ras activity leads to impaired synaptic plasticity and dendritic spine loss (Oliveira et al. 2014). Interestingly, in one particular study, the authors concluded that neurofibromin was the key RasGAP responsible for the inactivation of stimulus-induced Ras activity (Oliveira et al. 2014). When the neurofibromin level is reduced by shRNA knockdown in the CA1 region of the rat hippocampus, Ras activation in spines is sustained, leading to impairment of spine structural plasticity and loss of spines (Oliveira et al. 2014). Our results are consistent with this study in that reduced Nf1 RasGAP activity in the Nf1^{23alN/23alN} mouse brain leads to continued hyper-activation of Ras/ERK levels, resulting in defective memory formation. Although initial analysis of dendritic spines showed normal morphology and density in mutant cerebellar neurons, future studies will aim to understand the effects of the reduced Nf1 RasGAP activity of the Nf1^{23alN/23alN} mice on dendritic spine structure
and plasticity in brain regions important for spatial and fear conditioning memory formation.

2.4.3 Regulated alternative splicing of Nf1 exon 23a

Given the significant regulatory function of the expression of exon 23a in Ras/ERK signaling, it is not surprising that alternative splicing of this exon is tightly regulated. Our recent studies have led to the discovery of a complex regulation mechanism of tissue-specific alternative splicing of NF1 exon 23a that involves a set of cis-acting elements and at least four groups of trans-acting RNA-binding proteins. Of the four groups of proteins, Hu proteins and CELF (CUGBP1 and ETR3-like factor) proteins that are expressed in neurons function as negative regulators of exon 23a inclusion, thereby enhancing the production of the Nf1 protein isoform without this exon (Zhu et al. 2008, Barron et al. 2010, Zhou et al. 2011, Hinman et al. 2013); whereas TIAR (T-cell internal antigen 1-related) protein and MBNL (muscleblind-like) proteins function as positive regulators of exon 23a inclusion, promoting the production of the protein isoform that includes this exon (Zhu et al. 2008, Fleming et al. 2012). Hu proteins and TIAR interact with U-rich sequences and CELF and MBNL proteins interact with GU-rich sequences to regulate splicing of exon 23a (Zhu et al. 2008, Barron et al. 2010, Fleming et al. 2012). Interestingly, alternative splicing of exon 23a is also regulated by epigenetic mechanisms (Zhou et al. 2011, Sharma et al. 2014, Sharma et al. 2015). Particularly important is our finding that dynamic regulation of exon 23a inclusion is mediated by calcium levels in cardiomyocytes (Sharma et al. 2014, Sharma et al. 2015). Determination of whether similar dynamic
regulation of exon 23a inclusion occurs in neurons represents an important line of investigation in future studies.

2.4.4 The role of alternative splicing of *Nf1* exon 23a in learning

The Nf1 RasGAP is unique among the RasGAPs. It is regulated by alternative splicing of exon 23a, which is in the GRD. Studies from our laboratory and others have demonstrated that the expression status of *Nf1* exon 23a is a key regulator of the cellular Ras/ERK activity in cultured cells and in mice (Fig. 3A and 4A) (Andersen et al. 1993, Yunoue et al. 2003, Hinman et al. 2014).

We propose that alternative splicing of *Nf1* exon 23a plays an important role in learning and memory. Studies of two mouse models, including the current study, provide support to this hypothesis. In 2001, Costa and colleagues reported a very intriguing study in which they found that when exon 23a was deleted, the mutant mice, termed *Nf1*23a/−, developed learning deficits (Costa et al. 2001). Although exon 23a is generally skipped in CNS neurons, the authors detected its inclusion in the CA3 region of the hippocampus in wild type mice by immunohistochemical analysis. In *Nf1*23a/− mice, exon 23a was deleted in all cell types including hippocampal neurons (Costa et al. 2001). Thus, changes of the Nf1 RasGAP activity in the CA3 region in the mutant mice is likely at least partially responsible for the observed learning phenotypes.

The genetic mutations in *Nf1*23a/− and *Nf1*23aIN/23aIN models affect the Nf1 RasGAP functions in opposing ways, leading to constitutively high or low Nf1 RasGAP activity in cells, and thus low and high Ras/ERK activity, respectively (Fig. 3A and 4A) (Hinman et al. 2014). Comparing the learning phenotypes of
the two mutant mouse models led to several interesting conclusions and speculations.

First, both models show a clear deficiency in spatial learning (Fig. 5) (Costa et al. 2001), underscoring the significance of proper levels of Nf1 RasGAP as well as cellular Ras/ERK activities in hippocampus-based learning behavior (Lieberwirth et al. 2016). These results suggest either that spatial learning behavior is very sensitive to the precise levels of Ras/ERK activities in neurons or that proper spatial learning requires dynamic switching of the two Nf1 RasGAP isoforms in specific neurons.

Second, most of the other learning and motor tests, including ability to freeze, exploratory behaviors (open field), motor performance, and muscle strength, showed no significant differences between the $Nf1^{+/+}$ and the two different mutant mice, except that the $Nf1^{23a^{-/-}}$ mice exhibited motor coordination impairment in the accelerating rotarod test, which was not observed in the $Nf1^{23aIN/23aIN}$ mice (Fig. S3C) (Costa et al. 2001). These results are consistent with the notion that the Nf1 RasGAP activity is only important in brain regions relevant for specific learning tasks (Costa et al. 2001).

Third, the two mutant models both showed defective associative fear learning, suggesting that $Nf1$ exon 23a regulation is important for the function of the prefrontal cortex (Cestari et al. 2014). Interestingly, the two mutant models were defective in associative fear learning in opposite ways. The $Nf1^{23a^{-/-}}$ mice showed decreased freezing in contextual fear conditioning tests, while $Nf1^{23aIN/23aIN}$ mice showed increased freezing in both contextual and cued fear conditioning tests.
(Costa et al. 2001) (Fig. 6B and 6C). Although we cannot rule out the contributions of different genetic backgrounds, we speculate that the most likely explanation for the differences in the freezing behaviors is the differing Ras/ERK levels in these models. It appears that low Ras/ERK activity, which is expected in the \( Nf1^{23a/-} \) mice, leads to lower level freezing, and high Ras/ERK activity, as demonstrated in the \( Nf1^{23aIN/23aIN} \) mice, leads to a higher level of freezing. Interestingly, this speculation is consistent with a recent study in which offspring of out-bred mouse lines were selected for high and low fear behaviors in fear conditioning studies. Increased freezing time in the associative fear conditioning tests (the “high fear” group) was associated with high ERK activity in the lateral amygdala (Coyner et al. 2014). To further test this hypothesis, future studies can be conducted by infusing Ras or ERK inhibitors into specific brain regions and monitoring associative fear learning behaviors in the \( Nf1^{23aIN/23aIN} \) mutant mice.

In summary, we generated knock-in mice with constitutive \( Nf1 \) exon 23a inclusion and demonstrated that the regulation of \( Nf1 \) exon 23a inclusion in the brain leads to defective learning and memory formation in spatial and associative fear learning behaviors. This model will provide a new tool for studies of the role of Ras/ERK signaling in learning and memory.
2.5 Supplemental figures

Figure S2.1 The alternative splicing pattern of Nf1 exon 23a in developing brain and different regions of the adult brain in wild type mice. A. RT-PCR analysis using total RNA isolated from adult cerebellum, cortex, hippocampus, and brain stem tissues. Error bars represent standard error. N = 3. B. RT-PCR analysis using total RNA isolated from E14, P0, and adult mouse whole brains. Error bars represent standard error. N \geq 3. *P=1.05 \times 10^{-6}, **P=1.37 \times 10^{-5}
Figure S2.2 Brains dissected from 3 week-old wild type and mutant mice.
Figure S2.3 Additional sensorimotor and behavioral tests. Error bars represent standard error. A. In the sticky paper test, a piece of adhesive tape was placed on the hind limb paw. The amount of time it took for the mouse to lick its paw (latency) was used as a measure for tactile sensitivity. No significant difference was found between genotypes ($F_{2,27} = 0.024$, $P=0.9768$) B. The beam
walking test was used as a measure for motor coordination and balance. The amount of time it took to cross 50 cm long beams that either had a 16 mm round diameter, or a 16 mm or 9 mm square cross section was measured. *$Nf1^{23aIN/23aIN}$ is significantly less than $Nf1^{+/+}$ and $Nf1^{23aIN/+}$ ($F_{2,27} = 4.749$, $P=0.0171$). No significant difference was found between genotypes for 16 mm round ($F_{2,27} = 2.173$, $P=0.1333$) or 9 mm square beams ($F_{2,27} = 1.345$, $P=0.2774$). C. The accelerating rotarod test was performed as a measure of balance and coordination. The amount of time it took for mice to fall off a rod that was accelerating from 4 to 40 rpm was measured over three successive trials. There was no significant difference in performance between genotypes [Gene: $F_{2,27} = 0.312$, $P=0.7344$, Trial: $F_{2,54} = 11.953$, $P<0.0001$, Gene x Trial:$F_{4,54} = 0.698$, $P=0.597$] D-E. In the open field behavior test, mice were placed in a 50 cm square apparatus and their activity was recorded for ten minutes using video tracking software. The time spent immobile (D) and the time spent in the inner area of the box (E) were measured as indicators of anxiety. There was no significant difference between genotypes for time spent immobile ($F_{2,27} = 0.178$, $P=0.8375$) or time spent in inner area of the box ($F_{2,27} = 1.262$, $P=0.2991$). F. Recognition memory was measured through an object recognition test. The preference ratio for an unfamiliar object was measured immediately upon introduction of two identical unfamiliar objects (trial 1), 90 minutes later upon introduction of one object from trial one and one novel object (trial 2; a measure of short-term memory), and 24 hours later upon introduction of one object from trial one and one novel object (trial 3; a measure of long-term memory). Dotted line indicates novel object preference expected by chance. *For Trial 3, $Nf1^{23aIN/23aIN}$ was significantly less than $Nf1^{+/+}$ and $Nf1^{23aIN/+}$ ($F_{2,27} = 3.706$, $P=0.0378$). There was no significant difference between genotypes in Trials 1 ($F_{2,27} = 0.67$, $P=0.5198$) and 2 ($F_{2,27} = 0.41$, $P=0.668$).
Figure S2.4 Mutant cerebellar neurons display similar dendritic spine morphology and density as wild type neurons. Cerebellar neurons from P8 mouse pups were cultured and transfected with a GFP expression plasmid. Images were taken three days post transfection. Scale bar, 5 µm.
2.6 Materials and Methods

2.6.1 Generation of mutant mice

$N_{F1}^{23aINneo/+}$ mouse embryonic stem (ES) cells from the 129 background were obtained by gene targeting of R1 ES cells (Hinman et al. 2014). Chimeric 129:C57Bl/6J mice were generated by the Case Transgenic and Targeting Facility using the $N_{f1}^{23aINneo/+}$ cells and crossed with C57Bl/6J mice. A single founding $N_{f1}^{23aIN/+}$ mouse was obtained and genotyped by Southern blot analysis. The mutant mice contain a neomycin cassette in the intron downstream of $Nf1$ exon 23a, which does not affect gene expression, but are referred to as $N_{f1}^{23aIN}$ for simplicity. The mice were crossed for 10 generations onto the C57Bl/6J background. All mouse work was performed with the approval of the Case Western Reserve University Institutional Animal Care and Use Committee.

