MUSCLEBLIND-LIKE 2 IN RNA SPLICING REGULATION AND DISEASE

By

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To my family I left
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<td>atrioventricular</td>
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<tr>
<td>APP</td>
<td>amyloid precursor protein</td>
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<tr>
<td>C3</td>
<td>complement 3 locus</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<tr>
<td>CDM</td>
<td>congenital myotonic dystrophy</td>
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<tr>
<td>CLCN1</td>
<td>muscle-specific chloride channel</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>cTNT</td>
<td>cardiac troponin T</td>
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<tr>
<td>CUGBP1</td>
<td>CUG binding protein 1</td>
</tr>
<tr>
<td>DM</td>
<td>myotonic dystrophy</td>
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<tr>
<td>DMPK</td>
<td>dystrophica myotonica protein kinase</td>
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<tr>
<td>dsRNA</td>
<td>double stranded RNA</td>
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<tr>
<td>FAXTAS</td>
<td>fragile x tremor associated syndrome</td>
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<tr>
<td>FISH</td>
<td>fluorescence in situ hybridization</td>
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<tr>
<td>GI</td>
<td>gastrointestinal</td>
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<tr>
<td>GT</td>
<td>gene trap</td>
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<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
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<tr>
<td>HAS</td>
<td>human skeletal actin</td>
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<tr>
<td>HSA&lt;sup&gt;LR&lt;/sup&gt;</td>
<td>human skeletal actin long repeats</td>
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<tr>
<td>IR</td>
<td>Insulin receptor</td>
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<tr>
<td>Kb</td>
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<td>LTP</td>
<td>long term potentiation</td>
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<td>Mbnl</td>
<td>muscleblind-like</td>
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<td>MEF</td>
<td>mouse embryonic fibroblast</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>MRI</td>
<td>magnetic resonance imaging</td>
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<td>mRNA</td>
<td>messenger RNA</td>
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<td>NMDAR1</td>
<td>N-Methyl-D-Aspartate Receptor 1</td>
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<td>OCT</td>
<td>optimal cutting temperature</td>
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<tr>
<td>PKC</td>
<td>protein kinase C</td>
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<tr>
<td>POMA</td>
<td>paraneoplastic opsoclonus myoclonus ataxia</td>
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<tr>
<td>PTB</td>
<td>polypyrimidine tract binding protein</td>
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<tr>
<td>PTZ</td>
<td>pentylenetetrazol</td>
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<tr>
<td>RRM</td>
<td>RNA recognition motif</td>
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<tr>
<td>SF1</td>
<td>splicing factor 1</td>
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<td>ssRNA</td>
<td>single stranded RNA</td>
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<td>TA</td>
<td>tibialis anterior</td>
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<td>TFIIIA</td>
<td>transcription factor III A</td>
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<tr>
<td>TK</td>
<td>Herpes Simplex Virus thymidine kinase gene</td>
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<tr>
<td>UTR</td>
<td>untranslated region</td>
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Muscleblind-like 2 in RNA splicing regulation and disease

By

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December 2011

Chair: Maurice Swanson
Major: Medical Sciences – Genetics

Myotonic dystrophy (DM) is a multi-systemic, late-onset and dominantly inherited neuromuscular disorder with characteristic disease features which include myotonia, muscle wasting, mental retardation, memory impairment, hypersomnolence, apathy and cerebral atrophy. DM is caused by two different microsatellite expansions (CUG\textsuperscript{exp}, CCUG\textsuperscript{exp}) in the untranslated regions of the \textit{DMPK} and \textit{CNBP} genes, respectively. Transcription of these repeats result in the synthesis of toxic RNAs which sequester, and functionally repress, the activities of proteins in the muscleblind-like splicing factor family. Previous studies have shown that Mbnl1 is essential for the switch from embryonic to adult splicing patterns in skeletal muscle and this switch is blocked in \textit{Mbnl1} knockout mice which develop muscle pathology characteristic of DM. In brain, loss of Mbnl1 does not recapitulate the splicing alterations seen in DM, suggesting a role for other Mbnl family members in disease pathogenesis. Based on this possibility, I hypothesized that Mbnl2 was the primary splicing factor sequestered by toxic RNAs in the DM brain and that loss of this factor underlies the neurological features associated with DM. To address this hypothesis, \textit{Mbnl2} knockout mice (\textit{Mbnl2}^\Delta E2/\Delta E2) were generated and examined for DM-relevant phenotypes as well as aberrant alternative
splicing. Using these *Mbnl2* knockouts and splicing microarrays combined with RNA-seq, the alternative exons mis-spliced following loss of Mbnl2 were identified. Using this splicing information, I discovered that Mbnl2 promoted the switch from embryonic to adult alternative splicing during postnatal brain development. Using high throughput sequencing and crosslinking-immunoprecipitation, the direct binding sites for Mbnl2 were identified and confirmed that Mbnl2 binds preferentially to UGCU motifs to regulate alternative splicing. Surprisingly, *Mbnl2* knockouts showed impaired spatial memory formation and increased neuronal excitability which led to seizures upon treatment with a GABA antagonist. Epilepsy susceptibility has not been extensively reported in DM patients, but this result raises the issue of appropriate drug treatment and prolonged use. Importantly, *Mbnl2* knockout mice are an important new resource for investigations focused on characterizing the molecular events which regulate normal brain development and DM-relevant brain pathology and a useful model for ongoing drug development.
CHAPTER 1
INTRODUCTION

In the study of genetic disorders, it is common to identify mutations that result in a non-functional protein product (loss-of-function model) or a protein which has gained a deleterious function (gain-of-function model). In both cases, the affected gene leads to an abnormal phenotype usually associated with the normal function of the encoded protein. Many different types of mutations have been linked to known genetic disorders. These mutations include point mutations, deletions, insertions, duplications, inversions and microsatellite expansions.

A variation of the above scenario was recently introduced during studies on the molecular basis of myotonic dystrophy (dystrophia myotonica, DM), a disease caused by microsatellite expansions in untranslated regions of two unrelated genes, DMPK and CNBP. In DM, the expansions do not affect the function of the mutant genes or proximal genes, but rather act in an atypical fashion, now referred to as an RNA gain-of-function mutation. According to this dominant-negative RNA model, mutant RNAs accumulate in DM nuclei and sequester nuclear factors and impair a critical cellular pathway. The unique nature of this pathogenic mechanism is highlighted by the phenotypic outcome of these mutations since this disease is multi-systemic and leads to atrophy of a number of tissues. The discovery of the RNA gain-of-function model has provided a new dimension to ongoing research on other diseases caused by nucleotide repeat expansions including spinocerebellar ataxias, fragile X-associated tremor/ataxia syndrome (FXTAS) and a recently identified form of amyloid lateral sclerosis (ALS) linked to chromosome 9.
In this Introduction, I begin by discussing the main clinical features of myotonic dystrophy and the molecular events and mechanism that have been implicated in the disease progression. Then I will focus on the current mouse models that have been generated to study the disease and point out advantages, disadvantages and DM clinical features that are not phenocopied by these models. Finally, I conclude by stating and justifying the hypothesis that I am addressing in this thesis.

**Myotonic Dystrophy**

Myotonic dystrophy (dystrophia myotonica or DM) is a common form of muscular dystrophy with an unusual mechanism of pathogenesis that affects more than 1:8000 adults around the world. DM was first described more than a century ago as a multisystemic disease with the name myotonia atrophica (Batten and Gibb, 1909). Several case studies were reported in the early 1900s’ describing patients that developed different aspects of the disease including myotonia, muscle weakness and wasting, paralysis of vocal cords (Adie, 1923; Fox, 1910), cataracts, mental defects including hypersomnolence and cardiac defects (Evans, 1944). From the early days of the disease discovery, it was suspected that DM is a genetically transmitted late-onset disease that shifts into a more severe form with an earlier onset during vertical genetic transmission, a phenomenon that is termed genetic anticipation. There were cases described in 1910, where if they placed the child and the father next to each other, the diseased child showed similar symptoms and severity at an earlier age, a clear indication of genetic anticipation. Due to the anticipation phenomenon, the disease eventually appears in a pedigree as a congenital form of myotonic dystrophy (CDM) that affects newborns. To date, DM is divided in three different types with a similar
phenotype and possibly a similar molecular pathogenesis, DM type 1 (DM1), DM type 2 (DM2) and CDM.

**Genetics**

The genetic locus responsible for DM1 was the subject of intense scientific research since the 1970s, when scientists were focused on discovering a marker locus that was linked to the disease phenotype (DM locus) for indirect prenatal diagnosis. In 1971, J.H. Renwick and D.R. Bolling found that they could predict transmission of the disease with a 92% chance, by a three-locus linkage analysis of the DM locus, the ABH secretor locus and the Lutheran blood group locus (Renwick and Bolling, 1971). A decade later linkage of DM and complement 3 locus (C3) indicated that the locus that is responsible for the DM phenotype resides on chromosome 19 (Roses et al., 1986). Six years later (1992), a microsatellite repeat expansion was identified and correlated with the disease by several independent labs (Brook et al., 1992; Mahadevan et al., 1992) and the gene that carries the microsatellite expansion was identified to be a member of a protein serine/threonine kinase family. This finding, in addition to the finding that a CGG expansion is correlated with Fragile X syndrome, contributed to the identification of the molecular etiology of a number of diseases that have been found since then to be caused by unstable microsatellite repeats such as Huntington disease, six spinocerebellar ataxias, FXTAS and spinobulbar muscular atrophy (SBMA).

DM1 is now known to be an autosomal dominant late-onset neuromuscular disorder that is caused by the expansion of a CTG microsatellite repeat located in the 3’ untranslated region (UTR) of the gene *DMPK* (dystrophica myotonica protein kinase) located on chromosome 19q13.3. The onset of the disease correlates with an expansion of the CTG repeats above a certain threshold, where normal individuals have
5-37 repeats, individuals with mild DM phenotype that will develop mild myotonia and cataracts carry 50-150, and individuals with classical DM phenotype carry 100-1000 repeats. The severity and onset of the phenotype generally correlates with repeat number.

**Clinical Features of DM**

The most prevalent clinical features that DM1 patients develop are discussed below.

**Myotonia**

Myotonia is defined as the inability of muscle relaxation after a voluntary contraction. It is the hallmark clinical feature of DM patients and an overt sign that allowed physicians to characterize and categorize the disease. Electric stimulation was used extensively to reveal prolonged involuntary contraction of muscles and a failure of muscle relaxation that was independent of a compromised nervous system. In a 1909 case report of a patient who suffered from DM, the clinician reported:

> There is very marked reduction in irritability to the faradaic current amounting in the biceps and hand muscles almost to extinction. In the long flexors of the fingers there is an imperfect myotonic reaction shown both on volitional movement and electric stimulation. The patient is able to relax his grasp at once, but only to an extent that leaves the fingers somewhat in the position of tetany; from this they gradually relax. On faradaic stimulation the same occurs.

This case report not only describes myotonia characteristic of DM but also shows evidence that this patient was probably affected by myotonic dystrophy type 1 since the affected muscle groups were more distal to body axis.

Since 1992, a cause of myotonia in muscle diseases like myotonia congenital was known to result from mutations in the muscle specific chloride channel CIC-1 or CLCN1. *CLCN1* mutations caused either decreased levels of CIC-1 or alterations in the coding
sequence and the synthesis of channels with altered sensitivity. In 2002, myotonia in DM patients was found to be caused by $CLCN1$ gene defects (Mankodi et al., 2002). However, in DM the $CLCN1$ gene is not directly affected and instead the splicing of $CLCN1$ pre-mRNA is altered which leads to a premature termination codon, nonsense-mediated decay of the transcript and decreased levels of the chloride channel.

**Muscle wasting**

Muscle wasting and weakness is the second most prominent clinical feature of DM. Specific muscle groups atrophy and thus cannot generate sufficient force required for certain tasks. Unlike DM2 where proximal muscles (e.g., hip flexors) are affected, DM1-associated weakness and wasting is most prominent in distal muscles and also affects facial muscles such as sternomastoid as well as eyelid muscles which results in the characteristic facial weakness and eyelid ptosis, respectively. The molecular mechanism of muscle wasting has not yet been identified.

**Cataracts**

Most DM1 patients also suffer from an unusual type of cataract (Fearnside, 1915). Cataracts result in a loss of transparency of the eye lens but in the case of DM, the cataracts are dust-like opacities in the subcapsular region of the lens. The gene(s) and molecular events that are responsible for this eye phenotype are not understood.

**Heart problems**

As mentioned before the most prominent clinical feature of DM1 is myotonia and muscle wasting, but there is a group of clinical features unrelated to the skeletal muscle phenotype with severe consequences to DM1 patients. Heart conduction defects significantly affect the quality of life of DM patients and the severity of cardiac complications correlate with the length of the microsatellite repeat expansion.
arrhythmias are common in this patient population and may result in cardiac arrest and sudden death (Bache and Sarosi, 1968). A peculiarity of DM heart disease is that the conduction system is selectively affected with first degree atrioventricular block (AV block) and PR prolongation often accompanied by cardiomyopathy. The cellular/molecular mechanism underlying this defect is unknown but there is growing evidence that mis-splicing of several genes, possible the cardiac troponin T gene \( TNNT2 \) is the main mechanism (Philips et al., 1998). \( TNNT2 \) mutations have been implicated in reduced heart efficiency and cardiomyopathy (Thierfelder et al., 1994) and more interestingly a shift in splicing isoforms similar to the shift observed in DM patients has been correlated with changes in myofilament calcium sensitivity and heart failure (Townsend et al., 1995).

**Endocrine system**

Endocrine system defects are also present in DM. Patients with DM type 1 or type 2 have been reported to develop hyperinsulinism (Tevaarwerk and Hudson, 1977), diabetes, testicular atrophy, increased levels of luteinizing hormone (LT), estradiol and follicle stimulating hormone (FSH), decreased levels of testosterone, dihydrotestosterone (DHT) (Mastrogiacomo et al., 1996) as well as some abnormalities in growth hormone (GH) secretion with lower levels of GH during sleep compared to control individuals (Barreca et al., 1980). The endocrine system abnormalities show decreased penetrance with 80% for testicular atrophy and 70% for muscle specific insulin resistance (Barbosa et al., 1974; Moxley et al., 1978). The muscle specific insulin resistance is further supported by the finding that in skeletal muscle of DM1 patients alternative splicing of the insulin receptor pre-mRNA is aberrantly regulated resulting in a non-muscle isoform with lower-signaling capabilities (Savkur et al., 2001).
Gastrointestinal track

Gastrointestinal complaints from patients with DM are common. The symptoms result from the compromised gastrointestinal musculature. Patients suffer impaired lower bowel function leading to constipation, and delayed gastric emptying (Ronnblom et al., 2002). The majority (55%) of DM1 patients suffer from abdominal pain, 35% have upper GI tract movement inconsistencies leading to emesis, 33% have chronic or episodic diarrhea and 45% suffer from dysphagia (inability or difficulty to swallow) that rarely leads to parenteral feeding. In the DM patient community, 25% of the patients find the GI track symptoms to have a profound effect on their quality of life (Ronnblom et al., 1996).

Mature balding and calcifying epithelioma

The first association of DM and calcifying epithelioma or pilomatrixomata was first described by Cantwell and Reed (Cantwell and Reed, 1965) and is strongly associated with DM1 (Geh and Moss, 1999). It is one of the most common pediatric superficial tumors with an early onset, indeed prior to the onset of other typical DM features. DM patients also develop alopecia or pattern baldness that affects men more than women. The etiology of the balding pattern is believed to be androgenic as these patients suffer from testicular atrophy and decreased levels of circulating testosterone, as well as defects in androgen receptors in the hair follicles.

Brain abnormalities

Although DM patients develop a variety of different symptoms, the main concern is abnormal brain function, which diminishes quality of life. The most common complaints are altered sleep patterns early in disease progression and cognitive function due to cerebral atrophy at later stages. Patients suffer from executive function deficits, age-
related decline of frontal and temporal cognitive functions, excessive daytime sleepiness (hypersomnolence) and memory problems as well as severe apathy (Meola et al., 2003; Modoni et al., 2004). The central nervous system involvement in DM includes personality alterations, such as avoidant personality (Meola et al., 2003), obsessive-compulsive, passive-aggressive and schizotypic traits (Delaporte, 1998), as well as severe impairment of general intelligence and verbal fluency (Abe et al., 1994). Patients suffer from visuospatial, executive, arithmetic and attention ability deficits and score low on the general IQ test (converted Wechsler Adult Intelligence Scale Revised) (Turnpenny et al., 1994; Winblad et al., 2006). Hypersomnolence has not been attributed to hypercapnia (an increase in blood CO\textsubscript{2} due to hypoventilation) (van der Meche et al., 1994), it is not cataplectic (Phillips et al., 1999) and is not described as narcolepsy, even though anti-narcoleptic drugs such as Modafinil have been employed and successfully reduce the abnormal sleep symptoms (Talbot et al., 2003). Patients with DM1 fall asleep not due to loss of interest, but to their inability to remain engaged. One reason for DM sleep disorders may be a general atrophy of the corpus callosum (Giubilei et al., 1999).

