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Development of an *In Vitro* Neuronal Model of Myotonic Dystrophy Type 1

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Development of an *In Vitro* Neuronal Model of Myotonic Dystrophy Type 1

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Chao Lin
B.S., Jilin University, 2008
M.S. Fudan University, 2012

Advisor: Gary Bassell, Ph.D.

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Abstract

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By Chao Lin

Myotonic dystrophy (dystrophia myotonica, DM) is an autosomal-dominant multisystemic genetic disease. It has two subtypes, myotonic dystrophy type 1 (DM1) and myotonic dystrophy type 2 (DM2). DM affects not only muscles but also other tissues and systems including the central nervous system (CNS). DM1 is caused by the expansion of CTG repeats in the 3'UTR of *dystrophia myotonica protein kinase* (*DMPK*) gene, which are transcribed and form intranuclear RNA foci and sequestrate proteins. The sequestration of Muscleblind-like (MBNL) into RNA foci disrupts its function in regulating target RNA processing, including alternative splicing and localization. Previous studies have made significant progress in understanding the mechanism of RNA mis-splicing in DM1. However, a major gap exists in our knowledge of how RNA localization is disrupted in DM1. To investigate how DM1 affects MBNL-regulated RNA localization in neurons, I developed a novel *in vitro* model of DM1 through exclusively expressing expanded CTG repeats in primary mouse cortical neurons using AAV9 virus with a neuronal-specific synapsin promoter. My model successfully reproduced DM1 features including RNA foci and MBNL sequestration in the nuclei of AAV-transduced neurons expressing CTG repeats. While no obvious mis-splicing events were observed, the localization of *Snap25* mRNA in neuronal processes of transduced neurons was disrupted. I detected reduced dendritic arbor complexity in our DM1 model, which could be rescued by antisense oligonucleotides (ASO) treatment to degrade RNA foci and liberate MBNLs. Taken together, my results suggest that MBNL sequestration by RNA foci can impair distinct aspects of neuronal development. The mis-localization of MBNL target mRNAs like *Snap25* may contribute to the early neurodevelopmental defects independent of RNA mis-splicing in DM1.

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Chapter 1. Introduction

1.1 Myotonic Dystrophy

Myotonic dystrophy (dystrophia myotonica, DM) is an autosomal-dominant, progressive, multisystemic genetic disease, and is the most common form of adult muscular dystrophy (Gourdon and Meola 2017). Myotonic dystrophy was first described as a muscular dystrophy characterized with progressive muscle wasting, weakness and myotonia (Mishra, Singh et al. 2018). It is now recognized as a multi-systemic disease affecting not only skeletal and smooth muscle, but also heart, gastrointestinal system, endocrine system, skin, central nervous system (CNS), and other systems (Bird 1993, Machuca-Tzili, Brook et al. 2005, Turner and Hilton-Jones 2010). To date, there are two clinically and molecularly distinct types of myotonic dystrophy: myotonic dystrophy type 1 (DM1), which can affect the newborns, children, and adults; and myotonic dystrophy type 2 (DM2), which mainly affects adults (Ranum and Day 2002, Peric, Rakocevic Stojanovic et al. 2017). The estimated worldwide prevalence of DM is about 1 per 8000, but in some regions, such as Quebec, the prevalence may be approximately 1 to 500 (Yotova, Labuda et al. 2005, Turner and Hilton-Jones 2010). DM1 is usually considered as more prevalent than DM2. However, there are evidence indicated that the frequency of DM2 may be underestimated, at least in some regions like Finland (Suominen, Bachinski et al. 2011).