2.6.2 Genotyping

Tail clippings from 2-3-week old mice were genotyped by PCR using three primers, the sequences of which are as follows:

Forward 1 (5’-GCAACTTGCCACTCCCTACTGAATAAAGCT-3’)

Reverse 1 (5’–TACCCGGTAGAATTTCGACGA-3’)

Reverse 2 (5’–GAGAATGTTTCAATGTAACCTAATTCCAGG-3’)

Primers Forward 1 and Reverse 1 were used to detect the mutant $Nf1$ allele, whereas primers Forward 1 and Reverse 2 were used to detect the wild type $Nf1$ allele.
2.6.3 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Adult mice were sacrificed by cervical dislocation and snap-frozen tissues were homogenized in Trizol (Invitrogen). RNA was isolated from the dissected mouse tissues. RT-PCR was performed as described previously using the mouse Nf1 forward (5’ – GAACCAGAGGAACCTCCTTCAGATG – 3’) and mouse Nf1 reverse (5’ – CATACGGCAGACAATGGCAGGATT – 3’) primers and 23-25 PCR cycles (Hinman et al. 2014). Percent exon inclusion ([exon included/(exon included + exon skipped) X100] was measured with a Typhoon Trio Variable Mode Imager (GE Healthcare), and results represent averages of experiments from three or more mice. The percent exon inclusion is presented as mean ± the standard error of the mean (Papakonstanti et al.). Statistical analysis was performed using a Student’s t-test.

2.6.4 Western blots

Western blot analyses were performed using different amounts of total protein from mouse brain homogenates, ranging from 20 µg to 100 µg. The mouse brains were homogenized using a Mini-Beadbeater (Biospec Products). The primary antibodies used in this study include: anti-U1 70K (1:250, a gift from Dr. Susan Berget, Baylor College of Medicine), anti-γ-tubulin (1:10,000, Sigma T6557), anti-Ras (1:200, Thermo Scientific), anti-phospho-p44/42 MAPK (ERK1/2) (137F5) (1:1000, Cell Signaling #4695), anti-phospho-Akt (Ser473) (587F11) (1:1000, Cell Signaling #4051), anti-Akt (1:2000, Cell Signaling #9272), anti-phospho-44/42 MAPK (ERK1/2) (1:2000, Cell Signaling #137F5), anti-S6 ribosomal protein (5G10) (1:1000, Cell Signaling #2217), anti-
phospho-S6 ribosomal protein (Ser240/244)(D68F8) XP (1:1000, Cell Signaling #5364), and anti-neurofibromin(D) (1:200, Santa Cruz Biotechnology SC-67). Goat anti-mouse IgG (1:2000, Thermo Scientific) and goat anti-rabbit (1:5000, Thermo Scientific) were used as secondary antibodies. Western blot analysis was conducted using a Typhoon Trio Variable Mode Imager (GE Healthcare). The relative level of phospho-proteins/total proteins is presented as mean ± the standard error of the mean (Papakonstanti et al.). Statistical analysis was performed using a Student’s t-test.

2.6.5 Active Ras pull-down assay

Fresh mouse brain tissue was homogenized in ice-cold lysis buffer (25 mM Tris-HCl pH 7.2, 150mM NaCl, 5 mM MgCl₂, 1%NP-40, and 5% glycerol) containing Complete Mini EDTA-Free Protease Inhibitor (Horwood et al.). Homogenates were centrifuged at 16,100 g for 10 minutes at 4°C. Supernatants were used immediately to perform an active Ras pull down using the Active Ras Pull-Down and Detection Kit from Thermo-Scientific (Cat. No. 89855), and Ras was detected by western blot analysis. Mock pull-downs were also performed using GST alone.

2.6.6 Mouse behavioral tests

For the behavioral tests, we used 10 or more age- and sex-matched adult mice for each genotype. The experimenters were blinded to the genotype of each animal during the experiments. All animal experimental procedures were reviewed and approved by the Case Western Reserve University Institutional Animal Care and Use Committee.
2.6.6.1 T-maze test

We utilized a T-shaped maze for assessing short-term spatial memory. Mice were placed in a plexiglass T-maze (with arms 60 cm in length) and were allowed to explore the maze freely for 10 minutes while one of the arms was closed. The closed arm was switched between animals to avoid any arm preference bias (counterbalanced). Following a 10-minute exploration, mice were returned to their home cage for 2 hours and then put back in the T-maze with all three arms open. Once put in the T-maze, mice were video recorded for 8 minutes using the EthoVision XT tracking system (Noldus Information Technology). The time spent in each arm and total numbers of arm entries were counted by using the video-scoring software. The preference score for memory measurement was calculated as the time spent in the previously closed arm divided by the overall time spent in both arms, which was expected to be 50% by chance.

2.6.6.2 Morris water maze test

The Morris water maze test was used for evaluating long-term spatial memory. Mice were trained in a circular pool (120 cm), in a well-lit room replete of visual cues. The pool water was whitened with non-toxic white dye and the temperature was maintained at 23°C. A clear escape platform (10 cm in diameter) was placed 0.5 cm beneath the water level in the center of a quadrant (north, south, east, or west) of the pool in the same location relative to visual cues in the room. Animals were tested for three trials per day over 4 days. Prior to the beginning of testing, mice were allowed to swim freely in the pool for 30
sec and then allowed to sit on the escape platform for an additional 30 seconds. On days 1–4 of testing, the platform was in quadrant 4 (the northwest quadrant) for all three trials. Mice were placed in the water from one of the four start positions at the edge of each quadrant and allowed to swim for 60 seconds. If mice did not find the platform during the allotted time, they were guided toward it, and held for 15 seconds on the platform. The same procedure was followed for three trials (one from each start position), at which point the mouse was dried and placed back into its home cage (warmed with a heating pad) for 30-40 minutes until the start of the next trial block. Swim time and path length were recorded using a tracking software (ANY-maze, Stoelting Co.). Following the final session, the platform was removed for a probe trial to test for spatial strategy and retention. During the probe test, mice were allowed to swim for 60 seconds without the possibility of escape; the percentage of time spent in the quadrant where the platform was previously located was measured.

2.6.6.3 Fear conditioning test

We utilized a fear conditioning paradigm to assess fear-related memory and its extinction in mice. Mice were placed in a conditioning box (Med Associates, Fairfax, VT) and trained to associate an auditory cue (a pure tone of 5kHz, 80 dB for 30 seconds) with an electrical shock (0.5 mA for 1 second). This procedure was repeated four times with a 180 second accumulation and 60 second inter-stimulus-interval (ISI). The tone and shock were co-terminated. At the end of the trial, mice were taken out and placed back into the box 24 hours later to evaluate their learned aversion for an environment associated with the
shock (context-dependent fear). Mice were placed in the same box in which they were trained for the duration of 6 minutes, and freezing behavior in the absence of tone (conditioned stimulus, CS) or aversive stimulus (unconditioned stimulus, US) was measured. The animals were then removed, and the context was changed so that mice could no longer recognize the chamber in which they had been trained. Two hours later, mice were tested for cue-dependent fear conditioning by reintroducing them into the contextually altered box (shape, lighting, and odor), and freezing behavior was measured during the first 3 minutes to verify that the mice did not recognize the context. After 3 minutes, the tone (30 seconds, 5kHz, 80dB) was delivered 10 times without shock (US) exposure with a 60 second ISI, and freezing behavior was measured to determine cue-dependent fear conditioning and its extinction. Freezing data during each CS exposure were combined into one block of two CS exposures.

2.6.6.4 Sticky paper test

The sticky paper test was selected to test the general tactile sensitivity of the paws of the mice. Small adhesive stimuli (Avery adhesive-backed labels, 5 mm square) were placed on the palmar side of the hind paw, and the time to make contact and remove the adhesive tape was recorded. Each mouse received 1 trial in its home cage, and cage mates were temporarily removed during testing. If the mouse did not remove the tape within 300 seconds, the experimenter removed it.
2.6.6.5 Beam walking test

The beam walking test is the standard protocol for assessing motor coordination in mice. Mice need to traverse a graded series of narrow beams to reach an enclosed safety platform. We provided three types of beams consisting of long strips of wood (50 cm) with a 16-mm round, or 16- or 9-mm-square cross-section. The beams were placed horizontally 30 cm above the bench surface, with one end attached to an enclosed box into which the mouse could escape. Mice were placed at the start of the 16-mm-square beam and trained to traverse the beam to the enclosed box. Then they received three consecutive trials on each of the beams, in each case progressing from the 16-mm-square beam, to the 16-mm-round beam, and then to the 9-mm-square beam. Mice were allowed up to 60 seconds to traverse each beam. The latency to traverse each beam and the number of times the hind feet slipped off each beam were recorded for each trial. Analysis of each measure was based on the mean scores of the three trials for each beam.

2.6.6.6 Rotarod test

To measure the body coordination of mice, the rotarod test (Columbus Instruments, Columbus, OH) was conducted. For the habituation trials, mice were given 2 trials, with the rod (3 cm in diameter) rotating at 0 rpm and at a constant speed (4 rpm) for 60 sec. The habituation data were excluded from the results section. After habituation, each mouse was given 3 trials with a rotating rod accelerating by 0.1 rpm/s from 4 to 40 rpm. Three trials (40 min ITI) were
given during the same day. The latency for the mice to fall from the rotarod was measured.

2.6.6.7 Open field behavior test

The open field consisted of a 50 cm-long square plastic apparatus, closed with 50 cm-high walls, and activity was recorded using ANY-maze video tracking software. The field was digitally divided into inner area (30 cm x 30 cm) and peripheral area (10 cm wide gallery) and time spent in each area was scored. During 10 minutes of testing, data were collected including the distance traveled in meters, time immobile (defined as more than 2 seconds of non-locomotion), and time spent in the inner area. The percentage of time spent in the inner area was calculated as an anxiety index.