MRI studies have revealed an extensive loss of brain matter, including both white and gray matter loss as well as cortical atrophy (Damian et al., 1993; Hashimoto et al., 1995a; Hashimoto et al., 1995b). In addition to brain tissue and cell body loss, patients have developed neuronal inclusion bodies, decreased myelin sheathing and increased neurofibrillary tangles located mostly in the limbic system, brainstem, hippocampus, entorhinal cortex and temporal lobes (Ono et al., 1987; Oyamada et al., 2006).
In contrast to muscle, the molecular basis of brain-related defects is mostly unknown. It is well established that the pathogenic DMPK RNA in the brain form foci similar to the nuclear RNA foci present in myonuclei. These foci have been detected by RNA-FISH in a wide range of different regions of the brain and cell types, such as the dentate gyrus, hippocampus, cerebral cortex, thalamus, substantia nigra and the brainstem. The density and size of the RNA foci correlates with the size of the CTG repeat expansion in DM1 patients. In the DM brain, the molecular events that lead to clinical phenotypes may be simpler than in muscle since there is no evidence for sequestration of transcription factors, such as SP1, STAT1, STAT2 and the retinoic acid receptor gamma subunit. The only factors that have been found to bind strongly to CUG RNA foci in the brain are the two members of the MBNL family, MBNL1 and MBNL2, suggesting a splicing mis-regulation etiology that explains DM-related brain deficits (Jiang et al., 2004).

Sequestration of Mbnl1 and Mbnl2 in RNA foci is accompanied by a few well documented aberrant splicing events. In the current literature there are 14 alternative splicing events identified to be mis-regulated in DM patients, corresponding to 10 different genes throughout the tissues (Ranum and Cooper, 2006). As in muscle, aberrant splicing disrupts the developmental hierarchy in brain resulting in a reversion to an embryonic state. Despite the heterogeneity of the brain, only three genes have been found to be mis-regulated so far, the N-methyl-D-aspartate receptor 1 (NMDAR1), the amyloid precursor protein (APP) and Tau (MAPT) (Jiang et al., 2004; Leroy et al., 2006a; Sergeant et al., 2001). The association of mis-regulated events and disease is
yet to be established despite the fact that these genes have been already implicated in other brain diseases.

Exclusion of exon 2, 3 and 10 from MAPT transcripts generates alternative Tau isoforms with altered function. Exclusion of exon 2 results in an MAPT in which the N-terminal domain interacts with the axonal membrane and possibly stabilizes of microtubules as it was found to be enriched at the membrane of growth cones and distal axons of hippocampal neurons (Brandt et al., 1995). A similar role has been attributed to exon 10 that encodes an additional microtubule binding domain. MAPT exon 2 and 10 splicing has been reported to be regulated by an exonic silencer and enhancer, respectively, by providing binding sites for specific splicing factors (Wang et al., 2005). Another very interesting exon whose splicing is mis-regulated in DM patients is exon 6 that has been reported to lead to formation of neurofibrillary tangles and axonopathy and also corresponds to the fetal splicing pattern (Ishihara et al., 2001; Leroy et al., 2006b).

A gene that is mis-regulated that may be linked to a DM clinical feature is NMDAR1. NMDAR1 is a glutaminergic receptor in the brain and is required for normal long-term potentiation (LTP) in the hippocampus, an event that precedes memory formation and learning.(Brigman et al., 2010; Tonegawa et al., 1996) In patients with myotonic dystrophy, there is a substantial increase of NMDAR1 exon 5 inclusion in the temporal cortex, an event that dictates alterations in gating, subcellular localization of the receptor and pharmacological behavior. This change in the channel’s properties has been suggested as the main etiology of the memory impairment observed in DM (Traynelis et al., 1995).
The Molecular Etiology of Myotonic Dystrophy

For more than a decade, we have known that the DMPK gene with a triplet repeat expansion is responsible for the autosomal dominant vertical transmission of DM1. The generation of polyCUG transgenic mice, which carried a few hundred CTG repeats in a different gene (human skeletal actin, HSA), established the notion that the disease is caused by the repeat expansion and not by altered DMPK expression shown by the lack of classical DM manifestations in Dmpk<sup>-/-</sup> mice (Mankodi et al., 2000). (Reddy et al., 1996) The HSA long repeat (HSA<sup>Lr</sup>) mice showed severe myotonia and most of the muscle histopathological aspects of DM muscle, including centralized nuclei, split and pycnotic fibers, as well as fibrosis. These pathological features were only present in skeletal muscle because the HSA transgene is only expressed in this tissue. The fact that these repeats cause a DM-associated muscle phenotype when expressed in a different mRNA context suggested that DM pathogenesis results from the expression of the repeats alone and altered expression of the host gene is not an important aspect of the etiology. Many hypotheses were proposed to explain how these repeat expansions could lead to a multi-systemic disease but a few elegant experiments established the RNA gain of function mechanism. In earlier studies, RNA-FISH experiments using a hybridization probe against CTG repeats showed that these repeats and its associated transcript are sequestered in distinct nuclear foci in DM patient cells and tissues (Mankodi et al., 2001). Label transfer and filter binding experiments showed that these RNA species have the capability of binding with high affinity to a specific set of muscleblind-like or MBNL proteins (Miller et al., 2000; Yuan et al., 2007). These proteins belong to a family of proteins first described in a mutant line of Drosophila melanogaster (Miller et al., 2000). In this mutant line, the flies showed an unusual late
developmental phenotype with defects in muscle and eye terminal differentiation, thus the gene was named Muscleblind or mbl (Artero et al., 1998; Begemann et al., 1997). Knocking out Mbnl1 in mice results in myotonia and muscle histopathology similar to DM, suggesting that loss of MBNL protein function by the CUG\textsuperscript{exp} RNA gain of function and sequestration model is the main molecular event that leads to DM.

Since the establishment of the RNA gain-of-function and protein sequestration model, it has been shown that loss of function of the Mbnl1 protein leads to a mis-regulation of alternative splicing and recapitulation of certain aspects of DM in a mouse Mbnl1 knockout model (Kanadia et al., 2003a). These clinical aspects of myotonic dystrophy were rescued in a classical gene therapy/complementation experiment in HSA\textsuperscript{LR} mice, substantiating the Mbnl loss-of-function theory (Kanadia et al., 2006). The finding that Mbnl1 plays a major role in alternative splicing regulation by switching from an embryonic to an adult splicing pattern indicated that DM is a spliceopathy.

Using a different approach, but involving a similar molecular mechanism, considerable research has been done on another factor that binds to CUG repeats and is mis-regulated in patients with DM. The CUG binding protein (CUGBP1/CELF1) is an alternative splicing factor that was identified together with additional proteins from the CELF family of RNA-binding proteins. These proteins have a high affinity for UG dinucleotide repeats and CELF1 was the first factor to be considered as disease factor involved in DM1 pathogenesis (Timchenko et al., 1996). Although CELF1 binds to CUG repeats (Timchenko et al., 2001) this protein is not sequestered in RNA foci. Surprisingly, CELF1 steady state levels are elevated in DM1 tissues and cells due to PKC-mediated hyperphosphorylation of the CELF1 protein. This hyperphosphorylation
is due to activation of the PKC pathway via an unknown mechanism (Kuyumcu-Martinez et al., 2007). Studies to date have shown that CELF1 acts as an antagonist to MBNL1 since it promotes embryonic splicing patterns. In a mouse CELF1 overexpression model, myogenesis is inhibited as is the myofiber type switch from fast glycolytic to fast oxidative (Timchenko et al., 2004). The indirect relationship between CUG repeat expansion and CELF1 hyperactivity supports the hypothesis that Mbnl loss-of-function plays an important role in the pathogenesis of DM.

Muscleblind-Like Protein Family

The MBNL family consists of three known paralogous proteins with more than 80% sequence identity: MBNL1, MBNL2 and MBNL3. The proteins consist of an N-terminus that contains two pairs of zinc finger like, or CCCH, motifs and a C-terminus that is involved in homodimerization and other protein-protein interactions (Yuan et al., 2007). Proteins that bind RNA possess a variety of structural domains that have the ability to either bind to single stranded (ss)RNA by recognition of an RNA sequence or to double stranded (ds)RNA or to other more complex RNA conformations by recognizing a combination of RNA sequence and higher-order structures. One of the most common RNA binding domains, and one of the first to be identified, is the RNA Recognition Motif (RRM). It consists of four beta strands packed between two alpha helixes (βαβαβ-β-fold) and binds to ssRNA (Query et al., 1989) most likely via β strand-RNA interactions. Nova-1, is a well-characterized RNA binding protein that binds to a UCAU tetranucleotide via its 3rd KH domain (Buckanovich and Darnell, 1997) and regulates alternative splicing (Jensen et al., 2000a). Nova-1 is implicated in paraneoplastic opsoclonus myoclonus ataxia (POMA) (Buckanovich et al., 1996) and is expressed
exclusively in specific regions of the brain (Buckanovich et al., 1993; Buckanovich et al., 1996).

Another characteristic RNA-binding domain that has been implicated in dsRNA binding is the zinc finger (ZF), or C2H2, motif. TFIIIA was the first protein to be described which consisted of repetitive ZFs that could bind selectively to DNA and regulate gene expression in *Xenopus* oocytes. These DNA binding domains have a finger-like shape with the zinc ions stabilizing this conformation by binding to cysteines and histidines, amino acids that were previously known to bind zinc. In the case of TFIIIA, zinc ions are bound by nine 30-amino acid repetitive sequences that carry cysteines and histidines at the base of the fingers, in a C2H2 conformation which is the most common DNA binding ZF domain (Miller et al., 1985). Later investigations demonstrated that the same ZF motif of TFIIIA (zinc finger 5) bound 5S rRNA via direct interactions of the ZF with a double helical region of the RNA (Theunissen et al., 1998). A large number of different ZF motifs have been documented, including the CCCH, or C3H, motif that is found in the Mbl/MBNL proteins which bind to dsRNA. Mbl proteins in *Drosophila*, and Mbnl1 in mammals, have been shown by directed mutagenesis and synthetic CUG repeats to bind the dsRNA stem via recognition of pyrimidine mismatches bordered by a C-G and G-C base pairs (Goers et al., 2008; Yuan et al., 2007). The binding occurs via direct interaction of the protein with the G and mutation of this nucleotide eliminates binding. Most of the Mbnl splicing isoforms carry two pairs of C3H motifs that are separated from each other by a linker region. Crystallographic analysis has shown that the second pair of ZFs promotes RNA folding into dsRNA or an anti-parallel structure (Teplova and Patel, 2008).
The binding of RNA-binding proteins to pre-mRNA may regulate editing (Paul and Bass, 1998) and alternative splicing (Ho et al., 2004) while binding to mRNA may alter mRNA function by either promoting translation (Cuchalova et al., 2010; Kessler and Sachs, 1998), increasing stability (Ruiz-Echevarria and Peltz, 2000), directing localization (Adereth et al., 2005; Ross et al., 1997), or degradation (Lagos-Quintana et al., 2001; Peltz et al., 1993). The major function of MBNL1 is alternative splicing regulation. This protein promotes exon skipping when bound to conserved intronic enhancers upstream of an exon or exon inclusion when bound downstream of an alternative exon (Grammatikakis et al., 2011; Ho et al., 2004; Sen et al., 2010). Using minigene splicing assays and mutagenesis analysis of known DM mis-spliced targets, it has been shown that all three MBNL protein isoforms regulate splicing in a similar fashion and independently of CELF1 or other CELF proteins. This finding argues against the hypothesis of a synergistic binding and regulation of alternative splicing from MBNL1 and CELF1 on the same target (Kino et al., 2009). Mouse Mbnl1 and Mbnl3 bind to the same intronic elements upstream or downstream of excluded or included exons, (Grammatikakis et al., 2011). Many of the mis-spliced mRNA species that are found in DM patients, such as cardiac troponin T (TNNT2 exon 5), insulin receptor (INSR exon 11), chloride channel type 1 (CLCN1 exon 7a) and Troponin T type 3 (TNNT3 F exon) are also mis-regulated in Mbnl1 knockout mice (Kanadia et al., 2003a). The identification of mis-spliced genes in Mbnl1 knockout mice, and the generation of an RNA splicing map, revealed that Mbnl1 binds preferentially to YGCY clusters, particularly UGCU. Mbnl1 has been shown to directly bind to YGCY clusters to regulate
alternative splicing (Yuan et al., 2007). Interestingly YGCY clusters are a characteristic structural feature of CUG expansion RNA hairpins (Du et al.; Ho et al., 2004).

Most of the research on DM1 pathogenesis has been focused on Mbnl1 due to its relatively high expression level in muscle, a major affected tissue in DM. The limited research that has been conducted on the two other family members, Mbnl2 and Mbnl3, was done in conjunction to Mbnl1 studies to test if all three Mbnl genes can function in a similar way. All three family members regulate alternative splicing by promoting adult splicing patterns for same targets in cell culture splicing assays (Ho et al., 2004). Expression analysis shows a more uniform expression for mouse Mbnl2 throughout development and in different tissues, whereas Mbnl3 shows mostly embryonic expression with a few exceptions (Kanadia et al., 2003b; Lee et al., 2007). Even though Mbnl3 promotes an adult splicing pattern in transfected cells, the fact that Mbnl3 is primarily expressed during embryogenesis argues against the hypothesis that this family member plays a role in postnatal splicing regulation. Indeed, Mbnl3 is expressed in muscle progenitor cells (MPCs) including myoblasts and Mbnl3 overexpression inhibits myogenesis (Lee et al., 2007). During myogenic differentiation in C2C12 myoblasts, Mbnl3 promotes exclusion of the beta-exon of muscle transcription factor myocyte enhancer factor 2 (Mef2), leading to a less active Mef2. Mbnl3 is only expressed in the muscle precursor cells where it is presumed to inhibit their differentiation into mature skeletal myofibers (Lee et al., 2010).

On the other hand, Mbnl2 is widely expressed in most tissues throughout development suggesting a more universal role in alternative splicing regulation. To evaluate the role of Mbnl2 loss-of-function in DM, two gene trap mice were developed
that gave contradictory results (Hao et al., 2008; Lin et al., 2006) and these will be further discussed in the DM mouse model section. Even though Mbnl proteins are paralogs, Mbnl2 has been implicated in non-splicing functions that have not been addressed for the other two family members. Adereth et al. investigated the role of Mbnl2-assisted localization of integrin α3 in migrating tumor cells to understand how muscleblind loss leads to muscle detachment from the epidermis in Drosophila mbl mutants (Adereth et al., 2005; Artero et al., 1998). Mbnl1 or Mbnl3 were not included in this study to test the possibility that this function may be a shared among all three family members. A function that may be unique to Mbnl2 is in circadian rhythm regulation. Mbnl2 levels show circadian rhythm fluctuations in the rat pineal gland with Mbnl2 RNA increasing 7-fold during the dark cycle and its upregulation may be adrenergic-cAMP controlled (Kim et al., 2009). The pineal gland interacts with the suprachiasmatic nucleus of the hypothalamus to interpret visual inputs in circadian rhythm regulation.(Falcon et al., 2009) The effect of Mbnl2 level fluctuations in circadian rhythm maintenance, and the downstream target RNAs that may be affected have not yet been defined, but Mbnl2 loss-of-function could cause the hypersomnolence observed in DM patients.

**Mouse DM Models**

In an effort to understand the molecular basis of DM and design new therapeutic strategies, several mouse models have been created. Since DM is a genetic disease dependent on the expansion of microsatellite repeats, several attempts have been made to generate mouse models that bear the same mutation. This is a genetic challenge since the CTG repeat expansions are unstable in mice.
About a decade ago, Thornton and colleagues generated a transgenic mouse model that expresses 250 CUG repeats behind the human skeletal actin gene promoter (Mankodi et al., 2000). This muscle-specific DM model was generated to test the hypothesis that DM results from the CUG repeat expansion at the RNA level and not due to loss of function of \textit{DMPK} function, or mis-regulation of neighboring genes. In \textit{HSA}\textsuperscript{LR} mice, the CUG repeat is expressed at very high levels only in skeletal muscle. In addition, the trans gene is integrated at a site remote from the \textit{DMPK} genetic locus. These non-translated CTG repeats accumulated in nuclear foci and caused a similar muscle pathology including, but not limited to, splicing mis-regulation, chloride channel loss, myotonia and centralized nuclei. Mbnl1 protein was shown to be sequestered in these RNA foci but CELF1 levels are not up-regulated suggesting a secondary role of CELF1 to the disease pathogenesis. The severity of the phenotype was directly correlated to the expression level of the transgene and control mice with short repeats (\textit{HAS}\textsuperscript{SR}) failed to reproduce the phenotype suggesting that the effect of the repeats is at the mRNA level.

\textit{Mbnl1}\textsuperscript{ΔΕ3/ΔΕ3}

To test the hypothesis that Mbnl1 sequestration and loss-of-function results in DM, an \textit{Mbnl1} knockout (KO) model was generated by deleting \textit{Mbnl1} exon 3 (Kanadia et al., 2003a). \textit{Mbnl1}\textsuperscript{ΔΕ3/ΔΕ3} mice recapitulate faithfully a number of DM clinical features including myotonia, muscle pathology cataracts, cardiac conduction defects and aberrant splicing of the muscle specific chloride channel, cardiac troponin T and insulin receptor,. Due to the severity of myotonia and movement impairment, a brain-specific \textit{Mbnl1} KO mouse is necessary to address Mbnl1 functions in the brain. Importantly,
*Mbnl1* knockout mice do not recapitulate many of the mis-splicing patterns in the DM1 brain.