2.6.6.8 Object recognition test

We further evaluated the non-spatial, reference memory performance of mice using a novel object recognition paradigm. We conducted 3 trials (each 5 minutes) in the home cage. During the first trial, two identical objects were placed at the corner of the cage (T1) and the mouse was allowed to investigate these objects for 5 minutes. This session was followed by a 1.5 hour delay during which the animals were returned to their home cages with their cage mates. The animals then performed a 5 minute dissimilar stimuli session (T2, short-term memory). In this session, an object that was presented in T1 and another object that was unfamiliar were placed in the test cages. Mice were then returned to their home cages and 24 hours later, the third session was performed (T3, long-
term memory). In this 5 minute session, an object that was presented in T1 and T2 and another object that was unfamiliar were placed in the test cages. The objects were made of hard plastic and/or metal with apparently different shapes. The total amount of time spent to sniff and contact each object were recorded and scored using ANY-maze video tracking software.

2.6.6.9 Statistical Analysis

All data are presented as mean ± the standard error of the mean (Papakonstanti et al.). A two-way analysis of variance (ANOVA) with a within-factor of trials and a between-factor of genotype groups was used to analyze the acquisition data from the Morris water maze, fear conditioning training, cued fear conditioning extinction, beam walking, and rotarod tasks. To analyze the performance in the T-maze, Morris water maze probe trials, sticky paper, object recognition, context-fear conditioning and open field behavior tests, a one-way ANOVA was utilized for genotype group comparisons. Bonferroni corrections were used for post-hoc analysis if required. Statistical significance was set at p<0.05.

2.6.7 Dendritic spine analysis

Cerebella of 8-day-old mouse pups were dissected and neurons cultured as described previously (Zhou et al. 2011). 3x10^6 neurons were plated in 35mm FluoroDish Cell Culture Dishes (WPI). Twenty-four hours later, transfection was carried out using 1.44 ul Lipofectamine 2000 and 432 ng pMaxGFP, the GFP expression plasmid. Fluorescence imaging was conducted three days post transfection using a Leica DMI 6000 microscope.
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2.8 Conflicts of Interests

The authors have no conflict of interests to declare.
CHAPTER 3 – Discussion and future directions

3.1. Summary of my thesis research

In my thesis research, I used our newly generated knock-in mouse model to investigate the molecular and biological functions of \( \text{Nf1} \) exon 23a alternative splicing \textit{in vivo}, with a focus on cognitive behaviors. Specifically, we engineered mutations into the splicing signals surrounding endogenous alternative \( \text{Nf1} \) exon 23a in mouse ES cells, creating \( \text{Nf1}^{-23a\text{IN/23a\text{IN}}} \) mice in which the exon is constitutively included in all tissues (Figure 2.2). We showed that \( \text{Nf1}^{-23a\text{IN/23a\text{IN}}} \) mice displayed increased levels of active Ras compared to WT mice, which correlate with increased \( \text{Nf1} \) exon 23a inclusion (Figure 2.3). This suggests that the molecular activity of \( \text{Nf1} \) as a RasGAP is decreased when exon 23a is included (Figure 2.3). However, unlike \( \text{Nf1}^{-/-} \) mice, which were lethal, \( \text{Nf1}^{-23a\text{IN/23a\text{IN}}} \) mice are viable and appear to be physically normal (Figure 2.1) (Brannan \textit{et al.} 1994, Jacks \textit{et al.} 1994). I further showed that the level of the activated ERK1/2, pERK1/2, is dramatically elevated in the brains of \( \text{Nf1}^{-23a\text{IN/23a\text{IN}}} \) mice (Figure 2.4). These data suggest that alternative splicing of \( \text{Nf1} \) exon 23a functions to modulate its Ras regulatory activity \textit{in vivo}.

Notably, \( \text{Nf1}^{-23a\text{IN/23a\text{IN}}} \) mice performed poorly in both short- and long-term spatial learning, as well as in fear associative learning, compared to WT mice. Thus, our mutant mouse is a very helpful \textit{in vivo} model for future studies on the role of Ras/ERK signaling in learning and memory.

There are many open directions to follow-up my thesis research. It is unclear how, when, and where \( \text{Nf1} \) exon 23a alternative splicing modulates its Ras
regulatory activity in mice. In addition, there are many potential phenotypes of Nf1<sup>23aIN/23aIN</sup> mice that need to be further characterized and analyzed. Future studies addressing these questions will provide insights into the biological role of Nf1 exon 23a alternative splicing.

3.2. Hypotheses and future studies

In the following sections, I will discuss several hypotheses that are most exciting to me to continue this study in the future. I will also propose sets of experiments to test these hypotheses. I classify the future studies into two sections: 1) characterization of additional phenotypes of Nf1<sup>23aIN/23aIN</sup> mice and 2) biochemical and molecular analyses of the Ras activities mediated by expression of Nf1 exon 23a.

3.2.1 Further characterization and analyses of the phenotypes of Nf1<sup>23aIN/23aIN</sup> mice

In my view, learning and memory deficits appear to be the most severe phenotype of Nf1<sup>23aIN/23aIN</sup> mice due to the following reason. Nf1 is highly enriched in the whole brain, and the Nf1 exon 23a splicing in the brain is dramatically different between WT and Nf1<sup>23aIN/23aIN</sup> mice (Figures 2.2). Therefore, the brain functions such as learning and memory are predicted to have the most dramatic phenotypes in mutant mice.

Nf1<sup>+/−</sup> mice displayed impairments in various types of learning and memory such as spatial memory and social learning deficits (Silva et al. 1997) (Costa et al. 2001) (Molosh et al. 2014). These phenotypes were shown to be caused by elevated Ras/ERK1/2 signaling, and could be rescued by manipulations to
decrease Ras signaling (Costa et al. 2002, Li et al. 2005, Mainberger et al. 2013). In my thesis study, I showed that exon 23a inclusion decreases the RasGAP function of Nf1, leading to elevated levels of active Ras in the brain of Nf1^{23aIN/23aIN} mice. Therefore, it would be interesting to see if our mutant mice also displayed a wide range of cognitive phenotypes observed in Nf1^{+/−} mice. In addition, given that NF1 is a tumor suppressor gene and that the hallmark of NF1 disease is tumorigenesis, it is intriguing to determine if Nf1^{23aIN/23aIN} mice are prone to developing tumors. Described below are the details of these future studies to further characterize and analyze the phenotypes of Nf1^{23aIN/23aIN} mice.

**Question 1: Do Nf1^{23aIN/23aIN} mice have social learning deficits and autism spectrum disorder-associated phenotypes?**

A number of studies have demonstrated that 50% of NF1 patients display deficiencies in social information processing and social behaviors, which is a hallmark of autism spectrum disorders (ASDs). In a mouse study, Molosh et al. showed that Nf1^{+/−} displayed a selective social learning deficit and elevated activation of the MAPK in amygdala and frontal cortex, which are important brain structures for regulating social behaviors (Molosh et al. 2014). As shown in chapter 2, the Nf1^{23aIN/23aIN} mice also displayed an elevated level of Ras/p-ERK in their brains, just like what as shown in Nf1^{+/−} mice. Therefore, I hypothesize that our Nf1^{23aIN/23aIN} mice also have deficiencies in social learning ability.

To test this hypothesis, I would perform a three-chamber social memory test. The three-chamber paradigm test has been well employed to study general sociability and interest in social novelty in mouse models of CNS disorders.
(Figure 3.1). The principle of this test is based on the natural tendency that a normal, wild type mouse likes to explore and interact with a novel conspecific than with the familiar one.

To quantitate social tendencies of the test mouse, the main tasks are to measure the time the mouse spends with a novel conspecific and preference for a novel vs. a familiar conspecific. Thus, the experimental design of this test allows evaluation of two critical but distinguishable aspects of social behavior, social affiliation and social memory and novelty. "Sociability" is assessed by the likelihood that the test mouse spends time with another mouse, as compared to time spent alone in an identical but empty chamber. "Preference for social novelty" is defined as the likelihood that the test mouse spends time with a novel mouse rather than with a familiar mouse.
Figure 3.1 Overview of three-chamber test for social behavior. Each of these compartments contains a receptacle that allows an animal to see and smell its content but not to contact it directly. One receptacle contains a conspecific animal and the other is empty or contains an inanimate object.

(3a) In the three-chamber sociability test, the animal is placed in a chamber adjacent to two other chambers. The test monitors the percentage of time that the experimental animal spends exploring each receptacle.
(3b) The three-chamber social memory tests compare the time an experimental animal spends exploring an unfamiliar animal versus the time it spends exploring a familiar animal.
Based on what was shown with $Nf1^{+/}$ mice, I predict that our mutant mice are likely to display social learning deficits. If the $Nf1^{23aIN/23aIN}$ mice do demonstrate social learning deficits, I would like to investigate the molecular changes in amygdala which regulates social learning between WT and mutant mice such as the level of Ras, p-MAKP/p-ERK, LTP, glutamate, and if pharmacological inhibitors could rescue the social learning deficits.

Besides the three-chamber test, I would also like to perform other social behavior tests such as anxiety states in the elevated plus maze and depression or despair in the forced swim test. These social behavior assays will address the question if our $Nf1^{23aIN/23aIN}$ mutant mice recapitulate ASD-relevant phenotypes.

**Question 2: Do $Nf1^{23aIN/23aIN}$ mice have increased tumor susceptibility?**

If $Nf1$ exon 23a inclusion decreases the $Nf1$ RasGAP function in inactivating the oncogene Ras in mice, an obvious follow-up question is whether $Nf1^{23aIN/23aIN}$ mice have increased tumor susceptibility. Therefore, an important future experiment that should be performed is to age a group of mice and monitor them for tumorigenesis. The tumor types might be those that have been shown to previously associate with $Nf1^{+/}$ mice, including pheochromocytomas and leukemia, and other tumor types associated with NF1 disease including neurofibromas and astrocytomas (Jacks et al. 1994).

There is some evidence that $Nf1$ exon 23a inclusion may promote tumor formation. For example, in two distinct studies, it was found that $Nf1$ exon 23a inclusion is increased in sporadic glioblastoma, astrocytoma, and meningioma brain tumors (Suzuki et al. 1991, Tokuyama et al. 1995). However, it is not clear
whether increased \( Nf1 \) exon 23a inclusion alone would be sufficient to lead to tumorigenesis in \( Nf1^{23aIN/23aIN} \) mice. Loss of heterozygosity has been observed in many different tumors associated with human NF1 disease, indicating that the complete loss of NF1 is correlated with tumor formation (Xu et al. 1992, Gutmann et al. 1994, Sawada et al. 1996, Serra et al. 1997, Side et al. 1997, Kluwe et al. 1999, Lau et al. 2000, Rutkowski et al. 2000, Serra et al. 2000). Moreover, homozygous loss of \( Nf1 \) appears to be mandatory for the majority of NF1-related tumor formations in mice (Jacks et al. 1994). For instance, loss of both copies of \( Nf1 \) in astrocytes, as well as an \( Nf1^{+/}\) background, is required for astrocytoma formation in mice (Bajenaru et al. 2002, Bajenaru et al. 2003). Thus, I predict a low chance of seeing tumors in \( Nf1^{23aIN/23aIN} \) mice when aging them.

Nevertheless, it is likely that \( Nf1^{23aIN} \) mutations could speed up tumorigenesis in susceptible or sensitive mouse strains. For example, combining \( p53 \) and \( Nf1^{23aIN} \) mutants might lead to a higher chance of increased tumor formation than either mutant alone, similar to what has been observed in compound \( Nf1^{+/} \) and \( p53^{+/} \) mutants (Cichowski et al. 1999). \( Nf1^{23aIN} \) mutations might also have a high tumor incidence in mice that are sensitized by treatment with carcinogens.

Question 3. Do \( Nf1^{23aIN/23aIN} \) mice have LTP deficits in the hippocampus and amygdala? And could altered Ras signaling by pharmacological and genetic approaches rescue learning deficits observed in \( Nf1^{23aIN/23aIN} \) mice?

As discussed in chapter 1, long-term potentiation (LTP) is a persistent strengthening of synapses based on the recent patterns of activity that produce a long-lasting increase in signal transmission between two neurons. It is one form
of synaptic plasticity, the ability of chemical synapses to change their strength. Since memory is encoded by modifications of synaptic strength LTP is widely considered to be one of the major molecular mechanisms regulating memory (Karban et al. 2015)

Because \(Nf1^{+/−}\) mice displayed impairments in LTP caused by elevated Ras levels, I would also like to perform electrophysiological tests to determine whether amygdala or hippocampal slices of \(Nf1^{23aIN/23aIN}\) mice have decreased LTP (Costa et al. 2002). In addition, some PNS neurons of \(Nf1^{+/−}\) mice show changes in excitability or expression of ion channels (Wang et al. 2005, Hingtgen 2008, Wang et al. 2010, Hodgdon et al. 2012). Therefore, I would also like to test if there are alterations in excitability or expression of ion channels in hippocampal neurons isolated from \(Nf1^{23aIN/23aIN}\) mice.

As the learning deficits and impaired LTP seen in \(Nf1^{+/−}\) mice were rescued by decreasing the active Ras level, I would like to determine whether pharmacological treatment and genetic crossing to decrease Ras signaling could rescue any learning deficits observed in \(Nf1^{23aIN/23aIN}\) mice, or improve molecular indicators of learning, such as LTP in hippocampal slices.

To address these questions, \(Nf1^{23aIN/23aIN}\) mice will be first infused with Ras or ERK inhibitors such as lovastatin or FTase into specific brain regions. They will then be trained and tested with the different learning paradigms. Comparing the learning and memory ability of WT to mutant mice might suggest that the cognitive deficits are due to altered Ras signaling. To overcome the off-target effects of pharmacological inhibitor treatment, another approach is to cross
Nf1\textsuperscript{23aIN/23aIN} mice (elevated Ras) with the genetic background of Ras/ERK-deficient mice to test the learning and memory in the offspring mice.

3.2.2 Investigation into the biochemical and molecular mechanisms, and biological functions of Nf1 exon 23a alternative splicing as a RasGAP

My thesis study described in chapter 2 strongly suggests that skipping of exon 23a increases the inactivation of Ras by Nf1. In addition, previous studies from our lab have revealed that very complex molecular mechanisms are in place to regulate Nf1 exon 23a inclusion (Zhu et al. 2008, Barron et al. 2010, Zhou et al. 2011, Barron et al. 2012, Fleming et al. 2012). I therefore hypothesize that the tight regulation of Nf1 exon 23a inclusion evolved as a mechanism for providing appropriate Ras signaling to maintain the functions specific to certain cell types and developmental stages. Thus, follow-up studies to address this hypothesis should focus on elucidating when, where, and by which mechanism Nf1 exon 23a alternative splicing modulates its Ras regulatory activity in mice.

**Question 1: What is the differential expression of WT Nf1 exon 23a splicing isoforms in different tissues, cell types, developmental stages, and physiological conditions?**

As shown in chapter 2, skipping of exon 23a inactivates the RasGAP activity of Nf1 in in vivo (Figure 2.3). Therefore, it is expected that the suppression of Ras signaling by Nf1 is particularly functionally important in the cell types, tissues, developmental stages, and physiological states in which endogenous Nf1 exon 23a is skipped. Cells or tissues in which Nf1 exon 23a is normally skipped are likely to have the most dramatic phenotypes in Nf1\textsuperscript{23aIN/23aIN} mice.
Thus, to look for phenotypes in $Nf1^{23alN/23alN}$ mutant mice, it is critical to precisely understand when, where, and under which physiological conditions $Nf1$ exon 23a is skipped. The first step toward all these aforementioned follow-up studies is to gain a baseline of WT $Nf1$ exon 23a alternative splicing patterns.

Semi-quantitative RT-PCR is currently the most common method to analyze WT $Nf1$ exon 23a alternative splicing patterns. It is similar to what was done in the experiments of my thesis research. Shortly after $NF1$ was cloned, several studies examined $Nf1$ isoform expression in different tissues and cell types using non-quantitative RT-PCR, or an antibody specific to the peptide encoded by $NF1$ exon 23a (Mantani et al. 1994, Gutmann et al. 1995) (Costa et al. 2002). The experiments are reliable for detecting the presence or absence of a particular isoform in a tissue, but not for quantitating the relative abundance of the two $Nf1$ isoforms. It would be beneficial to repeat the experiments using semi-quantitative RT-PCR, similar to what was done in the experiments of my thesis research.

I would also be interested in precisely quantitating the differential expression of WT $Nf1$ isoforms in various cell types within the nervous system using RT-PCR. A number of previous studies and ours clearly showed that $Nf1$ exon 23a is predominantly skipped in most neurons (Mantani et al. 1994, Gutmann et al. 1995, Metheny et al. 1996, Zhou et al. 2011). However, it remains elusive that $Nf1$ exon 23a is predominantly skipped only in neurons, or in every neuron type within the nervous system. The inclusion of $Nf1$ exon 23a is barely detected in whole brain (Andersen et al. 1993, Danglot et al. 1994, Mantani et al. 1994, Gutmann et al. 1995). This suggests that either $Nf1$ exon 23a is also skipped in
other cell types of the brain, or that the total expression of Nf1 mRNA is much higher in neurons than in other cells. Thus, I would like to compare the levels of Nf1 exon 23a inclusion in primary neurons, astrocytes, and oligodendrocytes. One experiment using murine neocortical cultures found predominant exon 23a inclusion in mixed glia cultures, but did not use quantitative methods (Gutmann et al. 1995). In the PNS, a single experiment indicated that the inclusion of Nf1 exon 23a increases upon differentiation of a Schwann-like cell line. However, it would be useful to repeat these experiments using quantitative methods in primary Schwann cells, the cell type implicated in neurofibroma formation (Gutmann et al. 1993). Determining the expression pattern of Nf1 exon 23a in specific cell types might give hints as to in which cell types Nf1 exon 23a skipping is particularly important.

More importantly, I would like to determine and map the relative expression of Nf1 isoforms within specific regions of the brain where Nf1 has been shown to have important cognitive functions, such as the hippocampus and amygdala. Although exon 23a skipping predominates across various brain regions such as the cerebellum, cortex, hippocampus, and brain stem as shown in supplemental Figure S2.1A, it is not certain that this splicing phenotype is the same in every part of these brain structures. Indeed, CA3 region of the hippocampus was shown to have more exon 23a inclusion than other CA1 and DG regions using immunohistochemistry (Costa et al. 2001). Given the important function of the hippocampus in learning and memory, I would like to quantitatively examine the relative abundance of Nf1 exon 23a splicing isoforms in three hippocampus
regions CA1, CA3, and DG. A direct approach to address this question is to isolate three hippocampus regions CA1, CA3, and DG, then perform RT-PCR to quantitate two Nf1 exon 23a isoforms. However, one technical difficulty arising with this approach is that it is very difficult to accurately dissect out three hippocampus regions. To overcome this technical difficulty, I would like to perform the BaseScope Assays (Advanced Cell Diagnostics), a novel in situ hybridization (ISH) to visualize single RNA molecules for splice variants. This assay utilizes RNA-specific probes that align to exon junction and multi-step signal amplification to detect single RNA transcript. The experiment starts with properly prepared paraffin-embedded brain tissues from WT and mutant Nf1<sup>23aIN/23aIN</sup> mice, which will be sectioned by microtome. Brain sections of hippocampus mounted on glass are first pretreated, and then exon 23a junction-specific probes are hybridized to Nf1 transcripts. Under a common bright field microscope at 40-100X magnifications, each single RNA transcript appears as a distinct dot. Counting the dots from experiments using different probes specific to two Nf1 exon 23 splicing isoforms will allow an accurate quantitation of relative abundance of these isoform in distinct hippocampus regions.

In short, it is critical to gain an accurate quantitation of differential expression of Nf1 exon 23a splicing isoforms and a precise understanding of when and where wildtype Nf1 exon 23a is included.

**Question 2: What is the role of Nf1 RasGAP in the dynamic regulation of Ras/ERK during learning? Does Nf1 exon 23a alternative splicing change during learning? If so, what is its role in learning?**
Alternative splicing is a dynamic process and exon inclusion within a cell type can change in response to changes in physiological state (Xie et al. 2001, An et al. 2007, Lee et al. 2007, Schor et al. 2009). Indeed, our previous study showed that Nf1 exon 23a inclusion decreases in response to depolarization of cardiomyocytes caused by increased Ca\(^{2+}\) (Sharma et al. 2014).

My thesis research and other studies show that increased Ras/ERK signaling is required for various learning paradigms, such as spatial long-term memory tests. Therefore, I hypothesize that Nf1 exon 23a inclusion efficiency is altered upon various learning paradigms known to require increased Ras/ERK signaling.