**CELF1 Overexpression**

As stated previously, a major factor in the regulation of the embryonic to adult alternative splicing switch is CELF1, which has been shown to be upregulated in DM patients. Increase of CELF1 leads to retention of an embryonic splicing pattern, causing a delay in muscle maturation and cardiac defects (Koshelev et al., 2010; Timchenko et al., 2004). Two different transgenic mouse models have been generated which overexpress CELF1 in muscle and heart. In the doxycycline-inducible muscle-specific model, an 8-fold upregulation of CELF1 results in severe muscle pathology including myotonia, centralized myonuclei, split fibers, muscle wasting, a prominent DM feature absent from other models, and splicing defects.

In a second CELF1 overexpressor model, human CELF1 was tetracycline-inducible using a heart-specific reverse tetracycline trans-activator transgene. These mice show dilated cardiomyopathy, widespread degeneration, necrosis and loss of myocardial fiber, leading to death after 14 days of doxycycline administration (Koshelev et al., 2010). Most of the known mis-regulated alternative splicing targets in adults were also found to follow the embryonic splicing pattern.

**DM300, DM328XL and DMSXL**

In these transgenic mice, CTG repeats are contained in a 45 kb fragment encompassing the human DM1 locus which includes 300 (DM300), 800 (DM328XL) or 1200-1800 (DMSXL) CUG repeats (Gomes-Pereira et al., 2007; Seznec et al., 2000). These mice were generated to reproduce a more human DM1-like expression pattern of the CTG repeats. The DM300 mice develop muscle pathology, including muscle loss
and CELF1 upregulation, age-dependent defects in insulin metabolism (Guiraud-Dogan et al., 2007) and CNS pathology including the abnormal distribution of tau protein isoforms (Seznec et al., 2001) found in DM patients (Sergeant et al., 2001). Intergenerational instability of the CUG repeats of the DM300 mice led to progeny that had 1200-1800 CTG repeats with depressed expression levels compared to DM300 mice. In the homozygous state, these mice also develop the clinical features listed above but with an onset as early as 1 month of age. Despite the useful information that the above mice provided, there are concerns on the expression levels of the transgene due to random integration and epigenetic regulation of the locus (e.g., hypermethylation). Research is now focused on resolving the molecular pathways involved in DM1 pathogenesis and possible therapeutic strategies.

**Mbnl2 Gene Trap Mouse Models**

The success of the *Mbnl1* KO model in recapitulating a DM-relevant phenotype generated a controversy about the role of other MBNL proteins in DM. To verify a role of Mbnl2 in DM disease, two different knockout mice were generated by independent labs by introducing a gene trap in two different *Mbnl2* introns, intron 2 and intron 4 (Hao et al., 2008; Lin et al., 2006). The gene trap consists of a cassette which contains neomycin selection and β-galactosidase markers downstream of an engrailed 2 (EN2) splice site acceptor. The EN2 splicing acceptor and a strong polyadenylation signal downstream of β-gal terminates translation and reduces production of the protein encoded by the targeted gene.

The difference between the two *Mbnl2* gene traps is the number of C$_3$H motifs that were present in the final fused protein. Integration of the gene trap in intron 4 generated
a protein with fully functional pairs of C$_3$H motifs and most of the Mbnl2 linker region whereas integration in intron 2 generated only a small Mbnl2 fragment with one C$_3$H pair. Another difference between the two gene traps is that the GT2 mouse showed gene trap leakiness with possible splicing of $Mbnl2$ exon 2 to exon 3, skipping the splicing acceptor of the gene trap, as indicated by the residual WT mRNA shown on Northern blot analysis of total RNA of $Mbnl2^{GT2/GT2}$ mice (Hao et al., 2008).

Analysis of these gene trap mice showed different phenotypes (Figure 1-1). The GT4 mice were healthy and lived a normal life span with no signs of DM clinical features, but a cross with $Mbnl1$ KOs did not yield double mutant mice suggesting that Mbnl2 expression is essential following loss of Mbnl1 (unpublished data). On the other hand, the GT2 mouse showed a mild $Mbnl1^{AE3/ΔE3}$ related phenotype with very mild myotonia, mosaic loss of Clcn1 in muscle and very minor mis-splicing of Clcn1 pre-mRNA. No further analyses have been performed on these mice and the fact that GT2 mice show only a mild DM-like phenotype is intriguing suggesting that a low level of Mbnl2 protein is sufficient to maintain almost normal function.

**Discussion**

The missing DM-relevant features of the $Mbnl1$ KO model, the unlinked molecular cascade between CUG expansion and CELF1 upregulation and a more complete DM manifestation in CUG overexpressing mice points out possible roles for other proteins in DM pathogenesis. However, another possible interpretation is that recapitulation of the DM phenotype in mice requires coordinate sequestration of all three Mbnl proteins. In this study, I addressed this latter possibility by generating an $Mbnl2$ KO mouse model (Fig. 1-1) which shows widespread mis-regulation of alternative splicing in the brain.
Two families of alternative splicing regulators have been proposed to be responsible for the splicing regulation of these genes, the MBNL and the CELF family of proteins. CELF1 has been shown to regulate the splicing of at least one of the genes but a direct link between CUG expansion and CELF1 upregulation has not been established. On the other hand, loss of Mbnl1 in a mouse model failed to recapitulate the majority of mis-splicing events in the brain suggesting a possible role for Mbnl2, or a novel unidentified RNA binding protein that is also sequestered by CUG repeats.
Figure 1-1. Different models of Mbnl2 deficient mice show diverse phenotypes. A) Two different Mbnl2 gene traps were generated, one in intron 4 (upper) that leads to a mouse with no apparent abnormal phenotype and one in intron 2 (lower) that gave rise to a mouse model with mild myotonia, muscle histopathology and mis-splicing of Clcn1. B) Homologous recombination strategy to generate a null Mbnl2 mouse model targeting exon 2 that carries the only translation initiation codon.
CHAPTER 2
MBNL2 IS EXPRESSED IN MURINE ADULT BRAIN BUT NOT MUSCLE

The high degree of conservation between Mbnl2 and Mbnl1, the capability to bind and regulate alternative splicing of the same targets in vitro and expression pattern of Mbnl2 make this Mbnl protein a possible candidate to explain the brain related events in DM. In this chapter, I present a detailed Mbnl2 expression analysis in the mouse brain defining the major splicing isoforms and the cellular and subcellular distribution.

Results

**Mbnl2 Is Widely Expressed In the Murine Brain**

To define the regions of the brain, and the cell types, that express Mbnl2, I took advantage of the previously generated Mbnl2 GT4 mouse line that expresses β-galactosidase under the Mbnl2 promoter. β-galactosidase catalyzes the hydrolysis of lactose and other beta-galactosides including the model substrate of 5-bromo-4-chloro-3-indolyl-β-D-galactoside. The monomers of this indole derivative are colorless but after oxidization they produce a stable blue precipitate (Holt and Sadler, 1958a; Holt and Sadler, 1958b). Using this approach, I assayed the expression pattern of the Mbnl2 gene. The β-galactosidase cassette is integrated in intron 4 of the Mbnl2 gene. A splicing acceptor upstream of the cassette results in splicing of the Mbnl2 exon 4 to the cassette. A strong polyadenylation signal on the 3’ end of the cassette terminates translation of the fused mRNA transcript. This leads to a fused Mbnl2/β-galactosidase protein that is expressed where a full length Mbnl2 protein should be expressed. To visualize the expression pattern of Mbnl2 gene in the mouse brain I obtained coronal brain section from the GT4 mice and performed an X-Gal staining.
The neuronal populations that stained intensely were the cerebellar Purkinje cells, cells in the deep nuclei of the cerebellum as well as hippocampal granular and pyramidal neurons and the habenular nuclei, thus providing evidence for relatively high Mbnl2 expression levels (Figure 2-1). There were a plethora of other types of cells that showed β-gal staining, including glia within the white matter of the brain stem and cerebellum, ependymal cells in the ventricles as well as Golgi type 2 cells in the granular layer, but the signal was lower possibly due to lower levels of Mbnl2 transcription or due to the small size of the cells.

The nature of the β-gal staining technique does not address the issue of the subcellular localization of the Mbnl2 protein. To address this issue, we need to know the subcellular localization of Mbnl2, as well as the presence of DMPK-CUG expanded RNA in the nuclei of these cells.

**Generation of an Mbnl2 Specific Antibody**

To evaluate the role of Mbnl2 in the brain and its implications in DM pathogenesis, the levels of Mbnl2 protein expression and its sub-cellular localization need to be characterized by the development of a mono-specific antibody. The high degree of primary structure conservation among the three Mbnl paralogs did not allow the use of existing Mbnl antibodies to characterize the Mbnl2 protein expression pattern. To find a unique to Mbnl2 region that could be used to raise a homolog-specific antibody, I first performed an Mbnl2 isoform analysis and then aligned all the known isoforms of the three Mbnl homologs at a protein level.
**Isoform analysis**

In the current literature, several Mbnl1 splicing isoforms have been studied which include, or exclude, exons 5, 7 and 8 as well as a truncated isoform that skips exon 1-2 and produces an RNA species with an alternative 3’ UTR and a different potential translation initiation codon. To verify the different Mbnl2 isoforms that are expressed in the brain, I produced a cDNA library using RNAs isolated from different regions of the brain and sequenced a number of clones with primers complementary to exons 1 and 9. This analysis identified three Mbnl2 alternative spliced exons, one of which was described for the first time and annotated as Mbnl2 exon 8a. The alternative exons included: 1) exon 6, which is equivalent to Mbnl1 exon 5; 2) exon 8a, equivalent to Mbnl1 exon 7; 3) exon 8b, equivalent to Mbnl1 exon 8 (Figure 2-2A). The combination of these three exons produced 7 different spliced isoforms and the majority included exon 6. To define the major Mbnl2 isoform in brain and muscle, the level of inclusion and exclusion of the aforementioned exons was tested by RT-PCR (Figure 2-2C). In brain, a high percentage of the Mbnl2 mRNA population includes exon 6, as well as multiple isoforms that include only exon 8a, only exon8b or both. In muscle the majority of the isoforms skip all three of these alternative exons.

**Antibody production and testing**

Two regions that are unique to Mbnl2 were used to raise an isoform specific polyclonal antibody. The first region is part of the linker between the two pairs of C3H domains and is present in all Mbnl2 isoforms. The second region is isoform-specific and generated by the skipping of exon 8b. Exon 8b skipping generated a unique to C-terminus domain with unknown function (Figure 2-2B). Both regions were used to
design small immunoreactive peptides and generate polyclonal rabbit antibodies against the murine Mbnl2 protein.

The cross-reactivity of the resulting antisera was characterized to evaluate the degree of antibody specificity for Mbnl2. To test the cross-reaction to the other two members of the Mbnl family, plasmids that express one of the three Mbnl homologs fused to a C-terminal Myc-tag were transfected into CosM6 cells. The specificity of the purified antibody was tested by both immunofluorescence (Figure 2-3) and western blotting (Figure 2-4A). Finally, to verify that the antibody recognized endogenous Mbnl2, I employed C2C12 cells, which have been reported to express high levels of Mbnl2 (Holt et al., 2009). Treatment of these cells with siRNA against the endogenous Mbnl2 mRNA confirmed the specificity of the Mb2k antibody (Figure 2-4B).

The purified antibody which was elicited against the linker region recognized protein expressed from the myc-tagged Mbnl2 expression vector, but not from the myc-tagged Mbnl1 and myc-tagged Mbnl3 expression vectors (Figure 2-4A, upper row). To verify transfection efficiency and expression of the other two family member proteins in the cell lysates, western blot analysis was performed with an anti-Myc antibody (Figure 2-4A, middle row). The results show similar expression levels for all three myc-tagged proteins. For a loading control, anti-Gapdh antibody was used which demonstrated equal loading for all four cell lysates, including a myc construct where the myc-tag was out of frame (transfection control) and thus was not detected by the anti-Myc antibody (Figure 2-4A, bottom lane).

A similar detection pattern was observed by IF on these cells using the same transfection conditions (Figure 2-3). More importantly, Mbnl2, as well as the other Mbnl
proteins detected with the anti-Myc antibody, had the expected nuclear localization pattern. It is worthwhile to note that all three Myc-tagged Mbnl expressing constructs were constructed with the isoforms that include the exon responsible for the nuclear localization of the Mbnl protein.

All three Mbnl paralogs were expressed in a cell culture system to test the cross-reaction of the antibody with the other two Mbnl proteins, Mbnl1 and Mbnl3. A caveat this approach is that proteins are ectopically expressed at a relatively high level so it is important to test whether these antibodies recognize endogenous Mbnl2. To test antibody cross-reaction with other endogenously expressed proteins, siRNA knockdowns of Mbnl2 were performed in C2C12 cells. Western blot analysis of control versus siRNA-treated cells showed that the purified anti-Mbnl2 antibody recognized endogenous Mbnl2 protein (Figure 2-4B, upper lane). The results indicate that in a cell-based system, these anti-Mbnl2 antibodies specifically recognize Mbnl2.

**Generation of an Mbnl2 Knockout Mouse Model**

Based on the hypothesis that loss of Mbnl2 in the brain resulted in the CNS deficits associated with DM, I tested this hypothesis by generating *Mbnl2* knockout mice. The *Mbnl2* knockouts served also as a negative control in the Mbnl2 RNA and protein expression analysis.

**Generation of the Mbnl2 ES cell targeting vector**

The *Mbnl2* conditional knockout targeting vector was constructed using a new method of plasmid manipulation and genetic engineering, termed recombineering, to avoid complications associated with conventional cloning techniques. A major advantage of recombineering is that the introduction of extraneous sequences at the *Mbnl2* genomic locus was not included except for a few sequence elements that
allowed correct targeting in ES cells. A conditional targeted allele was generated to avoid a possible embryonic lethal phenotype due to loss of Mbnl2 during embryogenesis, as well as to tease out potential tissue-specific contributions of the protein to possible disease-relevant phenotypes. This strategy avoided a problem with the $Mbnl1^{\Delta E3\Delta E3}$ mouse model, which is a constitutive knockout model, so that an unbiased analysis of potential CNS deficits is compromised. All of the behavioral analysis tasks for mice require normal motor function.

To generate the $Mbnl2$ conditional targeting construct, a bacterial artificial chromosome (BAC) was obtained which included all of $Mbnl2$ exon 2 and ~10 kb of upstream and downstream genomic sequence. Exon 2 is the only exon that is utilized for translation initiation and there are annotated splicing events which lead to exon 2 skipping. A 10 kb fragment of the Mbnl2 BAC was cloned into a targeting vector (Figure 2-5, lane 1) that contained all the necessary selection markers and elements for ES cell targeting, including the negative selection marker thimidine kinase (TK). To generate a conditional KO gene, $Mbnl2$ exon 2 was flanked by loxP sites. The loxP site is a 34 bp DNA element composed by two 13 bp inverted repeats that provide enzyme specificity and an 8 bp linker that provides directionality. The loxP sites are recognized by Cre recombinase, which, depending on the orientation of the linker element, either promotes inversion or circularization and deletion of the genomic sequence between two loxP sites by homologous recombination (Sauer and Henderson, 1988).

I introduced the first loxP site about 250 bp upstream of the intron-exon junction of exon 2 (Figure 2-5, lane 2), to prevent inference with cis-acting splicing elements. In any DNA manipulation, a selection method/marker is necessary to isolate and/or enrich for
the desired targeting result. To target the 5' loxP, a commercially available plasmid that carries a neomycin selection marker (Neo) flanked by two loxP elements (floxed) was used. This cassette is driven by two promoters, a eukaryotic Pgk, and a prokaryotic Em7, promoter to drive selection in both ES cells and E. coli. After the correct targeting event and clonal selection, the selection cassette was deleted by introducing a lactose-inducible cre expressing plasmid that upon induction recombined the two loxP sites (Figure 2-5, lane 3). This step is necessary before the introduction of the downstream loxP site (3' to the exon) so that a second selection step can be performed using the same selection marker.

To target the 3' loxP site, a similar method was employed by selecting a site 250 bp downstream of Mbnl2 exon 2 and using a plasmid where Neo is flanked by two Frt sites and one loxP site at the 3' most end (Figure 2-5, lane 4). The frt sites belong to a yeast site-specific recombination system which promotes a balance between the levels of two different plasmid isoforms, plasmid A and B. The recombination is catalyzed by the enzyme Flp (Volkert and Broach, 1986).

The final targeting construct was tested for sequence integrity by genomic sequencing of the introduced DNA elements, as well as loxP function. To test loxP functionality, I introduced the final targeting construct to cells that express Cre recombinase and tested for exon 2 deletion (Figure 2-5, lane 5).

**ES cell targeting**

After complete verification, the targeting construct was linearized and targeted to 129 SvImJ ES cells following standard procedures (Kanadia et al., 2003a). Positive clones that survived G418 and Neo selection were picked and screened by Southern blotting to identify the correct targeting events. Two different probes were used for the screening,
one spanning a 200 nt region upstream of the 5' arm of homology (ARM) and one spanning a 300 nt region downstream of the 3' ARM. A correct targeted event on the 5' ARM should yield a band size of 5 kb after StuI digestion versus the 6.4 kb for the WT allele. A correct targeting event on the 3' ARM should give a band size of 5 kb after Scal digestion versus 31 kb for the WT allele. After selection, 432 colonies were picked and 390 of these were screened. We found 7 (1.8%) correctly targeted 5' ARM clones of which 5 (1.3%) also correctly targeted the 3' ARM. To verify functional loxP integration of the targeted Mbnl2 conditional allele, Cre was ectopically expressed in targeted ESCs and assayed for loss of exon 2.