To test this hypothesis, I would train a group of age and sex-matched WT mice based on a learning paradigm that was shown to require increased Ras/ERK signaling. A Morris Water Maze, a long-term spatial memory test, is a good learning paradigm to test this hypothesis. A time-course experiment will be performed to analyze the Nf1 RasGAP levels and Nf1 exon 23a alternative splicing patterns in specific brain regions. Western blots will be used to determine the Ras protein level. RT-PCR and in situ hybridization described earlier will be utilized to study the Nf1 exon 23a alternative splicing patterns.

**Question 3: Does the level of Nf1 exon 23a inclusion correlate with the active Ras level in vivo?**

In order to address this question, the levels of active Ras should be measured in more tissues, cell types, developmental stages, physiological states, and specific brain regions of Nf1\(^{23aIN/23aIN}\) mice, particularly those in which WT Nf1 exon 23a is skipped. For example, is there also an increase in active Ras levels
in the testes or muscle of Nf1^{23aIN/23aIN} mice, where levels of inclusion of WT Nf1 exon 23a are similar to those seen in brain (Figure 2.2)? Is there little change in active Ras levels in Nf1^{23aIN/23aIN} lung or kidney, where Nf1 exon 23a inclusion levels are already high in WT? If changes in Ras-GTP levels were to correlate with the magnitude of the difference in exon 23a inclusion between WT and Nf1^{23aIN/23aIN} tissues, it would argue more strongly for a role for Nf1 exon 23a inclusion in regulating Ras-GAP activity. However, the differences in total Nf1 protein levels must be taken into account when performing these analyses.

**Question 4. What is the molecular mechanism by which exon 23a inclusion into Nf1 GAP-related domain changes the RasGAP activity of Nf1?**

Another interesting question is how Nf1 exon 23a inclusion can change the RasGAP activity of Nf1. What is the precise mechanism by which this occurs? Is it because of gain of function of exon 23a?

One group that did a structural analysis of the Ras-GAP domain of NF1 noted that NF1 exon 23a lies in an exposed portion of the NF1 GAP domain. This finding together with the presence of many charged amino acids within the exon, led this group to hypothesize that the exon could be involved in protein-protein interactions (Scheffzek et al. 1998).

Thus, an interesting experiment is to immunoprecipitate (co-IP) two endogenous neurofibromin protein isoforms from brain lysates of Nf1^{23aIN/23aIN} and Nf1^{23a-/-} mice using a Nf1-specific antibody, then compare the interacting partners by performing mass spectrometry. Proteins that interact specifically with one isoform but not the other will provide hints on the functional differences
between the two protein isoforms. We can generate \( \text{Nf1}^{23a/-} \) mice by crossing the \( \text{Nf1}^{23aIN/23aIN} \) mice, which contain loxP sites surrounding exon 23a, with Cre-expressing mice. Very recently, high-quality NF1 antibodies provided by Abcam and Bethyl were used to detect NF1 protein–protein interactions in unaltered in vivo conditions by affinity purification coupled mass spectrometry (Li et al. 2017). These antibodies will be great reagents to perform my proposed co-IP experiment. Another way to do this experiment is to isolate neurons (nearly 100% exon 23a skipping) and glia cells (nearly 100% exon inclusion) from WT mouse brains. Neuron and glia cell lysates would then be used to perform co-IP and mass spectrometry. However, this experiment would need high-quality antibodies that have epitope tags specific to each Nf1 protein isoform.

Another question is to determine if \( \text{Nf1} \) exon 23a inclusion may impact RasGAP activity of Nf1 protein by affecting its localization. Nf1 is mainly present within the cytoplasm, but was shown to shuttle between the cytoplasm and nucleus (Vandenbroucke et al. 2004). Particularly in neurons, Nf1 is localized over the cell body and axons, but is highly expressed in dendrites (Nordlund et al. 1993). Nf1 also associates with microtubules through a portion of its GAP domain (Gregory et al. 1993, Xu et al. 1997). If exon 23a inclusion alters the localization of Nf1 by relocating it from the cytoplasm to the nucleus, it could alter the regulation of Ras in the cytoplasm. This localization shuttling in neurons would significantly affect the RasGAP activity in axons and dendrites, where all neuronal activities occur. One study pointed out that the sequence \( \text{Nf1} \) exon 23a shares the similarities with the nuclear localization signals of other proteins.
(Andersen et al. 1993). Thus, I would like to investigate the subcellular localization of Nf1 protein isoforms in different areas such as the hippocampus, amygdala, and prefrontal cortex.

One approach to study localization is to perform immunohistochemistry using antibodies specific to different Nf1 protein isoforms in the brain slices of three different mouse genotypes \((Nf1^{+/+}, \ Nf1^{23aIN/23aIN}, \ and \ Nf1^{23a/-} \ mice)\). Another approach is to culture neurons isolated from the brains of \(Nf1^{+/+}, \ Nf1^{23aIN/23aIN}, \ and \ Nf1^{23a/-}\), and then perform immunofluorescence experiments to compare the subcellular localization of Nf1 protein.

### 3.3 Conclusions

In summary, using our knock-in mouse models, I demonstrated that \(Nf1\) exon 23a alternative splicing plays an essential role in the regulation of Ras/ERK signaling and in proper learning and memory. Our mouse models will serve as useful tools for future studies into the potential downstream biological consequences of \(Nf1\) alternative splicing, such as alterations in learning and tumor susceptibility.
CHAPTER 4 – Appendices

Besides the main project, I also carried out other projects. They are preliminary data and need to be further studies.

4.1 Part 1: Calcium-mediated histone modifications regulate intron retention in cardiomyocytes

4.1.1 Introduction

Calcium is an important intracellular second messenger that regulates many biological processes. Many extracellular environmental cues lead to cellular calcium level changes, which impacts on the output of gene expression. In cardiomyocytes, calcium is known to control gene expression at the level of transcription, while its role in regulating alternative splicing has not been well studied (Xie et al. 2001, Black et al. 2003, An et al. 2007, Lee et al. 2007, Schor et al. 2009, Schor et al. 2013).

Alternative splicing is an important mechanism that increases functional diversity by allowing for the production of multiple different mRNAs from the same pre-mRNA, which can then be translated into proteins that have different functions. It was once thought that alternative splicing is rare, but it is now known that over 90% of human genes are alternatively spliced (Pan et al. 2008, Wang et al. 2008). Alternative splicing can take many forms, such as alternative 3’ or 5’ splice site usage, intron retention, mutually exclusive exons, and cassette exons (Black et al. 2003).

We have recently demonstrated that elevated intracellular calcium levels lead to chromatin, resulting in changes in alternative splicing patterns (Sharma et al. 2014) (Sharma et al. 2015). Specifically, increased calcium will cause histone
hyperacetylation along gene bodies, leading to faster transcriptional elongation rate and alternative splicing changes (Figure 4.1). These results are consistent with the kinetic coupling model proposed by the Kornbluht group suggesting that faster elongation rate promotes exon skipping.
Figure 4.1 Model of calcium-induced histone modification changes that regulate alternative splicing. A. Increased calcium levels activate CaMK and PKD, which phosphorylate class II HDACs, causing their translocation from the nucleus to the cytoplasm. B. The disrupted HAT-HDAC balance leads to histone hyperacetylation, which relaxes chromatin, resulting in an increase in RNA Poll II transcriptional elongation rate (Sharma et al., PNAS, 2014).
Exon skipping is one form of alternative splicing. We hypothesize that other forms of alternative splicing events may also be affected by a similar mechanism. Intron retention is receiving a lot of attention lately in the alternative splicing field. Genome-wide alternative splicing analysis has revealed a surprisingly high frequency of intron retention in transcripts of multiexonic genes in mammals. Intron retention-coupled NMD has been shown as a key regulator of gene expression (Braunschweig et al. 2013, Genome Res. 2014, and Guo, et al., Molecular Cell. 2014). In one part of my thesis research, I studied the mechanisms by which calcium-mediated regulation of intron retention at the epigenetic level.

4.1.2 Results

4.1.2.1. Intron retention events are affected by increased calcium.

In a high-throughput RNA-seq analysis comparing KCl- and NaCl-treated cardiomyocytes, we found that indeed, intron retention events are affected by KCl treatment. We filtered out significant alternative splicing events by setting the PSI (Percent Spliced In) value greater than 30% and found 91 introns that changed splicing pattern (Table 4.1). Notably, 94% of 91 intron retention events show more retention after KCl treatment.

4.1.2.2. Calcium induces intron retention of Dvl1 and Gdi1 in both ES-derived cardiomyocytes and primary cardiomyocytes

To validate the RNA-seq output, I selected two targets among 91 intron retention events, Dvl1 and Gdi1, to perform RT-PCR assays. They both showed more intron retention in KCl-treated cardiomyocytes, and their splicing was
restored when KCl is removed. Figures 4.2 and figure 4.3 show the results using ES-derived and primary mouse cardiomyocytes, respectively.
Table 4.1: KCl induces changes in alternative splicing events in cardiomyocytes

<table>
<thead>
<tr>
<th>Type of alternative splicing</th>
<th># of events</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cassette exons</td>
<td>202</td>
</tr>
<tr>
<td>Retention of introns</td>
<td>91</td>
</tr>
<tr>
<td>Alternative 3’ splice sites</td>
<td>37</td>
</tr>
<tr>
<td>Alternative 5’ splice sites</td>
<td>22</td>
</tr>
<tr>
<td>Mutually exclusive exons</td>
<td>12</td>
</tr>
</tbody>
</table>
Figure 4.2 KCl induces intron retention of Dvl1 and GdiI in ES-derived cardiomyocytes. Semiquantitative RT-PCR analysis of the alternative splicing of Dvl1 intron 3 and GdiI intron 5 using total RNA isolated from ES-derived cardiomyocytes treated with 100 mM NaCl, KCl, or KCl followed by recovery. The numbers indicate the % inclusion of retained intron transcripts relative to total ones. The arrows show the locations of RT-PCR primers.
Figure 4.3 KCl induces intron retention of Dvl1 and GdiI in primary cardiomyocytes. Semiquantitative RT-PCR analysis of the alternative splicing of Dvl1 intron 3 and GdiI intron 5 using total RNA isolated from primary cardiomyocytes treated with 100 mM NaCl, KCl, or KCl followed by recovery. The numbers indicate the % inclusion of retained intron transcripts relative to total ones. The arrows show the locations of RT-PCR primers.
4.1.2.3 Calcium-mediated intron retention is controlled by the CaMKIIgama and PKD1 signaling pathways.

To investigate the signaling pathways involved in the calcium-mediated changes in intron retention patterns, we treated the primary cardiomyocytes with inhibitors blocking the functions of calcium signaling molecules. Specifically, we used Gö6976 targeting PKC/PKD and KN93 targeting CaMKII, in conjunction with KCl treatment. Figure 4.4 shows that KCl-induced intron retention is mediated through either the CaMKIIgama or PKD1 signaling pathways, similar to the previously described exon skipping events (Sharma et al. 2014).
Figure 4.4 Calcium-mediated intron retention is controlled by the CaMKIIgamma and PKD1 signaling pathways. In addition to the treatments of 100 mM NaCl or KCl, primary cardiomyocytes were treated with the PKC/PKD inhibitor Gö6976 (10 μM) and the CaMK inhibitor KN93 (1 μM) individually or in combination. Semiquantitative RT-PCR analysis of the alternative splicing of Gdil intron 5 using total RNA isolated from primary cardiomyocytes with different treatment. The numbers indicate the % inclusion of the retained intron transcript relative to total ones. The arrows show the locations of the RT-PCR primer.
4.1.3 Discussions and future experiments

Intron retention is widely perceived as an aberrant splicing event with little or no functional consequence. However, recent work has now shown that intron retention is used as a means to regulate gene expression post-transcriptionally by coupling it to nonsense-mediated mRNA decay (NMD) (Wong et al., Cell 2013). Most importantly, intron retention coupled NMD is deregulated in disease. However, the mechanisms underlying this deregulation of intron retention coupled NMD are not well understood.

I hypothesize that the model shown in Figure 4.1 can also explain for intron retention events. Thus, I would follow the layout of our published paper (Sharma et al., PNAS 2014) to study the regulatory mechanism of intron retention by examining calcium signaling molecules, HATs and HDACs accumulation, and Pol II accumulation and elongation rates. Intron retention can cause non-sensed media decay (NMD), adding another layer of gene expression control. Therefore, I would like to study the expression level of proteins that are encoded by intron-retained transcript, compared to the control (NaCl treatment).
4.2 Part 2: DBC1-mediated epigenetic regulation of alternative splicing of the neurofibromatosis type 1 pre-mRNA exon 23a.

4.2.1 Introduction

The research on splicing regulation has been traditionally focused on cis-acting RNA elements such as splicing enhancers and silencers, and trans-acting protein splicing factors, which have been mostly studied independent of chromatin context. An important recent discovery is that epigenetic regulation is not limited to controlling what regions of the genome are expressed, but also how they are spliced. A number of studies have shown that nucleosome occupancy, histone modifications, and transcriptional elongation rate impact on splicing outcome. However, the opposite direction in which chromatin structure is altered by splicing machinery has not been well studied.

Recently, Close et al. have shown that DBIRD complex formed by DBC1 and ZIRD regulates alternative pre-mRNA splicing by increasing the RNAP II transcriptional elongation rate. Specifically, DBIRD complex increases the transcriptional elongation rate across a subset of A/T-rich alternative exons, promoting exon skipping. Knockdown of DBIRD results in a decrease in transcriptional elongation surrounding affected exon regions (Close et al. 2012).

A/T-rich DNA sequences will be transcribed into AU-rich sequences on pre-mRNA based on the principle of complementary base pairing. We previously showed that AU-rich sequences on pre-mRNA were binding sites for Hu proteins to suppress the inclusion of alternative exon 23a of the neurofibromatosis type 1 (NF1) (Zhu et al. 2008), (Zhou et al. 2011). This connection suggests the possibility that DBIRD is involved in the splicing regulation of NF1 exon 23a.
Moreover, we previously demonstrated a unique alternative pre-mRNA splicing mechanism by which Hu proteins directly interact with HDAC2 and inhibit its deacetylation activity to induce local histone hyperacetylation surrounding alternative exon 23a of the NF1 pre-mRNA. Consequently, Hu proteins increase the local transcriptional elongation rate around NF1 exon 23a, decreasing the inclusion of this exon (Zhou et al. 2011). Thus, both DBIRD complex and Hu protein were shown to actively modulate the transcriptional elongation rate around AU-rich alternative exons (Zhou et al. 2011), (Close et al. 2012).

These connections between DBIRD complex and Hu proteins prompted us to study the global targets of DBIRD-dependent alternative exons generated by a splicing sensitive microarray upon the knockdown of DBC1 and ZIRD. Among over 3000 targets found, alternative exon 23a of the NF1 pre-mRNA was identified to be one of the potential DBC1-dependent exons (Close et al. 2012). However, Close et al. did not perform experiments to verify the involvement of DBIRD complex in the regulation of NF1 exon 23a splicing. Therefore, one goal of my thesis work was to determine if DBIRD complex plays a role in regulating NF1 exon 23a splicing, and if so, by which mechanisms.

4.2.2 Background about Hu proteins and DBIRD complex

4.2.2.1 Hu proteins

4.2.2.1.1 Discovery of Hu proteins

The mammalian Hu protein family encompasses four closely related RNA binding proteins that share homology with the Drosophila ELAV protein: HuR, HuB, HuC, and HuD (Good 1995). The Hu protein family was named after a
patient with a form of paraneoplastic neurologic degeneration in which ectopic expression of these proteins in small cell lung cancer tumors results in the production of antibodies that attack the nervous system (Graus et al. 1986, Dalmau et al. 1990, Szabo et al. 1991, Musunuru et al. 2001). All four proteins share a similar domain structure, consisting of three highly conserved RNA recognition motifs, a hinge region, and a divergent amino (N)-terminus (Okano et al. 1997).

4.2.2.1.2 Biological functions of Hu proteins


Though the mechanisms underlying the cytoplasmic functions of Hu proteins have been studied extensively, the nuclear functions of Hu proteins, including splicing regulation, were discovered more recently and are poorly understood. Because of recent genome-wide studies involving HuR, HuC, and HuD, we now appreciate that the regulation of splicing by Hu proteins is widespread, but only a handful of splicing targets of Hu proteins have been studied in detail (Lebedeva et al. 2011, Mukherjee et al. 2011, Ince-Dunn et al. 2012). Hu proteins positively regulate the inclusion of some exons and suppress the inclusion of others, although they bind to similar RNA sequences surrounding both types of exons (Licatalosi et al. 2008, Xue et al. 2009, Witten et al. 2011, Charizanis et al. 2012). Studies so far indicate that Hu proteins suppress inclusion of target exons by binding to U-rich sequences within or surrounding the exon and blocking the action of core splicing factors. For example, the neuron-specific Hu proteins bind to U-rich sequences upstream and downstream of NF1 exon 23a and inhibit the interaction of key spliceosomal components including U1 snRNP, U6 snRNP, and U2AF65 with the Nf1 pre-mRNA. Similarly,
HuR binds to an exonic U-rich sequence to prevent the interaction of U2AF65 with the Fas pre-mRNA (Izquierdo 2008, Zhu et al. 2008). Hu proteins often antagonize the actions of TIA-1/TIAR, another group of splicing regulatory proteins that bind to U-rich sequences (Zhu et al. 2006, Izquierdo 2008, Zhu et al. 2008, Izquierdo 2010, Izquierdo 2010). Whether Hu proteins inhibit the activity of splicing factors by simply binding to and blocking their binding sites or through more complex mechanisms is not fully understood. Another layer by which Hu proteins can act is to affect local histone acetylation and transcriptional elongation rate to regulate exon inclusion (Zhou et al. 2011).

4.2.2.2 DBC1

4.2.2.2.1 Discovery of DBC1 protein

The DBC1 gene was first found to be absent in human chromosome 8p21 in breast cancer; thus, it was named Deleted in Breast Cancer-1 (DBC1) (Hamaguchi et al. 2002). DBC1 is easily confused with another DBC1 protein, which stands for deleted in bladder cancer-1. Additionally, DBC1 was named with many other abbreviations such as KIAA1967, p30 DBC protein, P30DBC1, and NET35. DBC1 is a paralogue of the cell division cycle and apoptosis regulator protein 1, named CCAR1 (Anantharaman et al. 2008).

4.2.2.2.2 Structure and functional organization of DBC1

DBC1 encodes a 923 amino acid protein, containing multiple-domains such as a nuclear localization signal (NLS), a RNA-binding domain and a leucine zipper (LZ) at the amino-terminus, an inactive EF hand (calmodulin-like calcium-binding region), a coil-coiled (CC) domain at its carboxyl-terminus, and two
globular regions (Nudix) as described in Figure 4.5. The presence of different domains suggests the involvement of DBC1 in numerous biological processes (Kim et al. 2009).

![Functional structure of DBC1](image)

**Figure 4.5 Functional structure of DBC1 (Kim et al. 2009)**

4.2.2.2.3 Molecular and cellular functions of DBC1

Due to its original identification, DBC1 was thought to play important roles in the tumor development and progression. To date, however, no direct evidence has been shown if DBC1 is a tumor suppressor or a tumor promoter. Indeed, some studies reported that DBC1 was found in several cancer cell types and tumor tissues (Hamaguchi et al. 2002) (Kim et al. 2009).

Although DBC1 was identified more than a decade ago, its various molecular and cellular functions have just been discovered.

* **DBC1 as an endogenous inhibitor of epigenetic modifiers and nuclear receptors**
  
  - DBC1 is a key regulator of SIRT1 and HDAC3

  DBC1 is well known for its association with the SIRT1 protein deacetylase, the mammalian ortholog of yeast sir2. DBC1 was shown to directly interact with...
SIRT1 and inhibit SIRT1 activity both \textit{in vitro} and \textit{in vivo} (Zhao et al. 2008) (Kim et al. 2008). Thus, it functions as an endogenous inhibitor of SIRT1 in human cells. The leucine zipper motif of DBC1 and the catalytic domain of SIRT1 are required for the DBC1-SIRT1 interaction (Zhao et al. 2008). Besides SIRT1, another member of the class I histone deacetylase family, HDAC3, was shown to directly interact with DBC1, and its deacetylase activity was also inhibited by DBC1 (Chini et al. 2010). The N terminus of DBC1 and the C terminus of HDAC3 are required for the DBC1-HDAC3 interaction (Chini et al. 2010). Besides SIRT1 and HDAC3, DBC1 also interacts with HDAC1 but not with HDAC4-HDAC7 (Kim et al. 2008). Notably, although DBC1 also associates with HDAC1, it does not inhibit HDAC1 activity (Chini et al. 2010). This demonstrates the specificity of DBC1 in inhibiting the activities of several certain deacetylase enzymes, including SIRT1 and HDAC3.