**ES cell blastocyst injections**

ES cells from positive clone 173 were injected in C57BL/6 pseudopregnant female mice (University of Michigan Transgenic Core) and chimeric male animals were acquired for mating and germline transmission of the conditional allele. After two blastocyst injections, 5 chimeric animals were shipped and received by our lab at 3 weeks of age. The chimeric percentage and coat color shows the contribution of the 129 SvImJ cells in the development of the embryo and the highest percentage chimera we obtained was 95% and the lowest was 40%.

**Mbnl2 conditional line generation and maintenance**

Chimeric mice carry a mixture of wild type cells and cells heterozygous for the conditional allele. The conditional allele is expected to contribute equally with the WT allele for Mbnl2 expression and the additional genetic information should not result in an aberrant phenotype. To obtain heterozygous Mbnl2^{+/con} mice, all 5 chimeras were mated to C57BL/6 female mice and the progeny were genotyped for germline transmission of the conditional allele. From the 5 chimeric mouse matings, only two
mated successfully (75% and 40%) and both liters from these matings gave germline transmission. Heterozygous conditional mice were further mated to obtain conditional allele homozygosity. To minimize genetic drift, mice were backcrossed to either C57BL/6 or 129SvlmJ mice (obtained from JAX).

**Mbnl2^ΔE2ΔE2 constitutive knockout mouse generation**

In the current model of DM pathogenesis, toxic CUG repeat RNAs sequester a number of proteins. To determine if constitutive Mbnl2 loss was embryonic lethal, I attempted to generate a line which lacked Mbnl2 expression in all the tissues. Constitutive *Mbnl2* knockout lines were generated by mating *Mbnl2^+/con* mice with B6.C-Tg(CMV-cre)1Cgn/J mice that express Cre in all tissues during embryogenesis. Under the CMV promoter, Cre is expressed in the germline which results in permanent deletion of *Mbnl2* exon 2 in the resulting line. *Mbnl2^ΔE2ΔE2* were routinely intercrossed and then backcrossed every 10 generations to minimize genetic drift. Deletion of *Mbnl2* exon 2 in *Mbnl2^ΔE2ΔE2* mice was confirmed by Southern blotting of WT, *Mbnl2^+/ΔE2* and *Mbnl2^ΔE2ΔE2* mice using a hybridization probe outside of the deleted region (Figure 2-6A) and RT-PCR analysis with primers in *Mbnl2* exon 2 and exon 3 (Figure 2-6B).

**Mbnl2 Protein Is Expressed and Mostly Nuclear In Brain, but Is Expressed at a Low Level in Muscle of Adult Mice**

The high expression of Mbnl2 RNA in the brain suggested a more specialized role in the development and maturation of the neuronal network. To be a viable candidate for sequestration and involvement in the DM brain, Mbnl2 protein should be expressed in specific cell populations that are affected by DM disease and the sequestration hypothesis dictates that Mbnl2 should be predominantly nuclear.
After verification of the genotype, $Mbnl2^{\Delta E2/\Delta E2}$ mice were examined for $Mbnl2$ expression at both RNA and protein levels. To assess loss of exon 2 at the RNA level, RT-PCR was performed using primer sets in exons 1 and 3, and 1 and 2, in and RNAs isolated from brain and muscle (Figure 2-7B). In both tissues, deletion of exon 2 was verified.

During the analysis of $Mbnl2$ protein expression, the Mb2k antibody described earlier in this thesis failed to detect $Mbnl2$ in mouse tissues likely due to relatively low affinity of this polyclonal for endogenous mouse $Mbnl2$. As an alternative, an anti-$Mbnl2$ monoclonal antibody (mAb 3B4), which was also elicited against the same linker region as Mb2k and should recognize all $Mbnl2$ isoforms, had recently become available (Holt et al., 2009) Therefore, we used mAb 3B4 to examine the expression levels of $Mbnl2$ in six different mouse tissues (hippocampus, cerebellum, heart, liver, lung, muscle, spleen) of WT and $Mbnl2$ knockout mice (Figure 2-7A. As expected, and in contrast to $Mbnl1$, $Mbnl2$ was not predominantly expressed in the muscle. Indeed, mAb 3B4 detected very low levels of $Mbnl2$ protein in adult skeletal muscle in contrast to $Mbnl2$ RNA levels detected by RT-PCR and Northern blot analyses. In contrast, $Mbnl2$ protein was readily detectable in the brain. The lack of $Mbnl2$ protein in skeletal muscle can be attributed to either a translational inhibitory mechanism, localization of the mRNA to regions of low translational efficiency, or post-translational modification of the protein at a region that masks the antibody epitope. A recent study also reported that $Mbnl2$ expression is developmentally regulated with high expression levels early in embryogenesis and during muscle regeneration and decreased levels in adult muscle tissue (Holt et al., 2009). Most surprising was the finding that $Mbnl2$ protein levels are
the highest in multiple regions of the brain. No Mbnl2 protein was detected in 
\( \text{Mbnl2}^{\Delta E2/\Delta E2} \) mice in any tissues confirming antibody specificity. No difference in Mbnl1 protein levels was detected. In summary, all tissues examined except skeletal muscle showed expression of Mbnl2 protein, including the heart which is affected in DM patients.

To identify the cell types which expressed Mbnl2, IHC was performed on coronal brain sections at the midbrain area and sagittal cerebellar sections of both WT and 
\( \text{Mbnl2}^{\Delta E2/\Delta E2} \) mice mAb 3B4. To evaluate the impact of the Mbnl2 loss on hippocampal area, Nissl staining was performed using both WT and \( \text{Mbnl2}^{\Delta E2/\Delta E2} \) brains to assess for changes in gross morphology and integrity of the hippocampal formation. In agreement with the Mbnl2 LacZ staining patterns, the IHC studies showed an intense staining of cells in the frontal cortex, dentate gyrus, CA1-CA3 pyramidal region of the hippocampus and Purkinje cells of the cerebellum of WT mice. No staining was observed in \( \text{Mbnl2}^{\Delta E2/\Delta E2} \) brain sections (Figure 2-8, 2-9). Mbnl2 protein co-localized with nuclear DAPI staining indicating that the majority of Mbnl2 is nuclear. In cells of the cerebral cortex of the frontal lobe, the protein was localized in both the nucleus and the cytoplasm (Figure 2-8) supporting a prior suggestion that Mbnl2 has a role in integrin RNA trafficking and localization role in growing axons (de Andrade and Jansen, 2005).

**Discussion**

The lack of \( \beta \)-gal staining in other regions of the brain, such as the neurons of the cerebellar molecular layer, the Bergmann glia and the choroid plexus epithelial cells, suggest that Mbnl2 function(s) are important for a specific population of cells in the CNS, and only these cells will be directly affected by loss of Mbnl2 function in the DM.
brain. DM patients do not suffer from ataxia which could be explained by the lack of DMPK expression in the cerebellum. Alternatively, MBNL2 protein may localize to the cytoplasm and is not trapped by CUG repeats. Another possibility is functional complementation by another protein that is not sequestered by CUG repeats and can compensate for the loss of Mbnl2. On the other hand, Mbnl2 seems to be highly expressed in hippocampal neurons, a region of the brain that is responsible for learning and memory which are functions affected in DM patients. The intense staining in the habenular nuclei is of great importance as these nuclei are known to be involved in circadian rhythm regulation. The most common complaint of DM patients is that they cannot control their susceptibility to falling asleep. The habenular nuclei are located above the thalamus at its posterior end proximal to the midline of the brain. This region has been considered to be a part of the epithalamus which interacts closely with the pineal body, which is the region where Mbnl2 RNA levels show circadian fluctuations (Kim et al., 2009). Recently, the habenular nuclei have been implicated in motivational control of behavior, such as reward-based action selection (Matsumoto and Hikosaka, 2007), spatial reference memory and cognitive function (Lecourtier et al., 2004), and attention disturbances (Lecourtier and Kelly, 2005).

During this study, several novel brain and muscle isoforms of Mbnl2 were discovered and a previous study indicated that alternative splicing of Mbnl1 exon 5 (also referred to as exon 7) is developmentally regulated and plays role in the intracellular localization of the protein (Terenzi and Ladd, 2010). The high percentage of Mbnl2 exon 6 inclusion suggests that Mbnl2 localizes to the nucleus in neurons of the CNS. This
possibility supports the hypothesis that Mbnl2 plays a pivotal role in the regulation of alternative splicing in the brain similar to the function of Mbnl1 in muscle.

A novel rabbit polyclonal antibody (Mb2k) was developed which can be used to detect Mbnl2 proteins. This antibody was used to evaluate Mbnl2 protein expression levels in different tissues by western blot and Mbnl2 localization by immunohistochemistry (IHC, data not shown). Unfortunately, the antibody gave a non-specific weak signal by IF and non-specific bands by western blotting of mouse tissues that were present on Mbnl2 knockout mice. During the generation of the Mbnl2 knockout mouse, a new Mbnl2 specific monoclonal antibody became commercially available that was tested in both WT and Mbnl2 knockouts and confirmed its specificity. The predominant nuclear localization of Mbnl2 and the high expression levels of this protein in all major neuronal cells of the hippocampus suggest that this Mbnl protein is the best candidate for a sequestered factor in DM. The sequestration of Mbnl2 by toxic CUG repeats has been verified in human DM1 fibroblast and myoblast cells (Holt et al., 2009). CUG\textsuperscript{exp} nuclear foci have been detected in neurons of autopsied DM1 brains in multiple regions including the, cerebral cortex, cerebellar Purkinje cells, hippocampus, dentate gyrus, substantia nigra, subcortical white matter, corpus callosum, brain stem and oligodendrocytes of the centrum semiovale (Jiang et al., 2004). No characterized nuclear structures have been found to colocalize with these RNA foci, including PML bodies, nucleoli, perinucleolar compartments or nuclear splicing factor compartments (SFCs or speckles).
Figure 2-1. *Mbnl2* is highly transcribed in the mouse brain. LacZ staining performed in mouse brain coronal sections indicating presence of *Mbnl2* transcripts. *Mbnl2* is highly expressed in Purkinje cells of the cerebellum (B) the hippocampal formation (C) and the medial habenula (D). More scattered expression is detected in the frontal cortex (A).
Figure 2-2. Extensive alternative splicing of *Mbnl2* occurs in the C-terminus of the protein. A) *Mbnl2* gene alternative splicing map. Untranslated exonic regions are in white, coding exons in black and alternative spliced exons in red. B) Amino acid sequence of the Mbnl2 isoforms produced by exon 8b skipping domain. C) RT-PCR analysis of *Mbnl2* alternative spliced exons 8a, 8b and 6 in different tissues (total brain, cerebellum hippocampus, tibialis anterior, gastrocnemius, soleus and quadriceps)
Figure 2-3. Mbnl2 polyclonal antibody does not cross-react with the other Mbnl protein isoforms. Cosm6 cells were transiently transfected with myc tagged Mbnl2, Mbnl1, Mbnl3 and an empty Myc expression vector. Immunofluorescence staining was performed with the new Mbnl2 polyclonal antibody (green) and an anti-Myc antibody (red). In blue is the nuclear stain DAPI.
The anti-Mbnl2 polyclonal antibody did not cross-react with other endogenous expressed proteins in cell culture. A) Cosm6 cells were transiently transfected with myc tagged Mbnl2, Mbnl1, Mbnl3 and an empty Myc expression vector. Western blot analysis was performed with the new Mbnl2 polyclonal antibody upper lane and an anti-Myc antibody (middle lane). Gapdh was used as a loading control (lower lane). B) MEF cells were transfected with Mbnl2 specific siRNA (right) and scrambled siRNA as control (left). Western blot analysis was performed with antibodies against Mbnl2, Mbnl1, Cugbp1, and Gapdh as loading control.
Figure 2-5. Generation of Mbnl2 KO targeting construct. To verify the Mbnl2 construct 1ug of plasmid from different stages of the targeting construct generation were cut with XbaI. Lane 1) Mbnl2 retrieval in the backbone construct PL253. Lane 2) targeting of the upstream LoxP site. Lane 3) cre mediated neomycin cassette excision. Lane 4) targeting of the downstream LoxP site. Lane 5) cre mediated deletion of exon 2. Digestion products were run in a 1% agarose gel and stained with ethidium bromide.
Figure 2-6. *Mbnl2*ΔE2/ΔE2 mice lack expression of exon 2. A) DNA from WT, *Mbnl2*ΔE2/ΔE2 and *Mbnl2*ΔE2/ΔE2 mouse tails was digested with *Stu*I and analyzed by Southern blotting. B) RNA from WT, *Mbnl2*ΔE2/ΔE2 and *Mbnl2*ΔE2/ΔE2 mouse brain was analyzed by RT-PCR for *Mbnl2* exon 2 expression (fwd primer in e2 and rev primer in e3) as well as expression changes of *Mbnl1* mRNA by analyzing levels of e3 (fwd primer in e3 and rev primer in e4). *Ppia* was used as loading control.
Figure 2-7. Mbnl2 is highly expressed in mouse brain but not in muscle. A) WT and Mbnl2ΔE2/ΔE2 mouse protein lysates from cerebellum, hippocampus, frontal brain, heart, lung quad and spleen were analyzed by western blotting with a monoclonal anti-Mbnl2 antibody (upper). Gapdh was used as a loading control (lower). B) WT and Mbnl2ΔE2/ΔE2 mouse RNA from total brain and quadriceps muscle was analyzed by RT-PCR for exon 2 exclusion with two different primer sets (fwd e1-rev e3 on the left and fwd e2-rev e3 on the right)
Figure 2-8. Mbnl2 shows nuclear and cytoplasmic localization in cells of the frontal cortex. Frontal cortex brain sections from WT and Mbnl2ΔE2/ΔE2 mice were used for IF with an anti-Mbnl2 antibody (green). DAPI served as a nuclear stain. Confocal imaging was taken at 20x (upper-scale bar 0-250μm) and 250x (lower-scale bar 0-7.5μm) zoom.
Figure 2-9. Mbnl2 is mostly nuclear in cells of the hippocampal formation. Hippocampal brain sections from WT and Mbnl2ΔE2ΔE2 mice were used for IF with an anti-Mbnl2 antibody (green). DAPI served as a nuclear stain. Confocal imaging was taken at 20x (upper scale bar 0-250μm) and 200x (lower scale bar 0-25μm) zoom.
CHAPTER 3
ANALYSIS OF THE $Mbnl^{\Delta E2/\Delta E2}$ KNOCKOUT MOUSE MODEL

In many cases, deletion of an essential gene that is implicated in multiple functions has a wide impact on the transcriptome and/or proteome of an organism and may lead to developmental defects, embryonic lethality or decreased life span or less severe phenotypes. Gene duplications which result in paralogous gene families provide functional redundancy to complex organisms, which assure the survival of the organism in case of deleterious gene mutations. The three paralogous $Mbnl$ genes possess a high degree of functional similarity and sequence conservation which suggests the possibility of functional complementation when another family member is compromised due to mutation. A prerequisite for functional complementation is that the spatial and temporal expression pattern of the paralogs should be similar.

The presence of RNA foci in the nuclei of cells of the hippocampus and dentate gyrus indicates that toxic RNA expression could lead to inhibition $Mbnl2$ function in these cells. If $Mbnl2$ is vital for the finely-tuned functional integrity of the brain, then we expect that loss of $Mbnl2$, if not embryonic lethal, it will compromise the computational capacity of the specific brain region/formation and result in a distinct phenotype. Prior to testing mice for brain-related phenotypes, we first confirmed that $Mbnl2$ knockouts did not show neuromuscular deficits that might impair their ability to complete a behavioral test. The previously reported $Mbnl2$ GT2 mouse model showed focal loss of the muscle-specific chloride channel CIC-1 and muscle pathology with centralized nuclei and mild myotonia assessed by EMG (Hao et al., 2008). Thus, it was necessary to determine if $Mbnl2^{\Delta E2/\Delta E2}$ mice showed any muscle defects before any comprehensive behavioral analysis.
Results

*Mbnl2*\(^{AE2/AE2}\) Mice Show Postnatal Growth Retardation

To test the hypothesis that *Mbnl2* is essential for normal development of the mouse, the Mendelian ratios of the progeny of *Mbnl2*\(^{+/AE2}\) x *Mbnl2*\(^{+/AE2}\) crosses was compared to the normal 1:4 ratio for a homozygous *Mbnl2*\(^{AE2/AE2}\) genotype. The genotype of mice was tested by genomic PCR amplification using primers in the region flanking exon 2. No statistically significant difference was found between the expected ratio and the actual ratio obtained from *Mbnl2*\(^{+/AE2}\) crosses (data not shown), suggesting that Mbnl2 is not an essential gene. Nevertheless, I noted enhanced death of *Mbnl2*\(^{AE2/AE2}\) mice around the weaning period. Only a few mice (n<10) that lived beyond weaning died prior to 5 months of age. Since constitutive deletion of *Mbnl2* does not result in embryonic lethality and the majority of the *Mbnl2* knockouts survive post sexual maturity, it was not necessary at this point to generate tissue specific *Mbnl2* knockouts.

During weaning an obvious difference in size was observed between pups in the same litter (Figure 3-1, left). Genotyping of these mice showed that the smaller pups were *Mbnl2*\(^{AE2/AE2}\) mice. To assess development of the *Mbnl2*\(^{AE2/AE2}\) lineage, the body weights of *Mbnl2*\(^{AE2/AE2}\) mice and littermates at several time points were obtained including weaning (P21) and P47(Figure 3-1, right). At P21, *Mbnl2*\(^{AE2/AE2}\) mice weighed <30% than WT littermates and this difference was more pronounced in males (data not shown). Both male and female *Mbnl2*\(^{AE2/AE2}\) mice attain a normal weight by P29.

No Aberrant Muscle Pathology Due to Mbnl2 Loss

The *Mbnl1*\(^{AE3/AE3}\) mice showed severe muscle histopathology including centralized nuclei, split fibers, as well as pycnotic nuclei but not muscle fiber loss. *Mbnl2*\(^{+/GT2}\) mice developed a similar but milder phenotype when 9 month old mice were evaluated. To
examine Mbnl2 knockouts, muscles were obtained from mice at ~5 months of age to test for any of the above defects. In a representative transverse section of tibialis anterior (TA) from WT and \textit{Mbnl2}^{ΔE2/ΔE2} mice H&E staining was used to assess overt histological changes in the mutant (Figure 3-2). Hematoxylin is a basic dye and stains the nuclei of cells (deep purple) and eosin is a basophilic dye that stains proteins (pink). In both WT and \textit{Mbnl2}^{ΔE2/ΔE2} mice, the muscle tissue appeared healthy with no obvious cytological differences between WT and \textit{Mbnl2} knockout muscle.

\textit{Mbnl2}^{ΔE2/ΔE2} Mice Do Not Show Muscle-Specific Chloride Channel Loss

In \textit{Mbnl1}^{ΔE3/ΔE3} mice, mis-splicing of the muscle specific chloride channel Clcn1 leads to inclusion of a premature termination codon and loss of Clcn1 protein from the muscle fiber membrane (Kanadia et al., 2003a). \textit{Mbnl2}^{+/GT2} mice have been reported to show a similar, but focal loss, of \textit{Clcn1} expression but not the mis-splicing of Clcn1 premRNA. To test if \textit{Mbnl2}^{ΔE2/ΔE2} mice showed any loss of Clcn1 protein expression from the muscle fiber membrane, IHC was performed using transverse quadriceps muscle sections from 3-5 month old WT and \textit{Mbnl2}^{ΔE2/ΔE2} mice and an anti-Clcn1 antibody (Figure 3-3A). The results showed no loss of Clcn1 from the muscle fiber membrane so at least up 5 months of age, Mbnl2 mice did not show any obvious muscle structural or functional changes.

\textit{Mbnl2}^{ΔE2/ΔE2} Mice Do Not Recapitulate Aberrant Splicing of Clcn1

To verify that my \textit{Mbnl2}^{ΔE2/ΔE2} mice do not show mis-splicing of \textit{Clcn1}, RT-PCR analysis was performed with primers in the upstream and downstream exons of the fetal exon 7a (Figure 3-3B). The analysis did not reveal an increase in the inclusion of Clcn1 exon 7a up to the age of 5 months in contrast to \textit{Mbnl1}^{ΔE3/ΔE3} mice.
In summary, \( Mbnl2^{\Delta E2/\Delta E2} \) mice up to 5 months of age did not develop a movement disorder and thus it was possible to test for behavioral abnormalities using tests such as the Morris water maze (MWM), open field and sensitivity to seizure development.

**Loss of Mbnl2 Does Not Lead to an Overt Hippocampal Pathology**

Despite the high levels of Mbnl2 expression, loss of the protein did not result in any overt structural changes in the hippocampal formation (Figure 3-4). Both WT and \( Mbnl2^{\Delta E2/\Delta E2} \) hippocampi displayed normal morphology without loss of any detectable cell population. Further analysis with more sensitive techniques is required to assess more subtle changes.

**\( Mbnl2^{\Delta E2/\Delta E2} \) Mice Show Impaired Spatial Learning and Memory**

The integrity of the hippocampus is essential for spatial pattern establishment and learning. The Morris water maze test is an assay which tests the effects of hippocampal degeneration, or lesions, in rodents. Most studies have focused on using this assay to test for deficiencies in the hippocampus, striatum, basal forebrain, cerebellum and neocortical areas and these as well as other CNS regions may influence the results. The Morris water maze tests the swimming pattern of mice and their ability to escape from the water onto a hidden platform. The test is based on the natural instinct of mice to avoid water and drowning. Two different versions of water maze test exist, the hidden platform test (spatial version) and the visible (non-spatial version). I performed the hidden platform test (spatial version) assesses to assess the ability of the mouse to read spatial cues, learn and memorize a spatial map of the platform location and retrieve that map to evaluate the visual cues at any time point to find the platform. The Morris water maze consists of a round swimming pool filled with colored water. The color of the water depends on mouse coat color since an adequate contrast is required
by the software to detect the animal during the procedure. A platform is located under
the water surface so that mice cannot see it but can climb onto this stage once it is
discovered. There are many training variations for the test but the most commonly used
requires a 5 day training period during which the mouse learns to find the platform and 1
trial day when the platform is removed. During platform removal, a mouse that has been
well trained and has no memory deficits should be able to remember where the platform
was and search for it by crossing over the original platform position. The water maze is
divided in four quadrants and the platform is located in the middle of one of the
quadrants. If a mouse has a defect in learning and memory of spatial orientation then, in
the most severe case, they will fail to restrict their platform search to the correct
quadrant and during the five training days they will fail to reach the platform. In less
severe forms, the mouse will reach the platform during the training period, but during
the trial day when the platform is removed, the number of cross-overs over the platform
is low.

To test if loss of Mbnl2 leads to spatial learning and memory deficits, WT and
$Mbnl2^{ΔE2/ΔE2}$ mice were tested in the hidden Morris water maze test. During the 5 day
learning period the $Mbnl2^{ΔE2/ΔE2}$ mice showed a significant learning delay at Day 3, but
recovered by Day 4 and performed as well as the WT at Day 5 (Figure 3-5). Mutant
mice did not show any difference in quadrant occupancy after removal of the platform
from the target quadrant but $Mbnl2^{ΔE2/ΔE2}$ mice spent more time in the quadrant next to
the target quadrant showing quadrant confusion in their searching path. In contrast, WT
mice showed an increase in target quadrant occupancy (Figure 3-6). Most importantly
on Day 6 after platform removal, $\textit{Mbnl2}^{\Delta E_2/\Delta E_2}$ mice showed a significantly decreased number of platform crossovers compared to WT (Figure 3-7).

To test if the decreased performance shown by the $\textit{Mbnl2}$ knockout mice was due to impaired swimming speed or a decrease in the searching pathway, the average speed and total distance traveled to the platform of WT and $\textit{Mbnl2}^{\Delta E_2/\Delta E_2}$ mice were compared (Figure 3-8 and 3-9) and no significant differences were found. To test if elevated stress response could affect the searching pathway, the time that mice spent in the thigmotaxis zone, defined as the perimeter region <10 cm from the tank wall, was used to assess stress level. Mice are introduced to a novel environment tend to display an elevated stress response due to their intrinsic escape behavior and rodents tend to limit their locomotor activity to an area close to the walls of a novel open space during the initial 5-10 minutes of initial placement. Subsequently, they slowly begin to explore the center of the open area. This induction of exploration and search for an escape is impaired by use of anxiolytic drugs (Simon et al., 1994) linking thigmotaxis to stress response. When the platform was removed on Day 6, $\textit{Mbnl2}^{\Delta E_2/\Delta E_2}$ mice remained in the thigmotaxis zone compared to WT mice (Figure 3-10).

**Loss of Mbnl2 Results in Increased Seizure Susceptibility**

During normal breeding and maintenance of the colony, a few $\textit{Mbnl2}^{\Delta E_2/\Delta E_2}$ mice developed a hyperexcitability phenotype near P21 followed by death with 24 hours. These mutants showed limited mobility movement and then nose twitching and sudden rapid/uncontrolled movement followed by tonic seizures. The etiology of the seizure onset and death was unclear but a number of heterozygous $\textit{Mbnl2}^{+/\Delta E_2}$ and homozygous $\textit{Mbnl2}^{\Delta E_2/\Delta E_2}$ animals died near weaning age.
Epilepsy and seizure induction results from abnormal neuronal network synchronization that has a multi-factor pathogenesis that is attributed mainly to loss of inhibitory networks and less frequent to a gain of excitatory networks in the brain. Over 70 different gene mutations and expression level changes have been implicated to an epileptic phenotype (Noebels, 2003). There are two main networks in the brain the glutaminergic or excitatory network and the GABAergic or inhibitory network. Neurons of the glutaminergic pathway release the neurotransmitter glutamate from the pre-synaptic membrane to the synaptic cleft. Glutamate binds to post-synaptic receptors and induces depolarization of the post-synaptic membrane inducing membrane excitation and generation of axon potentials. Neurons of the GABAergic network release GABA, a neurotransmitter that causes hyper-polarization or repolarization of the post-synaptic membrane, thus inhibiting membrane excitation and generation of axon potentials. Disruption of the balanced excitation and inhibition of neurons can lead to hyper-excitability and seizures.

In most cases, synchronous neuronal excitation has been linked to channelopathies that disrupt normal synaptic inhibition (McCormick et al., 1999; Spampanato et al., 2001), but also to a constellation of perturbations in other mechanisms that lead to membrane excitability, such as vesicle docking and neurotransmitter synthesis (Kash et al., 1997; Rosahl et al., 1995). Cells that are not part of the main brain circuitry have also been implicated in hyper-excitability and seizures. Decreased glutamate uptake by glia cells that lack expression of the glutamate transporter GLT-1 leads to seizures in GLT-1 knockout mice (Tanaka et al., 1997). Channelopathies that result in presynaptic hyperexcitability can be caused by
mutations in either voltage or ligand gated channels that control the flow of potassium, sodium and calcium (Jouvenceau et al., 2001; Lau et al., 2000; Spampanato et al., 2001). The mutations interfere with the normal function of the channel by either altering the actual channel pore or any of the ligand binding domains and protein-protein interaction sites. Even a small percentage change in the sensitivity of these channels is capable to promote repetitive firing of neurons and seizure breakout. To test the hypothesis that $\text{Mbnl2}^{\Delta E2/\Delta E2}$ mice were prone to seizure induction, the seizure-inducing drug pentylenetetrazole (PTZ) was used. PTZ is a GABA antagonist and acts as an inhibitor of the inhibitory (GABAergic) pathways of the brain, exacerbating even modest changes and defects in the excitatory pathways. $\text{Mbnl2}^{+/\Delta E2}$ mice were also included in this study due to the unexpected result that these mice showed higher susceptibility in seizure induction than WT mice.

A pilot study was designed to test the effectiveness of different doses of PTZ in WT versus mutant $\text{Mbnl2}$ mice. This study determined that 40 mg PTZ per kg mouse body weight, injected intraperitoneally, was an ideal dose for this investigation since WT mice showed very limited seizure activity while $\text{Mbnl2}$ knockouts were profoundly affected. Seizures were assayed using a modified Racine scale (see methods for explanation). PTZ injection in $\text{Mbnl2}^{\Delta E2/\Delta E2}$ homozygous knockouts had a dramatic effect with an early onset (Level 1) with impaired movement and nose twitching during the first minute post-injection. $\text{Mbnl2}^{+/\Delta E2}$ mice showed a more delayed response (~2 minutes post-injection) while WT showed a more delayed response (Figure 3-11). A more profound difference was also observed between WT and mutants when the PTZ response was measured by the Racine scale (Figure 3-12). All $\text{Mbnl2}^{\Delta E2/\Delta E2}$ mice
reached Racine Level 6 and the majority of them died within 2-3 minutes whereas
*Mbnl2*+/ΔE2 heterozygotes showed a wide range of response with few animals reaching
the Level 6 followed by death while some only attained Level 2-3. WT mice remained at
Level 1 (Figure 3-12). Another measure is the time interval between drug introduction
and attainment of at least Level 5. In this case, *Mbnl2*ΔE2/ΔE2 mice reached this threshold
much earlier (~150 sec) compared to *Mbnl2*+/ΔE2 mice (>400 sec) (Figure. 3-13).

**Discussion**

Growth retardation in *Mbnl2* knockouts can result from multiple molecular and
behavioral changes which occur during early development and postnatal growth. Mbnl2
may be important for the splicing of genes responsible for normal development and
growth, such as the IGF family, effectors that regulate pre- and early postnatal growth
(Liu et al., 1993). For example, *Igf2* knockouts show a 40% decrease in birth weight but
this difference remains as the mice age (DeChiara et al., 1990). Another pathway that
regulates growth during the period between birth and P21 is the leptin pathway which
controls the hypothalamic food intake circuitry (Cottrell et al., 2009). It is known that
leptin normally inhibits the activity of orexigenic neurons that express neuropeptide Y,
promote hunger and stimulates the activity of anorexigenic neurons that express pro-
opiomelanocortin (POMC) resulting in a robust decrease in food intake (Balthasar et al.,
2004). Perinatally, leptin levels increase dramatically in the blood (Ahima et al., 1998).
This increase in leptin has been implicated in the development and maturation of the
food intake regulatory circuitry including regions of the brain such as the hypothalamus
and paraventricular nucleus which is progressively established between postnatal day
P6 and P16 (Bouret et al., 2004). During the perinatal period the increased leptin level
does not inhibit food intake since the body weight of young mice rapidly increases
(leptin resistance) (Mistry et al., 1999). Disruption of the leptin resistance mechanism could potentially lead to decreased food intake and retarded growth in $Mbnl2^{AE2\&E2}$ mice. In addition, an early postnatal leptin blockage has been correlated to long term leptin resistance of mature animals leading to obesity in rats (Attig et al., 2008). Preliminary data (not shown) show a tendency of increased body weight in 5 month and older $Mbnl2$ knockout mice which provides some supporting evidence for this hypothesis.

The Morris water maze test showed spatial learning and memory impairment of the $Mbnl2$ knockout mice by a modest delay in learning during training and decreased spatial precision. A concern to this study is the increased thigmotaxis $Mbnl2$ knockouts showed as a result of platform removal. This result could explain the altered platform cross-over performance of these mutants as the platform was placed away from the periphery of the water maze. Another potential explanation is that $Mbnl2$ knockouts never got accustomed to the new environment (pool arena) due to an elevated stress response. The Morris water maze is one of the most stressful tests for mice because they correlate water submersion with a danger of drowning. A different test to assess thigmotaxis and stress response such as open field should be also performed. The growth retardation could be attributed to similar behavioral abnormalities of $Mbnl2$ knockout mice for example an inability to compete for food, or even an inability to associate lactation with food intake. All of the above possible scenarios require further investigation.