Studies conducted in DBC1 knockout mice indicated that the enzymatic activities of HDAC3 and SIRT1 were higher in brain, liver, pancreas, and spleen tissues from DBC1 knockout mice than in wild-type animal counterparts (Escande et al. 2010). These studies demonstrated that DBC1 is a potential tumor suppressor, making it an attractive candidate for pharmacological targeting (Escande et al. 2010).

Very recently, DBC1 has been shown to interact with acetylated histone H4 at lysine 12 to protect it from the activity of HDACs (Nakayasu et al. 2013). This finding also reveals a new function of DBC1 as a checkpoint of gene expression control in inflammatory response (Nakayasu et al. 2013).
DBC1 regulates the methyltransferase SUV39H1.

Interestingly, DBC1 was shown to directly associate with the catalytic domain of SUV39H1, a histone H3K9-specific methyltransferase, and inhibit its ability to methylate histone H3 both in vitro and in vivo (Li et al. 2009) This study showed that DBC1 disrupts SUV39H1-SIRT1 interaction and inactivated the enzymatic activities of both SUV39H1 and SIRT1. Therefore, DBC1 is proposed as a regulator of chromatin modifications (Li et al. 2009).

*DBC1 as a regulator of nuclear receptors*

DBC1 interacts and regulates the stability and function of several nuclear receptors including the estrogen (ER), the androgen (AR), and the retinoic receptors (Garapaty et al. 2009) (Fu et al. 2009) (Koyama et al. 2010) (Trauemicht et al. 2008). In general, DBC1 functions as a co-activator for these receptors and these interactions could be either ligand-dependent or ligand-independent. Notably, DBC1 was demonstrated to enhance the AR DNA-binding activity in vitro but did not show the DNA-binding activity itself (Fu et al. 2009).

Very recently, DBC1 has been shown to interact with Rev-erbα to modulate its stability and function in circadian regulation of cell metabolism and clock genes (Chini et al. 2013). Notably, DBC1 has been shown to directly interact with a number of proteins but does not modulate the function and/or stability of all its interacting partners. Specifically, DBC1 was reported to directly associate with ZIRD but does not required for its stability (Close et al. 2012) Similarly, DBC1 does not significantly important for AR stability (Fu et al. 2009). This demonstrates the specificity of DBC1 in regulating protein stability.
*DBC1 as a splicing regulator*

As described earlier, DBC1 is also involved in the regulation of alternative pre-mRNA splicing as shown by Close *et al.* The presence of S1-like RNA-binding domain suggests an emerging role of DBC1 in splicing regulation that is still poorly understood.

In short, DBC1 is a multi-tasking protein; yet its molecular and cellular functions remain to be elucidated.

![Diagram showing various functions of DBC1](image)

**Figure 4.6 Various functions of DBC1**

4.2.2.3 ZIRD

Zinc-finger-containing protein ZNF326 interacts with nuclear mRNPs and DBC1; thus, it was named ZIRD (Close *et al.* 2012). ZIRD has not been studied in human cells before; however, its mouse protein homologue was shown to enrich in neurons (Lee *et al.* 2000)
4.2.3 Results

4.2.3.1 DBC1 regulates the alternative splicing of NF1 exon 23a

Since NF1 exon 23a was identified to be one of the potential DBC1-dependent alternative exons (Close et al. 2012), I am interested in determining if DBC1 and ZIRD by themselves regulate the NF1 exon 23a splicing.

To address this question, I overexpressed Flag-DBC1 and Flag-ZIRD with NF1 reporter in HeLa cells to study the splicing of NF1 exon 23a reporter. The human NF1 exon 23a reporter construct was made by inserting exon 23a with its flanking region into the first intronic region of HMT gene. The NF1 construct was linked with virus promoter to facilitate its expression.

Overexpression of DBC1 in HeLa cells increases skipping of NF1 reporter exon 23a. In addition, DBC1-knockout brain tissues show increased inclusion of Nf1 exon 23a. Therefore, DBC1 alone is sufficient to regulate NF1 exon 23a splicing.
Figure 4.7 Abundant expression of DBC1 represses the NF1 reporter exon 23a inclusion in HeLa cells. HeLa cells were transfected with the increasing amount of Flag-DBC1 plasmid from 0 µg to 6 µg with 0.5 µg NF1 reporter. Control plasmid was added to make 6.5 µg plasmids in total for each transfection. Upon two days of transfection, HeLa cells were harvested to isolate total RNA and proteins. A. Western blot analysis using anti-Flag antibody to detect Flag-DBC1. GAPDH was used as a loading control. B. RT-PCR assay to measure the splicing of the NF1 reporter exon 23a. Error bars represent SD, N = 2.
Figure 4.8 DBC1-knockout brain tissues show increased inclusion of Nf1 exon 23a
4.2.3.2 ZIRD alone does not regulate the alternative splicing of NF1 exon 23a

The titration experiments of ZIRD were conducted similar as those of DBC1. The result showed that high expression of ZIRD alone does not regulate the splicing of the NF1 reporter exon 23a (Figure 4.9).
Figure 4.9 ZIRD alone does not regulate NF1 exon 23a alternative splicing. HeLa cells were transfected with the increasing amount of Flag-ZIRD plasmid from 0 µg to 6 µg with 0.5 µg NF1 reporter. Control plasmid was added to make 6.5 µg plasmids in total for each transfection. Upon two days of transfection, HeLa cells were harvested to isolate total RNA and proteins.

- A. Western blot analysis using anti-Flag antibody to detect Flag-ZIRD (GAPDH was used as a loading control.)

- B. RT-PCR assay to measure the splicing of the NF1 reporter exon 23a (Error bars represent SD, N = 3.)
4.2.3.3 The N terminal 800 amino acids of DBC1 are required for the regulation of NF1 exon 23a splicing

To pinpoint which domain of DBC1 protein is the most important for the regulation of NF1 exon 23a splicing, I overexpressed the Flag-DBC1 and NF1 reporter in HeLa cells. My data showed that the 1-800 amino acids of DBC1 are sufficient for NF1 exon 23a splicing. The C terminus (coiled coil domain) of DBC1 is not important for NF1 exon 23a splicing.
A. Domain mapping of DBC1

B. The amino acids 1-800 of DBC1 are required for the regulation of NF1 exon 23a splicing.

Figure 4.10 RT-PCR assay to measure the splicing of the NF1 reporter exon 23a. HeLa cells were transfected with the full-length Flag-DBC1 and truncation mutant Flag-DBC1 plasmids with NF1 reporter. Upon two days of transfection, HeLa cells were harvested to isolate total RNA and proteins.
Figure 4.11 All full-length and truncation mutants Flag-DBC1 protein express in HeLa cells. HeLa cells were transfected with the full-length Flag-DBC1 and truncation mutant Flag-DBC1 plasmids with NF1 reporter. Upon two days of transfection, HeLa cells were harvested to isolate for total proteins. Anti-Flag antibody was used for western blot.
4.2.3.4 DBIRD complex interacts with Hu proteins

Given that DBIRD complex was shown to present at several AT-rich alternative exon regions (Close et al. 2012) and Hu proteins were shown to enrich at the NF1 exon 23a areas (Zhou et al. 2011), I am particularly interested in determining if DBIRD complex presents at NF1 exon 23a and interacts with Hu proteins, and if they act together to regulate the splicing of the NF1 exon 23a.

I started with co-immunoprecipitation (co-IP) assays to test if HuC interacts with DBC1 and ZIRD by co-overexpressing Myc-HuC plasmids with Flag-DBC1 and Flag-ZIRD plasmids in HeLa cells. The result showed that HuC interacts with both DBC1 and ZIRD in an RNA-independent manner (Figure 4.12).

Hu proteins were shown to directly interact with HDAC2 (Zhou et al. 2011). Thus, I detected HDAC2 in the bound proteins as a positive control. The results showed that Myc-HuC did pull down HDAC2 in HeLa cell lysate (data not shown).

I also conducted reciprocal co-IP assays using Flag beads for IP and anti-Myc antibody for western. However, rabbit anti-Myc antibody was very strong and gave a lot of background signals even though I used a very diluted antibody ratio (data not shown).
Figure 4.12 Myc-HuC interacts with Flag-DBC1 and Flag-ZIRD in co-IP assays. HeLa cells were co-transfected with Myc-HuC and Flag-DBC1 or Flag-ZIRD plasmids. Upon two days post-transfection, HeLa cells were harvested to obtain protein extract for co-IP. The cell lysate was incubated with anti-Myc antibody-coated agarose beads for 2 hours. The normal rabbit anti-IgG antibody was used as a negative control. The beads were then extensively washed with 300 mM NaCl wash buffer. The bound proteins were separated by SDS-PAGE and analyzed by immunoblot with anti-Flag antibody. 20 mg/mL RNase A was added in both the cell lysate and the wash buffer. The experiment was replicated three times.
4.2.3.4 DBC1 complex interacts with HDAC2

DBC1 was shown to interact with several members of class I of HDACs, HDAC1 and HDAC3, but not with class II HDACs, HDAC4-HDAC7 (Chini et al. 2010). To date, no studies have reported if DBC1 interacts with HDAC2, a member of class I HDAC. To test if DBC1 interacts with HDAC2, I carried out a co-IP assay using anti-DBC1 antibody-coated agarose beads to pull down HDAC2 using HeLa cell lysate. The result shows that DBC1 interacts with HDAC2 (Figure 4.13).
Figure 4.13 DBC1 interacts with HDAC2 in HeLa cell lysates

The co-IP assays were performed using anti-DBC1 antibodies and HeLa cell lysates containing HuR and DBC1. The HeLa cell lysate was incubated with anti-DBC1 antibody-coated agarose beads for 2 hours. Normal rabbit anti-IgG antibody was used as a negative control. Upon binding and extensive washing with 300 mM NaCl wash buffer, bound proteins were separated by SDS-PAGE and analyzed by immunoblot with anti-DBC1 and anti-HDAC2 antibodies.
4.2.3.4 The hinge domain of HuC is required for DBC1-HuC interaction.

To pinpoint which domain of HuC protein is the most important for the interaction of HuC protein with DBC1, I carried out the co-IP assay by overexpressing the truncated mutant HuC plasmids with Flag-DBC1. My data showed that the hinge domain of HuC is required for DBC1-HuC interaction.
Figure 4.14 The hinge domain of HuC required for the DBC1-HuC interaction.