Seizure susceptibility has not been carefully studied in DM patients and very few unpublished incidents have been reported. There are several differences between the
Mbnl2ΔE2/ΔE2 and DM patients. First, DM requires the production of toxic C(C)UG RNAs, so only those cells that transcribe either the DMPK or CNBP mutant genes will be affected in contrast to the Mbnl2 knockout model where Mbnl2 protein is ubiquitously absent. Second, in DM there is partial loss of MBNL proteins from toxic RNA and the level of sequestration and loss depends on the expression and size of the repeats. Spontaneous seizures were a rare event in the Mbnl2 knockouts where Mbnl2 is absent. The genetic basis of the seizure prone phenotype in the Mbnl2ΔE2/ΔE2 mouse model is not known and further experiments should be conducted to identify the pathways which are responsible for this phenotype. Additionally, mouse models of DM that express CUG expansion repeats should be tested for seizure susceptibility. However, existing poly(CUG) and poly(CCUG) models do not show sufficient expression of C(CTG expansions in the brain. If transgenic mice that express polyC(C)UG repeats in the brain show a similar seizure susceptibility phenotype then it is possible that seizure susceptibility is a common, but subclinical phenotype, in the DM population.
Figure 3-1. Growth retardation in $Mbnl2^{ΔE2/ΔE2}$ mice. 21 old WT and $Mbnl2^{ΔE2/ΔE2}$ day mice were photographed (left). Weight was recorded between P21 and P47.
Figure 3-2. Mbnl2 loss does not lead to muscle histopathology. TA muscle cross sections from WT (left) and Mbnl2ΔE2/ΔE2 (right) mice were stained with hematoxylin (purple nuclear stain) and eosin (pink cytoplasmic stain).
Figure 3-3. Mbnl2 loss does not lead to chloride channel loss due to Clcn1 aberrant splicing. WT and Mbnl2$^{ΔE2/ΔE2}$ quadriceps muscle was used for IF (A) or alternative splicing RT-PCR (B). Mbnl2$^{ΔE2/ΔE2}$ mice did not show any reduced staining for the muscle specific chloride channel protein Clcn-1 (red) compared to WT. Clcn1 fetal exon inclusion was not up-regulated in Mbnl2$^{ΔE2/ΔE2}$ mice comparing to WT and as seen in previously characterized Mbnl1$^{ΔE3/ΔE3}$ mice (Kanadia et al., 2003a).
Figure 3-4. No overt histological changes in the hippocampal formation of $Mbnl2^{ΔE2/ΔE2}$ mice. Coronal mid-brain sections of the hippocampal formation from WT and $Mbnl2^{ΔE2/ΔE2}$ mice were treated with Nissl stain to visualize RNA (purple) and eosin to visualize protein (pink).
Figure 3-5. Latency in learning and memory formation in the Morris water maze test. 
*Mbnl2ΔE2ΔE2* and WT mice were trained for 5 consecutive days (4 trials/day) to reach a submerged platform. The average time to platform was recorded for each day; WT (n=16), *Mbnl2ΔE2ΔE2* (n=13), *p<0.005*
Figure 3-6. Quadrant occupancy in the Morris water maze test. On Day 6, and after the platform removal, mice were allowed to swim in the maze for 60 seconds. The total time they spend in each quadrant was measured. Abbreviations are (T) target, (AR) adjacent right, (AL) adjacent left, (O) opposite quadrant using WT (n=16) and Mbnl2^{ΔE2/ΔE2} (n=16). *p<0.05
Figure 3-7. Decreased spatial memory and precision in Mbnl2\textsuperscript{ ΔE2/ΔE2} mice. On Day 6 and upon platform removal the mice were allowed to swim for 60 seconds in the maze. During the swim, the number of times that mice crossed over the platform was counted: WT (n=16) Mbnl2\textsuperscript{ ΔE2/ΔE2} (n=13), *p<0.05.
Figure 3-8. No significant difference in total path swim length between WT and Mbnl2ΔE2/ΔE2 mice. WT and Mbnl2ΔE2/ΔE2 mice were allowed to swim until they reached the platform, which was removed on Day 6. The total swimming path to reach the platform (< 60 seconds) on Day 6 was recorded. for WT (n=16) and Mbnl2ΔE2/ΔE2 (n=13) mice.
Figure 3-9. No significant difference in swimming speed between WT and $Mbnl2^{\Delta E2/\Delta E2}$ mice. On Day 6, mice were allowed to swim for 60 seconds without the platform and the average speed was calculated by dividing the total distance swam by total swim duration (60sec): WT ($n=16$), $Mbnl2^{\Delta E2/\Delta E2}$ ($n=13$).
Figure 3-10. *Mbnl2^{ΔΕ2ΔΕ2}* mice show increased thigmotaxis. A thigmotaxis zone was defined as 10 cm from the periphery of the MWM wall. Mice were allowed to swim 60 seconds after platform removal and the time they spent in that zone was recorded: WT (n=16) and *Mbnl2^{ΔΕ2ΔΕ2}* (n=13), *p*<0.05.
Figure 3-11. Latency to seizure onset is reduced in both $\textit{Mbnl2}^{\Delta E2/\Delta E2}$ and $\textit{Mbnl2}^{+/\Delta E2}$ mice. WT, $\textit{Mbnl2}^{+/\Delta E2}$ and $\textit{Mbnl2}^{\Delta E2/\Delta E2}$ mice where injected with 40 mg/kg PTZ and the latency scored on a modified Racine scale: WT ($n=4$), $\textit{Mbnl2}^{+/\Delta E2}$ ($n=6$) and $\textit{Mbnl2}^{\Delta E2/\Delta E2}$ ($n=4$), *p<0.0005 (WT), **p<0.005 ($\textit{Mbnl2}^{+/\Delta E2}$).
Figure 3-12. Severity of convulsion is increased on both in $Mbnl2^{\Delta E2/\Delta E2}$ and $Mbnl2^{+/\Delta E2}$ mice. WT, $Mbnl2^{-/\Delta E2}$ and $Mbnl2^{\Delta E2/\Delta E2}$ mice were scored for seizure severity during a 1 hour observation period following PTZ injection: WT ($n=4$), $Mbnl2^{+/\Delta E2}$ ($n=6$) and $Mbnl2^{\Delta E2/\Delta E2}$ ($n=4$), *p<0.005, **p<0.02.
Figure 3-13. Reduced time to peak severity in both $Mbnl2^{ΔE2/ΔE2}$ and $Mbnl2^{+/ΔE2}$ mice. The time to maximum seizure score (>Level 5) was recorded for both $Mbnl2^{+/ΔE2}$ and $Mbnl2^{ΔE2/ΔE2}$ mice: $Mbnl2^{+/ΔE2}$ ($n = 6$), $Mbnl2^{ΔE2/ΔE2}$ ($n = 4$), *p*<0.05.
Previous studies have shown that Mbnl1 acts as a developmental switch, by adapting the transcriptome for the demands of adulthood through the regulation of alternative splicing. Our discovery that Mbnl2 is a nuclear protein in neurons of the brain suggested that this Mbnl family member might serve a similar role in the CNS.

To test the hypothesis that Mbnl2 regulates alternative splicing in the brain, the WT and Mbnl2ΔE2ΔE2 transcriptomes were analyzed by splicing microarrays and RNA-seq using hippocampal RNAs to detect all possible RNA targets affected by Mbnl2 loss. The reasons why I focused on the hippocampus were: 1) Mbnl2 is localized to the nucleus in the hippocampus supporting the hypothesis that Mbnl2 is an alternative splicing factor; 2) the hippocampus is a small region in the brain with well-defined function in learning and memory. While this experimental strategy may have missed RNA processing events that occur in other regions of the brain, hippocampal cells have a closely related function which can be tested by behavioral assays. To validate the targets that were found by both splicing microarrays and RNA-seq, splicing sensitive PCR analysis was performed for the top targets.

Results

Alternative Splicing Dysregulation in Mbnl2 Knockout Brain

The microarray analysis revealed hundreds of genes (Figure A-1) with alternative splicing abnormalities in the adult hippocampus but only a handful in the muscle and in both arrays, the number of genes with expression level changes was insignificant (data not shown). More than 38% of the genes that were revealed by the microarray analysis were also shown to be mis-regulated by RNA-seq analysis and subsequent RT-PCR
analysis (Figure 4-1, 4-2). Interestingly, grouping the top microarray and RNA-seq targets according to their function and known disease association by gene ontology (Figure 4-2) revealed the possible functional implications of Mbnl2 loss in learning and memory formation as well as channel sensitivity that results in seizure formation. Overall, this analysis shows that the majority of the genes that are affected by loss of Mbnl2 are involved in axonal growth and neuronal plasticity.

**Intronic Enrichment of YGCY Clusters in Mbnl2 Knockout Mis-Regulated Exons**

Previously it has been shown that Mbnl1 regulates alternative splicing of muscle specific genes, such as the muscle specific chloride channel Clcn-1, by direct binding to YGCY clusters (Goers et al., 2010). Other RNA binding proteins show similar short motif specificity, such as NOVA that binds UCAY motifs (Jensen et al., 2000b) and the polypyrimidine track binding protein (PTB) that binds YCTY (Garcia-Blanco et al., 1989). Mbnl2 shows a great degree of conservation with Mbnl1 and has a similar zinc finger domain structure so I hypothesized that Mbnl2 might also bind preferentially to YGCY motifs. To test this hypothesis, we searched for YGCY motif enrichment, and other RNA binding motifs that have been previously found to be cis acting elements for other RNA binding proteins, in the intronic regions upstream and downstream of the cassette exons mis-regulated in Mbnl2 knockouts (Figure 4-3). We found a significant increase only in YGCY clusters in the mis-regulated genes providing supporting evidence that Mbnl1 and Mbnl2 bind RNA via the YGCY motif. In contrast, only a few targets were found to be mis-regulated and these did not enrichment for YGCY motifs (M. Cline, personal communication).
Direct Binding Sites for Mbnl2 on Mis-Regulated Targets

To test the hypothesis that Mbnl2 binds directly to target transcripts identified by microarray and RNA-seq analyses, high throughput sequencing-crosslinking immunoprecipitation (HITS-CLIP) (Licatalosi et al., 2008) was used to identify direct binding targets. Briefly, hippocampi from WT and Mbnl2ΔΕ2/ΔΕ2 mice were dissected, pulverized in liquid nitrogen and the resulting powder was crosslinked with UV-light to generate covalent crosslinks between RNA and associated proteins. After labeling the RNA with 32P and immunoprecipitation of Mbnl2 with mAb 3B4, the RNA was partially digested with RNAse A and then the protein was removed with Proteinase K. The released RNA was reverse transcribed, and subjected to high throughput sequencing. The sequencing reads (30-60 nt long), or CLIP tags were subjected to filtering for imperfect matches, exact duplicates and multiple genomic hits to achieve greater specificity. Mbnl2ΔΕ2/ΔΕ2 reads were used as negative, non-specific binding tags. Sequence tags were then mapped back to the mouse genome using Novoalign as described previously (Zhang and Darnell). The majority of the reads (>50%) were located in 3'UTR of coding genes, followed by reads in intronic and exonic regions of known genes (22% and 9% respectively) (data not shown). To define sequences where Mbnl2 binds in protein coding genes, overlapping tag sequences (>2) were grouped to clusters using the UCSC genome browser (http://genome.ucsc.edu/) as previously described (Yeo et al., 2009) (Figure 4-4A). Unique clusters (20,441) were found to overlap with protein coding genes which under higher stringency (Bonferroni correction p<0.01) yielded 4,792 peaks. The majority of these peaks were located in the 3'UTR and intronic regions of the genes verifying an alternative splicing role for Mbnl2. After combining the findings of splicing microarrays, RNA-seq and CLIP, an RNA splicing
map was generated to correlate binding of Mbnl2 and splicing regulation (Licatalosi et al., 2008) (Figure 4-4B). To generate the normalized Mbnl2 splicing map, all the CLIP tags for each transcript were normalized to 1.0 and the fraction of the normalized tags presented in 50 nucleotide windows upstream and downstream of the aberrant spliced transcripts were clustered, summed and multiplied by the number of transcripts they were present. This normalization will strengthen tags that occurred in multiple transcripts in the same region and resulted in a similar shift in alternative splicing. We found that intronic binding of Mbnl2 adjacent to the 3' splice site generally promotes exon skipping while binding 60-70 nt downstream of the 5' splicing site promotes exon inclusion.

To validate the hypothesis that Mbnl2 binds to YGCY clusters we employed crosslinking-induced mutation site (CIMS) analysis of the peak CLIP targets as previously described (Zhang and Darnell). Clustering of 62,932 deletions in unique tags yielded 557 CIMS (FDR<0.0001) with deletions observed in at least 4 tags (n≥4) with base composition 4%A, 33%C, 22%G, 41%U (Figure 4-5A). To reveal motif enrichment, de novo motif analysis was performed by assessing 21 bp around the deletion site. The majority of CIMS clusters were located in a UCGU motif which showed 16 fold enrichment completed to flanking sequences used as controls (Figure 4-5B).

**Discussion**

The microarray and RNA-seq data revealed a set of mis-spliced RNAs encoding ion channels previously implicated in spatial memory and seizure prone phenotypes. Deletion of a different alternative splicing factor, Rbfox1, affects the alternative splicing of same genes in a similar manner and results mice that are susceptible to spontaneous and kainic acid induced seizures (Gehman et al., 2011). Two genes that are highly
altered in both mouse models are Cacna1d and Grin1, a calcium ion channel and an NMDA receptor respectively. Involvement of Grin1 in seizure formation has never been reported and will not be further discussed in this chapter. On the other hand, Cacna1d is not only involved in seizure susceptibility but also in memory consolidation. According to RT-PCR analysis, Cacna1d shows the highest splicing mis-regulation with >60% shift in alternative splicing of exon 12a (Figure 4-1). Cacna1d is a fast voltage gated L-type calcium channel that controls presynaptic glutamate release. Its role is to decode sound-evoked depolarization of inner hair cells to increased calcium influx resulting in glutamate vesicle fusion at the presynaptic membrane, excitation of the postsynaptic membrane and propagation of the signal through the auditory pathway. Cacna1d is also highly expressed in atrial myocytes and cells of the sinoatrial and atrioventricular node, controlling the pacemaker activity of the heart (Mangoni et al., 2003). Recently a trinucleotide insertion in alternative exon 8b has been linked to deafness and irregularities in the sinoatrial node, the heart pacemaker of patients with SANDD syndrome (Baig et al., 2011). Individuals with SANDD syndrome suffer from hearing loss and bradycardia. In a mouse model study, Cacna1d^{-/-} mice were viable and suffered from sinoatrial dysfunction and congenital deafness (Platzer et al., 2000) similar to humans with SANDD.

In the hippocampus, the role of Cacna1d has not been yet elucidated, despite its high expression levels and wide cellular distribution pattern. Some studies have reported protein-protein interactions of the C-termini of Cacna1d channels with Shank, a postsynaptic adaptor protein (Zhang et al., 2005) and the N-termini with the ryanodine receptor type 2, which plays a role in Ca^{++} release from intracellular storage region (Kim
et al., 2007). Isoform expression level changes have been reported after pilocarpine-induced status epilepticus involving Ca^{++} channels in neuronal plasticity after epilepsy (Xu et al., 2007).

Two more genes with potentially high impact on the seizure prone phenotype are *Dlg2* and *Kcnma1*. *Dlg2* belongs to a family of intracellular scaffolding proteins known as membrane-associated guanylate kinase (MAGUK) protein family. The role of the MAGUKs is to provide a postsynaptic scaffolding network that drives localization and specific density of different receptors, for example NMDA receptor NR2B (Sans et al., 2003) and AMPA receptor subunits (Rumbaugh et al., 2003), to neuronal spines. To provide binding sites for protein-protein interactions, MAGUKs are composed of several different binding domains, including a triple PDZ domain, and a non-enzymatic guanylate kinase fragment. The diverse spatial and temporal expression of different MAGUK isoforms provides a mechanism to alter the cellular and sub-cellular localization of receptors in neurons changing the sensitivity and downstream pathway of signaling cascades. MAGUK protein change-of-function has been reported to be involved in many neurologic and neurodegenerative diseases such as Alzheimer’s disease (Lacor et al., 2004), L-DOPA induced diskynesia of Parkinson patients under L-DOPA treatment (Gardoni et al., 2006) and Huntington disease (Sun et al., 2001). Specific isoforms of *Dlg2* has been found to bind the inwardly rectifying potassium channel Kir2.1 of the postsynaptic membrane (Leyland and Dart, 2004). The density and intracellular localization of Kir proteins determine the resting membrane potential of neurons. Additional evidence also indicates alteration of Dlg2, and its binding partner NR2B, expression levels in temporal lobe related epilepsy (Liu et al., 2007). A change in
Dlg2 splicing isoform levels and potential change in their cellular localization or even binding preferences for NMDA, Kir and AMPA receptor subunits may underlie a change in postsynaptic sensitivity and hyperexcitability.

Kcnma1, or Slo1, belongs to the Slo family of potassium channels characterized by high single-channel conductance with a voltage range of activation that is modifiable in response to many factors and ions. Kcnma1 is expressed in numerous tissues including the mammalian CNS, pancreas, hair cells and smooth muscle and can be activated by either a membrane depolarization, an increase in the intracellular calcium concentration or by a synergistic effect of both (Magleby, 2003). The gating characteristics are subject not only to changes due to extrinsic factors but also by pre- and post-translational modifications including alternative splicing, phosphorylation and heteromultimer formation (Joiner et al., 1998; Ramanathan et al., 1999; Schubert and Nelson, 2001). In our report, we show a change in alternative splicing of Kcnma1 in the hippocampus which could lead to an abnormal sensitivity to calcium levels and/or to the kinetics of the channel. Studies in C. elegans have shown that deletion of Kcnma1 leads to increased neurotransmitter release (Wang et al., 2001) whereas in a mouse Kcnma1 knockout model the phenotype was more diverse including high-frequency hearing loss, erectile dysfunction, ataxia and vascular hypertension (Ruttiger et al., 2004; Sausbier et al., 2004; Werner et al., 2005). In humans, missense mutations lead to epilepsy (Du et al., 2005).

The three most highly mis-regulated genes in this study that influence learning and memory are Cacna1d, Tanc2 and Ndrg4. Tanc2 and Tanc1 are paralogous genes. Tanc1 is a scaffold/adaptor protein that interacts directly with PSD-95, a member of the
MAGUK family of proteins, and with other proteins via its multiple protein-protein interaction domains (Suzuki et al., 2005). Tanc1 and Tanc2 overexpression in neurons in vitro, results in an increase of spine density and excitatory synapses whereas Tanc1 knockout mice show impaired spatial memory and learning. Tanc2 deletion is embryonic lethal (Han et al.).

Similar spatial memory impairment was shown in an Ndrg4 knockout mouse model. The Ndr4 (N-Myc downstream-regulated gene 4) protein is the only member of the Ndrg family that is specifically expressed in brain and heart (Zhou et al., 2001) and undergoes extensive isoform regulation during development (Nakada et al., 2002). Downregulation of Ndr4 results in decreased neurite growth and process length in an in vitro cell system (Ohki et al., 2002), and Ndr4 levels are downregulated in Alzheimer's disease (Zhou et al., 2001) suggesting a role in neuronal growth and development. Indeed, Ndr4 deletion in mice resulted in inferior spatial learning and memory formation as well as increased neuronal damage and lesions following focal ischemia (Yamamoto et al.).