Full-length Myc-HuC and truncation mutant Myc-HuC plasmids were co-expressed with Flag-DBC1 in HeLa cells. The HeLa cell lysate was incubated with anti-Myc antibody-coated agarose beads for 2 hours. Normal rabbit anti-IgG antibody was used as a negative control. Upon binding and extensive washing with 300 mM NaCl wash buffer, bound proteins were separated by SDS-PAGE and analyzed by immunoblot with anti-Flag antibodies.

Fig 4A3. Full-length Myc-HuC and truncation mutant Myc-HuC plasmids were co-expressed with Flag-DBC1 in HeLa cells:
Figure 4.15 Full-length Myc-HuC and truncation mutant Myc-HuC plasmids were co-expressed with Flag-DBC1 in HeLa cells.
4.2.3.4 HuC protein interacts with histone in yeast two-hybrid screen assay

Figure 4.16 Interactions between HuC and Histone H3 in yeast

AD: activation domain, BD DNA-binding domain

Since yeast two-hybrid screens may generate false positives, the HuC-H3 interaction needs to be verified by other high confidence assays.
5.2.3.5 DBC1–Hu complex interacts with histone H3 peptides

The histone peptide binding assay showed that H3 peptides interact with HuR, the only protein of the Hu family expressed in HeLa cells (Fig. 4.17).

ASF/SF2 was shown to directly interact with H3 peptides (Loomis et al. 2009). Thus, I also detected ASF/SF2 in the bound proteins as a positive control. The results showed that H3 and H4 peptides did pull down ASF/SF2 in HeLa nuclear extract (data not shown).

![Histone peptide binding assay](image)

Figure 4.17 Histone peptide binding assay

Human synthetic histone H3 peptides were used to test if H3 interacts with HuR, which is ubiquitously expressed in HeLa cells. H3 peptide corresponds to amino acids 1-21 of histone H3, and is followed by a linker and biotinylated lysine to facilitate pulling down HuR by streptavidin beads. To conduct the experiment, HeLa nuclear extract was used as a source of HuR protein. Upon addition of the extract to the aforementioned beads and peptides, proteins were incubated with the beads and washed with high salt buffer (200 mM NaCl). Then, the bound proteins were separated by SDS-PAGE and analyzed by
immunoblot with anti-DBC1 antibody anti-HuR antibody. Anti-biotin antibody was also used to detect the presence of histone peptides.
5.2.3.6 All members of Hu proteins family interact with histones in GST pull-down assay

Each Hu protein has three RNA recognition motifs (RRMs 1-3) that share more than 90% amino acid sequence identity. To test if all members of Hu protein family interact with H3, I conducted a GST pull-down assay using all recombinant GST-Hu proteins made in bacteria. The result showed that all members of Hu protein family interact with H3 (Figure 4.18)

![Figure 4.18 GST pull-down assay](image)

All recombinant GST-Hu proteins were pre-incubated with histone core extract and pulled down by glutathione-coated agarose beads. Upon binding and extensive washing with high salt buffer (400 mM NaCl), bound proteins were detected by western blot. Beads only and beads pre-incubated with GST protein were included as negative controls.
4.2.3. Discussions and future work

4.2.3.1 The molecular mechanism by which DBC1 regulates the NF1 exon 23a splicing

My preliminary data showed that DBC1 interacts with both Hu and histone proteins, which prompted me to hypothesize that Hu proteins and DBC1 form a complex interacting with histones to prevent HDAC enzymes from accessing histones. Consequently, it induces local histone hyperacetylation and increases transcriptional elongation rate surrounding the Nf1 exon 23a regions to regulate NF1 exon 23a (Figure 4.19).

Specifically, my working hypothesis is that DBC1 acts in a cooperative manner with Hu proteins to actively modulate the chromatin structure around NF1 exon 23a areas by interacting with histone modifying enzymes and inhibiting their enzymatic activities, leading to a local increase in transcriptional elongation rate to regulate the splicing of the NF1 exon 23a (Figure 4.19).
Figure 4.19 Working model of DBC1-mediated epigenetic regulation of NF1 exon 23 splicing
To test this working hypothesis, I plan to perform the following experiments.

1. **Determine if DBC1 regulates the NF1 exon 23a splicing**

   (1) Our collaborator will be sending the DBC1 knockout mouse tissues for us soon. Upon getting the DBC1 knockout mouse brain tissue, I will determine the splicing phenotype of the NF1 exon 23a in the DBC1 knockout mouse brain compared to that of wildtype mouse by RT-PCR. Western blot with anti-DBC1 antibody will be performed as a control experiment.

   (2) Determine if DBC1 are present at NF1 exon 23a region by RNA-Immunoprecipitation (RIP) and/or ChIP

   (3) Determine the domains of DBC1 important for regulation of the NF1 exon 23a splicing

   (4) Knockdown HuR proteins in HeLa cells, then overexpress Flag-DBC1 and Flag-ZIRD with NF1 reporter to test the splicing of NF1 exon 23a

   (5) Determine the splicing of the NF1 exon 23a upon knockdown of DBC1 and ZIRD in neurons

   (6) Test the transcriptional elongation rate and chromatin structure around NF1 exon 23a upon knockdown of DBC1

   I will study the local histone acetylation and methylation status around NF1 exon 23a by ChIP.

   To assess the local transcriptional elongation rate around exon 23a, I will conduct RNP II ChIP assay and analyze the accumulation of nascent Nf1 pre-
mRNA at different exons using the BrU incorporation method established in our laboratory.

(7) Determine if DBC1-dependent alternative exons are also Hu-targets.

2. **Determine the nature of DBC1-Hu interactions**

Given that we have just obtained the full-length and truncated HA/Myc-DBC1 plasmids from our collaborator, I plan to perform a lot of biochemical assays to determine the nature of DBC1-Hu interactions and the functional significance of these interactions.

(1) To verify these interactions, I plan to perform more co-IP experiments using HeLa cells:

- Overexpress HA/Myc-DBC1 and Express-HuC, then co-IP using Myc beads or HA beads and western blot with anti-Express antibody

- Overexpress Myc-HuR, Myc-HuB, Myc-HuC, and Myc-HuD with Flag-DBC1/Flag-ZIRD to conduct co-IP assays using Myc beads and western blot with anti-Flag antibody

- Overexpress HA/Myc-DBC1 only, then co-IP using Myc beads or HA beads and western blot with anti-HuR and anti-HDAC2 antibodies

(2) To pinpoint the domains of HuC protein important for Hu-DBC1 interactions, I will overexpress the full-length and mutant truncation Myc-HuC with Flag-DBC1, and then perform co-IP assays using the Myc beads and western blot with anti-Flag antibody.

(3) To pinpoint the domains of DBC1 protein important for DBC1-Hu interactions, I will overexpress the full-length and mutant truncation HA/Myc-
DBC1 with Express-HuC, then perform co-IP assays using the Myc beads or HA beads and western blot with anti-Express antibody.

(4) I will be purifying GST-DBC1 proteins to conduct GST pull-down experiments. I plan to test DBC1-Hu interactions in an in vitro binding assay by incubating GST-DBC1 with MBP-HuC/His-HuR, and then blot with anti-MBP or anti-HuR antibodies.

3. **Determine if DBC1 inhibits HDAC2 activity**

   (1) Overexpress full-length and mutant truncation Myc-DBC1 in HeLa cells to perform co-IP assays to pinpoint the domain of DBC1 required for DBC1-HDAC2 using Myc beads.

   (2) I will conduct GST pull-down assays to test if GST-DBC1 interacts with MBP-HDAC2.

   (3) I will perform in vitro HDAC2 activity assay to test if DBC1 inhibits HDAC2 activity. In this assay, I will include HDAC3 as a positive control and HDAC1 and mutant DBC1 that does not interact with HDAC2 as negative controls.

   (4) To test if DBC1 acts together with Hu proteins to inhibit HDAC2 activity in vitro, I can perform in vitro HDAC2 activity assay using combining both DBC1 and Hu proteins.

4.2.3.2 Alternative Hypotheses

Besides the main working hypothesis, I have other alternative hypotheses to determine the functional significance of DBC1-Hu interactions and have designed experiments to test these alternative hypotheses.

1. **Determine if DBC1 modulates the stability of Hu proteins**
Given that DBC1 was shown to regulate the protein stability of several proteins it interacts with, I would like to test if DBC1 regulates the stability of Hu proteins.

When performing western blot for both wild-type and knock-out DBC1 mouse brain using anti-DBC1 antibody, I will blot for HuR using anti-HuR antibodies to compare the expression level of Hu proteins in wild type and that of knockout DBC1 mouse brains. I will blot for anti-ZIRD as a positive control.

If the expression level of Hu proteins in DBC1 knockout mouse brain is significantly lower than that of wild-type mouse brain tissue, I will pursue further experiments to determine the molecular mechanisms of Hu protein stability regulated by DBC1.

a. Look at gene expression
   - Do real-time RT-PCR to look at mRNA level of Hu proteins in wild-type and DBC1 knockout brain tissues
   - Over-express Myc-HuC only, and Myc-HuC with Flag-DBC1, and then perform RT-PCR to look at the expression level of Hu proteins.

b. Look at protein stability
   - Overexpress the control vector and Flag-DBC1 in HeLa cells and measure the half-life of Myc-HuC. I will treat the cells with cycloheximide (Chx) first to inhibit translation, then MG-132 to inhibit proteasome.
   - To determine if DBC1 interacts with Hu proteins to prevent their degradation, I will co-overexpress Myc-HuC and HA-ubiquitin.
plasmids in HeLa cells alone and with Flag-DBC1. Then, I will perform co-IP assays using Myc beads and western blot with anti-HA antibody. Comparing the ubiquitin signals between without and with overexpressing Flag-DBC1 to test if DBC1 prevents the decay of Hu proteins.

2. Determine if DBC1 enhances the binding of Hu proteins to RNA

DBC1 was shown to interact with AR and promote its DNA-binding activity. It would be interesting to see if DBC1 interacts with Hu proteins to enhance the binding of Hu proteins to RNA. To test this hypothesis, I will conduct a gel mobility shift using the titration of recombinant DBC1 protein and NF1 exon 23a reporter that has the binding sites for Hu proteins.


Hodgdon, K. E., C. M. Hingtgen and G. D. Nicol (2012). "Dorsal root ganglia isolated from Nf1+/- mice exhibit increased levels of mRNA expression of voltage-dependent sodium channels." Neuroscience 206: 237-244.


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defects in the Drosophila melanogaster neurofibromatosis-1 growth deficiency."