Overall, the splicing microarray and RNA-seq findings agree with the phenotype characterization of the Mbnl2ΔE2ΔE2 mouse model. Changes in the coding sequence of a protein isoform can alter its function and alter downstream molecular cascades. For instance, expression of a fetal isoform might lead to enhanced nonsense-mediated decay and thus phenocopy a null mutation. HITS-CLIP and CIMS analyses validated the direct binding of Mbnl2 to aberrantly spliced targets These results are similar to the binding map of Nova (Licatalosi et al., 2008) which suggests a common mechanism by which RNA binding proteins act to regulate alternative splicing. Binding of a splicing
factor upstream of an exon promotes skipping of the exon and binding of the splicing factor downstream promotes inclusion.
Figure 4-1. Mbnl2 regulates neonatal to adult alternative splicing changes in the hippocampus. Five highly scored targets were tested by splicing sensitive RT-PCR analysis with primers flanking the alternative exon. (Upper) For Mbnl2 alternative splicing, Ndrg4 and Tanc2 splicing was tested in WT, Mbnl1ΔE3/ΔE3 and Mbnl2ΔE2/ΔE hippocampi, including the developmental splicing change in postnatal (P) day 6 and 42 of mouse forebrain (fb) and hindbrain (hb). (Lower) to assess possible splicing changes due to Mbnl2 haploinsufficiency, alternative splicing of Canca1d, Kcnma1 and Dlg2 were tested in WT, Mbnl2ΔE2/ΔE hippocampi (courtesy of Kuang-Yung Lee).
Figure 4-2. RNA-seq and microarray data show possible learning/memory and epileptic defects in $Mbnl2^{\Delta E2/\Delta E2}$ mice. A) 104 splicing micro-array targets with a sep-score $\geq 0.7$ were compared with 179 RNA-seq targets (FDR $< 0.05$, DI $\geq 0.1$). B) Top RNA seq and microarray targets are grouped according to their function and gene ontology.
Figure 4-3. Mbnl2 regulates exon exclusion in the hippocampus for exons that show YGCY enrichment in the upstream introns. Enrichment of YGCY motifs was assayed for introns upstream (left) and downstream (right) of the misregulated exons in $\text{Mbnl2}^{\Delta E_2\Delta E_2}$ hippocampi. Each point represents the average frequency of the motifs present at the specific site upstream of the intron exon junction (0). Error bars indicate $+2/-2$ s.d. of the mean frequency distribution for the population of background exons (Courtesy of M. Cline).
Figure 4-4. Mbnl2 RNA splicing map. (A) UCSC genome browser representation of Ndrg4 alternative exon 14 showing CLIP tags (above) in different colors for different biological replicates and DNA conservation in blue bars for high and red bars for low (below). B) Normalized complexity Mbnl2 RNA map showing exon activation (red) and repression (blue) (Courtesy of Chaolin Zhang and Yuan Yuan).
Figure 4-5. Mbnl2 binds directly to UGCU clusters. A) Alignment of 557 CIMS (FDR<0.001) with deletions observed in at least 4 tags (n≥4). The base composition of these CIMS is 4% A, 33% C, 22%,G, 41% U. B) UGCU enrichment around CIMS (blue line) and CLIP cluster peaks (orange) compared to surrounding sequences used as control (Courtesy of Chaolin Zhang and Yuan Yuan).
CHAPTER 5
CONCLUDING REMARKS AND FUTURE DIRECTIONS

Microsatellite repeat expansion mutations are responsible for many neurological abnormalities including certain types of spinocerebellar ataxias (SCAs), amyloid lateral sclerosis (ALS) and Alzheimer’s disease. The expansion mutations can be toxic at a DNA, RNA or protein level depending on their location relevant to the affected gene. A recently described RNA gain of function model implicates repeat expansion in untranslated regions of mRNAs, in sequestration of splicing factors leading to DM. Sequestration of these alternative splicing factors causes aberrant splicing of genes, adding DM in the group of diseases termed spliceopathies. By studying the molecular events that lead to DM and similar diseases, we can create universal models of how expansion mutations interfere with normal cell function. These universal models are useful in understanding the molecular basis of newly described disorders caused by expansion mutations, as well as a framework to design better therapeutic strategies. Also by identifying and associating specific gene perturbations with certain clinical features of diseases can speed up narrowing down the genetic basis of many unidentified so far genetic disorders.

In this study, I report the functional role of Mbnl2 in the brain and also demonstrate the mechanism of action by determining the RNA binding sites. The Mbnl protein family shows remarkable genetic and protein similarity among the three paralogs and a high degree of functional domain conservation. The high degree of similarity suggests functional redundancy in vivo but my studies indicate temporal or spatial functional specialization for the three family members. Mbnl2 appears to play a predominant role in developmentally regulated alternative splicing in the brain while Mbnl1 subserves the
same function in skeletal muscle. For Mbnl2, the majority of the RNA targets identified by splicing microarrays and RNA-seq are involved neuronal plasticity, synaptic and post-synaptic function and neurogenesis. Thus, this study has identified key elements that could reveal novel brain-related defects in DM patients. Identification of the UGCU binding motif by HITS-CLIP strengthens the hypothesis that Mbnl2 binds and is sequestered by toxic CUG repeat expansions. In this study, I also investigated a possible role for Mbnl2 loss in the pathogenesis of brain-related DM clinical features by learning and memory defects in Mbnl2 knockouts. The results show that loss of Mbnl2 in mice affects their memory formation and spatial precision, a higher function of the brain suggesting possible executive function deficits. The unexpected discovery of seizure susceptibility in Mbnl2 knockouts raises an issue about drug administration to DM patients. Mbnl2 knockout mice show a dramatic increase in sensitivity to the GABAergic antagonist PTZ, suggestive of hyperexcitability in the DM nervous system. Although few reports of DM-associated seizures exist, seizures could undermine the mental health of DM patients and also be induced by anti-depressant or anti-arrhythmic drugs.

Despite the success of the Mbnl2 knockout model to recapitulate the DM brain aberrant splicing and reveal hundreds of new possible target genes implicated in the disease, there are certain drawbacks. There are phenotypes and molecular events that could be unrelated to DM and the expression pattern of the human DMPK and CNBP, which carry the expansion mutation. One possible scenario for the unexpected seizure susceptibility that these mice showed is loss of Mbnl2 in neuronal cells that don’t express Dmpk or Cnbp. Also in the constitutive Mbnl2 knockouts, the protein is
completely absent during embryogenesis and adulthood. In DM we have partial but not complete sequestration of Mbnl proteins as shown by the strong correlation of repeat length and severity of the phenotype. A more relevant to the disease mouse model would be a conditional Mbnl2 mouse where exon 2 deletion is driven under either Dmpk or Cnbp promoters. Another possible scenario and drawback of the Mbnl2 knockout and any other mouse model is the genetic difference between humans and mice. Genes that can be affected in mice may not be in humans due to sequence variation in coding regions and regulatory cis acting elements that regulate gene expression and alternative splicing.

Future studies should focus on identifying the neuronal circuitry that is affected in the Mbnl2 KO mice by direct electrode stimulation of hippocampal slices to study changes in LTP formation and consolidation as well as NMDA and AMPA sensitivity changes. In addition, the contribution of mis-regulated targets identified during this study to seizure formation should be tested using blocking morpholinos to force fetal splicing patterns in adults. To establish a stronger connection between the seizure prone phenotype and myotonic dystrophy, the spatial expression C(C)UG repeats should be examined in further detail in the DM brain and in DM mouse models that express the repeats under DMPK or CNBP promoters.

Beyond teasing out the molecular etiology of already identified behavioral effects of Mbnl2 loss, the Mbnl2 knockout mice are a great tool to address questions relevant to other clinical DM features. Another area of focus is DM-associated hypersomnia. The microarray data revealed several candidate genes that are implicated in circadian rhythm establishment and maintenance. Thus, Mbnl2ΔE2ΔE mice should be tested for
sleeping or circadian rhythm perturbations. Also, DM is not only considered to be a late onset neuromuscular disease, but also a premature aging disease. Most DM clinical features are natural signs of aging such as cataracts, loss of brain and muscle mass as well as heart conduction defects. In concordance with this notion, the weak muscle defects present in the Mbnl2 GT2 mice were observed in 9-10 month old animals, suggesting a potential aging effect in disease progression due to Mbnl2 loss. For that reason, it is of great importance to study the aging process of the Mbnl2ΔE2/ΔE mice by analyzing the survival rate, late onset neuromuscular disorders, and possible declines in brain function in aged mice.

On the other hand, DM is thought to be caused by a combinatorial loss of function of all three Mbnl proteins. The generation of each isoform specific Mbnl knockout mouse line serves to understand the unique functions and contribution to disease pathogenesis of each Mbnl2 isoform, but does not mimic the DM sequestration model. To achieve a more complete DM mouse model, all three Mbnl knockouts should be mated and triple Mbnl knockout lines should be generated. A drawback of this strategy is the likelihood that embryonic lethality will result from double or triple knockout generation. The severity and onset of DM is correlated with the expansion of the repeats suggesting a partial protein sequestration. Complete ablation of these splicing factors is probably incompatible with life. To overcome this problem and recapitulate partial sequestration, tissue specific knockouts can be generated to avoid embryonic lethality and triply heterozygous knockouts could recapitulate the partial loss of functions of these proteins.

Finally, both isoform specific and triple knockout models could serve as a clinical tool for drug discovery and gene therapy testing. Mbnl1 upregulation by gene therapy
reverts most of the muscle-related abnormalities in mice that express the toxic repeats in muscle (Kanadia et al., 2006). Mbnl2 and Mbnl3 upregulation has not been tested, but according to the structural and functional similarity between these proteins, similar results are anticipated. In conclusion, the muscleblind family of RNA-binding proteins shows structural and functional similarity and each paralog is responsible for the regulation of transcriptome plasticity either during different developmental periods or in different tissues (Fig. 5). Mbnl1 and Mbnl2 regulate the embryonic to adult shift of specific alternative exons in muscle and brain, respectively, and are active during postnatal life. In contrast, Mbnl3 functions during the embryonic period. This functional distinction serves as a robust paradigm for fine-tuning the transcriptome by related RNA-binding proteins during development.
Figure 5-1. Functional diversion and tissue distribution among the three Mbnl proteins.
CHAPTER 6
MATERIALS AND METHODS

Lac-Z Staining

Mixed C57Bl6/129 male $Mbnl2^{+/-\text{GT4}}$ mice (3-5 months of age) were obtained from Charles Thornton (University of Rochester). These mice were generated by integration of the GT vector pGTOpfs in $Mbnl2$ intron 4. Brains were dissected and incubated overnight at $4^\circ\text{C}$ in 0.2% PFA in PBS followed by 30% sucrose solution in PBS overnight at $4^\circ\text{C}$ and embedded in OCT (Tissue-Tek). Transverse cryostat sections (15 μm) were dried (30 min, RT) and incubated overnight in X-Gal staining solution (0.1M sodium phosphate, pH 7.4, 0.1% sodium deoxycholate, 2 mM MgCl, 0.2% NP-40, 1 mg/ml X-Gal, 0.1 mM KFe(CN)$_6$, 0.1 mM K$_4$Fe(CN)$_6$) at $37^\circ\text{C}$ in a humidified chamber. The stained sections were further fixed for 1 hr in 2% PFA for 10 min at RT. For counterstaining, the sections were incubated 30 s in water, 1 min in 95% EtOH, 20 s in eosin solution (Fisher Richard-Allan Scientific Cat# 71311, Eosin-Y with phloxine), 3 times in 95% EtOH for 1 min each, 3 times in 100% EtOH for 3 min each, 3 times in Citrisolv (Fisher Cat# 22-143975 Citrisolv) for 5 min each and finally mounted on a slide with Permount.

Generation of an $Mbnl2$ Polyclonal Antibody

Plasmid Transfections

COSM6 cells were grown in growth media containing DMEM (Invitrogen), 10% FBS (Invitrogen), 1% L-glutamine (Invitrogen), and 1% penicillin/streptomycin (Invitrogen) in a humidified 37 °C, 5% CO$_2$ incubator. The day before transfection, cells were seeded in each well of a 6-well plate. We avoided antibiotics in the growth media prior to plasmid transfections. After 16 hr, a mixture was added consisting of
transfection reagent (6 μL Fugene 6 [Roche] in 180 μL OptiMEM I [Invitrogen]) followed by vortexing for 5 s, incubation at RT for 5 min, addition of 2 μg of plasmid followed by vortexing 2 sec and incubation at RT for 15 min and expression vectors (empty vector pSP72, myc-Mbnl1, myc-Mbnl2 and myc-Mbnl3) that were provided by Mike Poulos and described previously. After 24 hr, the cells were washed with PBS and the antibiotic-free media was replaced. All wells were processed 48 hr post-transfection for western blot analysis or immunofluorescence.

Western Blotting

For western blot analysis, each well was washed with PBS followed by cell lysis in HEPES buffer (20 mM Hepes, 100 mM KCl PH=8.0, PicD, PicW) at 4°C, centrifugation at 16,000 RCF at 4°C and collection of the supernatant. To test the specificity of the new polyclonal antibody, 50 μg of protein lysate from each transfection was fractionated on a 12.5 Tris-glycine polyacrylamide gel, transferred to nitrocellulose membrane, blocked for 30 min with 5% skimmed milk in PBS and dried overnight. For immunoblotting, the nitrocellulose membrane was incubated for 5 min in PBS followed by overnight incubation at 4°C with the new Mbnl2 specific antibody (1:1000) in 5% milk/0.05% Igepal/PBS while two more replicates were incubated with anti-Myc antibody (1:500) for expression levels of the myc-tagged proteins and anti-Gapdh (1:5000) (loading control). After washing 3 times with PBS/0.05% Igepal, the membranes were incubated with a horse radish peroxidase linked secondary anti-rabbit (Mbnl2) or anti-mouse (anti-Myc and anti-Gapdh) antibody (1:5000 GE) in 5% milk/0.05% Igepal/PBS for 2 hr, washed three times with PBS/0.05% Igepal and processed with ECL reagents according to
manufacturer’s protocol (GE Bioscience). Visualization of chemiluminescence was achieved using BioMax film (Kodak).

\[\text{Mbnl2}^{\Delta E2/\Delta E2} \text{ Mouse Generation}\]

\textbf{ES Cell Targeting Construct}

The genomic background of the \textit{Mbnl2} targeting vector sequence was obtained from an SV129 BAC clone and generation was performed with standard recombineering techniques using protocols 1-4 (http://web.ncifcrf.gov/research/brb/protocol.aspx) and the following reagents: bacterial strains SW102, SW10. Plasmids PL253, PL451, PL452. Briefly, isolated BAC plasmid was electroporated in SW102 cells. The targeting backbone PL253 that carries the negative selection marker Herpes Simplex virus thymidine kinase (HSV-TK), was linearized by PCR amplification with primers that carry arms of homology (AH) to a 10 kb fragment that contains \textit{Mbnl2} exon 2

Gap repair fwd:

5’-agcctgccgtgagagagtgaagtcatcagcctccagccacctgacttccgcggacagtggtctgctctccca

GCCAGGGTTTTCCACGTACGACGTTGT-3’

Gap repair rev:

5’-ttggctcacctccacctttacctgccttatgtcattttcgcgtataaaaggggaaacgtacctccctcgtccgtagtgtgtgtgtccga

AACCTCAATCGCTTGCAGCAGCATCC-3’

Black small letters indicate the homologous region to \textit{Mbnl2} and capital bold letters indicate the homologous region to the plasmid backbone. All targeting events to generate the construct were performed in SW102 after heat induction of the P1 phage recombination system and correctly targeted plasmid clones were verified with \textit{XbaI} restriction analysis. The 10 kb \textit{Mbnl2} fragment retrieval via gap repair was also verified by PCR amplification of the PL253-\textit{Mbnl2} junction points. To introduce the 5’ loxP site,
targeted a neomycin selection marker floxed by two loxP sites, 250 bp upstream of
*Mbnl2* exon 2. This cassette was PCR amplified from plasmid PL452 with primers that
have AH for the exon 2 upstream region.

1<sup>st</sup> LoxP targeting fwd:

5'-tcctctaagtacagacgacgcaagtgcatactgttttAGGCCTataactgtataatgtgc

tacgaagtttatCGACCTGCAGCCTGTTGA-3'

1<sup>ST</sup> LoxP targeting rev:

5'-ggtgccaatgagagtgatctccgggacaactttaaaccacaaacctgcatactcgtagatagcatatat
acgaagttatatGTCGAGGCTGATCAGCGA-3'

Capital letters indicate a *Stul* restriction site that we introduced and was used to
screen ES cells for positive recombination events of the 5' AH by Southern blotting. Cre-
mediated recombination and excision of the neo cassette followed, by electroporation of
the positively targeted plasmid into arabinose-inducible Cre-expressing bacteria
(SW106). Targeting of the 3' loxP site was done similarly to the 5' by PCR amplifying a
neo cassette flanked by two Frt sites and one loxP site directly from plasmid PL451 with
the following primers

2<sup>nd</sup> loxP targeting fwd:

5'-aggtttatgtgtcttttggttctttgcactgaaacttatttatacGAAGTT CCTATTCTCTAGA

AAGTATAGGAACCTCAGGCTGAAGGAGGATTT-3'

2<sup>nd</sup> loxP targeting rev:

5'-taattttaacacagatggaaaaaaat ttggtgtgcctaatGTA CTATAACTTCGTAT

AGCATACATTATACGAAGTTAT ATT ATGACCTGACTG-3'
In black capital letters is an exogenous Scal site that was introduced to screen ES cells for positive recombination events of the 3’ AH by Southern blotting. The functionality of both final loxP and Frt sites were tested in arabinose-inducible Cre- or Flp-expressing cells, respectively (SW106 and SW105).

**ES Cell Targeting**

The targeting construct was linearized with NotI and electroporated in 129 SvlmJ ES cells. ES clones were cultured and selected as previously described. Positive clones that survived G418 and neomycin selection were picked and screened by Southern blotting to identify correctly targeting events. To test 5’ recombination events, ES cell genomic DNA was digested with StuI whereas to test 3’ recombination events I used Scal. For the 5’ AH, a 300 base probe was used that was PCR amplified from genomic DNA with primers upstream of the 5’ AH (fwd primer 5’-CTCTCCTCCTCCAGTTTGGCTTTG-3’, rev primer 5’-GTGTTCAGACGTGAGCACTGTAAC-3’). The targeted allele yields a 5 kb band and the WT allele a 6.4 kb band. For the 3’ AH we used a 250 base probe that was PCR amplified with primers downstream of the 3’ AH (fwd primer 5’-TCGACTTTCCATTCTGGGAGAAC-3’, rev primer 5’-GCATCCAGGGGACAATTCACATAG-3’). The targeted allele gives a band of 5 kb and the WT yields a band of 31 kb. ES cells from a positive clone were injected in C57BL/6 pseudopregnant female mice (University of Michigan) and chimeric male animals where acquired and mated to C57BL/6 females. Germline transmission of the conditional allele should yielded agouti pups. To further test for correct germline transmission, agouti pups from the F1 generation were genotyped with two forward primers (one in the neo cassette 5’-CGCCTTCTATCGCCTTCTGAGAGTTTCTTCTGAG-3’and one in Mbnl2 exon 2 5’-GTAGGGCTCTCAAGGGAGAGCAGTGACATTGAGC-3’ in 2:1 ratio) and one
reverse primer in *Mbnl2* intron 2 (5'-AATGTCAAACCAGACCAGAAATACACCACCACCAC-3'). Amplification was performed for 33 cycles (each cycle consisting of 94°C for 30 sec, 64°C for 30 sec and 70°C for 50 sec).

**Mating Scheme and Genotyping**

To create a constitutive *Mbnl2* knockout mouse line, *Mbnl2*<sup>-/-</sup>con mice were mated with B6.C-Tg(CMV-cre)1Cgn/J mice. F1 progeny was genotyped for CMV-Cre by PCR using primers in the Cre cassette and for *Mbnl2* exon 2 deletion with two forward primers (one upstream of exon 2 5’-GTACCACCTTCCTTGTGATACTGAAAGCTCTGAGGTC-3’ and one in *Mbnl2* exon 2 5’-GTAGGGCTCTCAAGGAAGCACTGCATTGAGC-3’) and one reverse in *Mbnl2* intron 2 (5’-AATGTCAAACCAGACCAGAAATACCACCATG-3’). Amplification was performed in 33 cycles (each cycle consisting of 94°C for 30 sec, 65°C for 30 sec and 72°C for 30 sec). To obtain *Mbnl2*<sup>ΔE2/ΔE2</sup> mice, heterozygous *Mbnl2*<sup>+/ΔE2</sup> mice were crossed and the pups were genotyped with the same primers.

**RNA Analysis of *Mbnl2*<sup>ΔE2/ΔE2</sup> Mice**

Loss of *Mbnl2* exon 2 was also assayed at the mRNA level. Male Bl6/129 *Mbnl2*<sup>+/+</sup>, *Mbnl2*<sup>+/ΔE2</sup> and *Mbnl2*<sup>ΔE2/ΔE2</sup> mice (5 months of age) were sacrificed and total RNA was isolated from the brain and TA muscles by homogenizing the tissue in Tri-reagent (Sigma) according to the manufacturer's protocol. First-strand cDNA synthesis was performed using reverse transcription of 2.5 μg RNA in a buffer containing 0.5 mM dNTPs, 0.5 μg oligo-dT, 0.01 M DTT, 40 U RNasin, 200 U of Superscript III reverse polymerase (20 μl total reaction volume). The RT mixture was incubated at 25°C for 5 min followed by 42°C for 1 hr and 72°C for 15 min. For *Mbnl2* expression analysis, 2 μl of cDNA was used for PCR with a forward primer in exon 2 (5’-
CAGTCAAGAGACTAGAACCCTGG GAGC-3') and reverse in exon 3 (5'-GGCGTTCCTGGAAACA
TAAA-3'). Ppia was used as a loading control with forward primer (5'-GCGGCAGGTCCATCTACG-3') and reverse (5'-GCCATCCAGCCATTCTAGTCT-3'). Mbnl1 levels were also tested with a forward primer in exon 3 (5'- GTTAGTGTCAACCAATTCAGAC-3') and a reverse primer in exon 4 (5'-GGGATCATGATCGGCTTAAC-3'). The products were resolved on a 1% agarose gel and visualized with ethidium bromide.

**Protein Expression Analysis of Mbnl2ΔE2/ΔE2 Mice**

Female Bl6/129 Mbnl2+/+ and Mbnl2ΔE/ΔE mice (2-3 months of age) were sacrificed and protein isolated by homogenizing the different tissues (cerebellum, hippocampus, frontal lobe, heart, lung, quadriceps and spleen) in HEPES buffer as described above. Western blot analysis was performed with a commercial anti-Mbnl2 antibody (1:1000 mAb 3B4 Santa Cruz Biotechnology, 1:5000 secondary anti-mouse antibody) as described above. Gapdh was used as a loading control (1:10000 mAb 6C5 primary, 1:5000 secondary anti-mouse antibodies).

**Muscle Immunofluorescence and Histology**

Female Bl6/129 Mbnl2+/+ and Mbnl2ΔE/ΔE mice (2-3 months of age) were sacrificed and quadriceps muscles were dissected, mounted on wooden dowels with 10% gum tragacanth (in PBS) and frozen in a liquid nitrogen precooled isopentane bath. For histological examination of the muscle sections, I employed H&E staining. Briefly, 10 μm cryosections mounted on microscope slides were air dried for 30 min, followed by a standard H&E staining protocol and mounted with Permount (Fisher cat# sp15-100 Permount). For all light image visualization of sections and digital photography, we used
a Leica DM 2000 inverted light microscope with a Qimaging MicroPublisher 5.0 RTV digital camera attached.

**Brain Immunofluorescence**

BL6/129 Mbnl2<sup>+/+</sup> and Mbnl2<sup>ΔE/ΔE</sup> mice (5 months of age) were sacrificed and the brains dissected and fixed at 4°C overnight in 4% paraformaldehyde in PBS. Sectioning was performed on a Vibratome at 4°C and 50 μm brain sections were obtained from different regions of the brain (sections were obtained at 70Hz vibration frequency, 0.2mm/sec cutting speed and sections were kept floating at 4°C in PBS). For immunostaining, sections were washed briefly in water and microwaved in a 0.1M Urea solution 3 times for 15sec each followed by 2 min cooling down intervals in a conventional microwave oven. Sections were blocked overnight in a 0.3% Triton-X100, 0.2% goat serum, PBS solution and then incubated in primary anti-Mbnl2 antibody (1:200 of mAb 3B4) in blocking solution for 3 days followed by washing (X 20 min) in PBS and incubation in secondary anti-mouse antibody (1:400 Alexa 488) for 48 hr. All incubations were performed at 4°C. Sections were counterstained and mounted on slides with VectaShield mounting media with DAPI. Microscopy was performed on a Zeis Axioskope II inverted fluorescence microscope and a Leica TCS-SP5 confocal microscope.

**Nissl Staining**

Vibratome sections were obtained as previously described from male Bl6/129 Mbnl2<sup>+/+</sup> and Mbnl2<sup>ΔE/ΔE</sup> mice (5 months of age). Sections were dried on microscope slides for 15 min and then submerged in the following sequence of solutions: 95% EtOH 15 min, 70% EtOH 1 min, 50% EtOH 1 min, dH₂O 2 min, dH₂O 1 min, Cresyl violet stain 3.5 min (0.25 gm cresyl violet acetate, 170 μl glacial acetic acid in 50 ml dH₂O), dH₂O 1
min, 50% EtOH 1 min, 70% acid EtOH (1 ml glacial acetic acid in 100 ml 70% EtOH),
95% EtOH 2 min, 95% EtOH (few dips), 100% EtOH 1 min, Citrisolv 5 min and then
mounted with Permount.

**Morris Water Maze Test**

**Animals**

I used mixed WT and *Mbnl2^{AE2/ΔE2}* mice (2-5 months of age) that were housed in
cages with littermates in a room maintained at 22°C and on a normal 12/12 hr light/dark
cycle. The genetic background is C57BL/6 and 129SvImJ.

**Water maze:** The water maze was a round galvanized trough, 1.22m in diameter,
silver color and 0.6 meters tall, filled with water at 25±2°C. Water transparency was
reduced with white tempera paint (RichArt) for contrast purposes between the mouse
body and the water surface. The maze was located in a dedicated test room with
reduced noise where screens and 2D and 3D cues were strategically placed around it.
The platform was made from clear Plexiglas, (10cm diameter) and was submerged 1.5
cm below the water level so that it is not visible to a viewer on the surface of the water.
The surface of the platform was scraped so that mice could obtain grip and climb on it.
A water pump was used to pump out water from the trough and add fresh water every
day.

**Training**

For each training day the mice were acclimatized before the test in the water maze
room in their original cage for 30 min. Each mouse was placed in a clear bucket with
long handle to avoid direct interaction with the animals and placed in the tank facing the
tank wall. The location that the mice were released was randomly chosen from one of
the three opposite compass points from the quadrant where the platform was located.
This point was matched for all the mice on the same trial. Mice were trained for 4 consecutive days. Each mouse had 4 trials per day in sets of two consecutive trials with 1 min interval. Each set was separated by 4 hours. Each mouse was allowed to swim for 60 s per trial or until it reached the platform. Mice that did not reach the platform within the first 60 s were guided to and allowed to rest on it for 10 s. After the end of each trial, mice were allowed to sit on the platform and orient themselves for 10 s. After each trial mice were returned to a separate cage to dry under a heating bulb. The last day (Day 6) the platform was removed and mice were allowed to swim for 60 s and then removed from the maze.

Data Acquisition and Analysis

Data was collected with a wired camera mounted on the ceiling above the tank. Data acquisition was recorded automatically by a computer. The software (EthoVision 3, Noldus information Technology) began recording 1 s after the mouse was released in the tank and stopped recording after the mouse was located on the platform for more than 1.5 s. The software recorded the total path length the mouse swam and the latency time to the platform.

The maze was divided in 4 quadrants (NW, SW, NE, and SE) and a 10 cm thigmotactic zone was defined with a circle that was drawn 10 cm inside of the tank wall. The software zoning was used to calculate latency to platform, percentage of target quadrant occupancy, thigmotactic occupancy, swimming speed and total number of platform cross-overs when the platform was removed. The platform was located in the SW quadrant.
Statistical analysis: A two sided student T-test was performed to obtain statistically significant difference between the two groups. The total number of mice that were used in this study is \( n=16 \) for WT and \( n=13 \) for \( Mbnl2^{ΔE2/ΔE2} \) mice.

**PTZ Seizure Susceptibility Test**

Mixed WT, \( Mbnl2^{ΔE2} \) and \( Mbnl2^{ΔE2/ΔE2} \) mice (2-5 months of age) were tested for seizure susceptibility by injection with pentylenetetrazol (PTZ). Each mouse was weighed to determine the PTZ solution (4 mg/ml) injection volume. Minimal PTZ dose concentration was determined by injecting 3 WT mice with different concentrations of PTZ (40, 60, 70 mg/kg). At 40 mg/kg, WT mice showed no reaction (Racine score=1) and all further injections were carried out under these conditions. WT and \( Mbnl2^{ΔE2/ΔE2} \) mice were injected at the same time. \( Mbnl2^{ΔE2} \) mice were injected separately. Following each injection, the mice were placed in an observational area (cage) for a maximum time of 60 min and the time of onset of convulsive behavior and nature/severity of the convulsion was scored. Seizure severity was determined using a modified Racine 0-6 scoring scale and statistical significant values were determined with a Mann-Whitney test. Racine scale: 0, no motor seizures; 1, freezing, staring, mouth or facial movements; 2, head nodding or isolated twitches and rigid posture.; 3, tail extension unilateral-bilateral forelimb clonus; 4, rearing and mice sit in an immobile state on their rear haunches with one or both forelimbs extended; 5: clonic seizures with loss of posture, jumping, falling; 6) tonic seizures with hindlimb extension and death. The observation period was immediately followed by euthanasia.

**Splicing Microarray**

Quadriceps muscle and hippocampi RNA was extracted from 3 month old WT and \( Mbnl2^{ΔE2/ΔE2} \) mixed male mice as previously described. To identify differential alternative
splicing between WT and \( Mbnl2^{\Delta E2/\Delta E2} \) mice, we processed the RNA samples for hybridization to Affymetrix “A-chip” oligonucleotide microarrays according to the standards of the manufacturer (Sugnet et al., 2006).

Data analysis: The analysis of the microarrays was performed as described previously (Sugnet et al., 2006). The separation score was calculated as the ratio between the skipped to included exon ratio of the mutant by the WT.

\[
\text{Sepscore} = \log_2\left[\frac{\text{Mut}(\text{skip/include})}{\text{WT}(\text{skip/include})}\right]
\]

Analysis of sequence motifs: the analysis of sequence motifs was performed as described previously (Du et al., 2010). Briefly, the 150 bp upstream and downstream intronic region of the differentially-spliced exon was analyzed with Improbizer. As background sequence we used the upstream and downstream intronic regions of exons that did not show any difference between WT and KO animals. P-value was set at <0.05.

**RT-PCR Splicing Analysis of Mbnl2 Targets**

Hippocampi from 2-5 month old male or female WT, \( Mbnl1^{\Delta E3/\Delta E3} \) and \( Mbnl2^{\Delta E2/\Delta E2} \) mice was dissected and processed as described above to generate cDNA. The primer set that was used to test for splicing changes of mis-regulated exons are shown in Table 6-1. To test developmental shifts in alternative exon utilization, we used cDNA generated from WT and \( Mbnl2^{\Delta E2/\Delta E2} \) forebrain and hindbrain at P6 and P42.

**HITS-CLIP**

Hippocampi were dissected from WT mice (11-12 weeks of age) as described above and snap frozen in liquid nitrogen in a pre-cooled metal mortar. Each biological replicate (4 hippocampi from 2 mice) was processed separately on different days to account for diurnal changes in \( Mbnl2 \) expression. Hippocampi were ground to a fine
powder using a pre-cooled pestle and the tissue slurry in liquid nitrogen was transferred to a glass petri dish kept on dry ice. After liquid nitrogen evaporation, the powdered hippocampi were crosslinked three times at 4000 x 100 μJoules in the UV stratalinker 1800 (Stratagene). The biological replicates were saved at -80°C. CLIP was performed as previously reported (Jensen and Darnell, 2008; Licatalosi et al., 2008; Ule et al., 2005) with modifications described below. The anti-Mbnl2 antibody mAb 3B4 was used for CLIP: 5 μg of mAb 3B4 bound to 100 μl of Dynabeads-Protein A (Invitrogen) completely cleared the Mbnl2 from each biological replicate (equivalent to 2 mg total protein). Hippocampi from Mbnl2ΔE2/ΔE2 mice were used as a control for the immunoprecipitation. RNase A concentrations were optimized and final concentrations of 5000U/mL and 0.06U/mL were used as high and low RNase levels, respectively. The cDNA libraries were generated using RNA linkers and primers described for Ago CLIP (Chi et al., 2009). The 5’ RNA linker contained a 4 nt degenerate sequence at the 3’end to enable better quantitation of CLIP tags. Library concentrations were estimated with Quant-iT dsDNA assay kit, high sensitivity (Invitrogen, Catalog #Q33120). Libraries were diluted to 9 nM before submitting for clonal cluster generation with an Illumina cBot followed by sequencing using an Illumina Genome Analyzer IIx. Raw sequence reads were filtered to remove unmappable, rRNA and PCR duplicate reads. Unique reads were aligned to the mm9 mouse genome database on the UCSC Genome Browser for visualization.
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<th>Gene</th>
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### APPENDIX

**MICROARRAY SPlicing RESULTS**

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Figure A-1. Top targets of splicing sensitive microarray with sepscore 2.38-1.00
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Figure A-1. Continued
Figure A-1. Continued
LIST OF REFERENCES


Cuchalova, L., T. Kouba, A. Herrmannova, I. Danyi, W.L. Chiu, and L. Valasek. 2010. The RNA recognition motif of eukaryotic translation initiation factor 3g (eIF3g) is required for resumption of scanning of posttermination ribosomes for reinitiation on GCN4 and together with eIF3i stimulates linear scanning. Mol Cell Biol. 30:4671-86.


BIOGRAPHICAL SKETCH

Konstantinos Charizanis was born in Athens Greece in 1979. He is the younger of two children born to Ioannis and Paraskevi Charizanis. Konstantinos attended the University of Patras from 2000-2005, where he studied and earned a Bachelor of Science degree in biology. After graduation, Konstantinos moved to Gainesville Florida and joined the Department of Exercise Physiology and Kinesiology at the University of Florida and obtained the MS degree in Human Performance in 2006. He then joined the Interdisciplinary Program in Biomedical Sciences at the University of Florida College of Medicine. Konstantinos did his doctoral thesis studies in Dr. Maurice Swanson’s laboratory in the Department of Molecular Genetics and Microbiology and completed his Ph.D. dissertation in December 2011. Konstantinos plans to work in the pharmaceutical industry for the next two years and then pursue an MBA degree.