1.2 Clinical presentation

1.2.1 Subtypes of DM1 and clinical features

Based on the clinical features and the onset age of patients, DM1 can be divided into several subtypes: mild, congenital, childhood onset, and classic (Bird 1993, Turner and Hilton-Jones 2010). Table 1.1 summarized the clinical features of DM1 subtypes. Mild (or asymptomatic) DM1 is the

least severe subtype with onset between 20 and 70 years of age. The patients may only have cataract, mild myotonia, or diabetes mellitus, and their life span may be relatively normal (Arsenault, Prevost et al. 2006, Turner and Hilton-Jones 2010). Congenital DM1, mostly transmitted from the mother, is characterized by polyhydramnios and reduced fetal movements before birth (Zeesman, Carson et al. 2002). Congenital DM1 can cause neonatal deaths, and survived infants have generalised weakness, hypotonia and respiratory failure postnatally. Congenital DM1 infants also have facial weakness including a typical clinical feature, tented upper lip or carp-mouth (Machuca-Tzili, Brook et al. 2005). Other symptoms include movement disorder, autism, intellectual disability, vision problem, sleep disorders, and other symptoms (Ekstrom, Hakenas-Plate et al. 2009). In their 30s or 40s, congenital DM1 patients often develop lethal cardiorespiratory problems (Turner and Hilton-Jones 2010).

Childhood onset DM1, with onset between 1 and 10 years of age, is a subtype that is underappreciated and easily misdiagnosed in the patients due to the lack of neurological issues and family history of disease. Childhood onset DM1 shares some common features with congenital DM1, such as movement disorder, intellectual disability, and visual issues. However, besides the different onset age, childhood onset DM1 patients have some special features. In contrast to congenital DM1, childhood onset DM1 can be transmitted from both parents. The patients usually have facial weakness, but no tented upper lip. Periodic limb movements, motor development delay, and psychosocial issues are common characters of childhood onset DM1 patients (Ho, Cardamone et al. 2015).

1.2.2 Clinical features of classic DM1

Classic DM1 is the most well-known and well described subtype of myotonic dystrophy type 1. The onset age of classic DM1 is between 10 to 30 years old, which means that this subtype can evident in both childhood and adulthood. Classic DM1 is characterized by myotonia, muscle weakness, cataract, conduction defects, respiratory failure, and so on (Turner and Hilton-Jones 2014). Because of the progressive severity of the disease, patients can be severely physically disabled in 50 or 60 years of age. Patients can also have sudden death due to cardiac rhythm disorders (Bassez, Lazarus et al. 2004). All of these symptoms induced relatively shortened life span of individuals with classic DM1.

Classic DM1 is a multi-systemic disease that affects different tissues and organs, such as muscle, heart, gastrointestinal system, endocrine system, and CNS. Muscle weakness, caused by dystrophic process, and myotonia, caused by defect of muscle membrane excitability, are the predominant symptom of classic DM1 (Machuca-Tzili, Brook et al. 2005). The weakness of facial and levator palpebrae muscles together with ptosis and premature hair loss result in a typical facial appearance of patients. The weakness of neck flexors and extensor caused neck weakness or 'dropped head' is also a common feature of classic DM1. The weakness of long finger flexors can cause substantial disability, whereas ankle dorsiflexion weakness is the main reason causing foot drop of the patients (Turner and Hilton-Jones 2014). Muscle weakness progresses slowly over years and it affects the daily activity such as using tools and movement (Bouchard, Cossette et al. 2015). Classic DM1 can cause heart problems, such as conduction disturbances and tachyarrhythmias (Chebel, Ben Hamda et al. 2005). About 30% of deaths of the DM1 patients, including sudden death, are caused by cardiac complications (Mathieu, Allard et al. 1999). The atrial tachyarrhythmia, progressive left ventricle dysfunction and ischaemic heart disease are the

potential reasons of cardiac complications induced deaths (Groh, Groh et al. 2008). Gastrointestinal system is also affected by classic DM1. The patients may have dysphagia, emesis, chronic or episodic diarrhea, constipation, megacolon, anal incontinence, and so on. These symptoms are assumed to be caused by myotonia or dysfunction of smooth muscle in gastrointestinal system (Ronnblom, Forsberg et al. 1996). Endocrine organs, including thyroid, pancreas, hypothalamus, gonads, and parathyroids, in classic DM1 patients are disordered (Orngreen, Arlien-Soborg et al. 2012). Infertility can occur in male classic DM1 patients due to testicular atrophy and the seminiferous tubules atrophy. Habitual abortion and menstrual irregularities can be detected in female patients (Garcia de Andoin, Echeverria et al. 2005). Insulin resistance induced hyperinsulinemia is also a common feature of the patients (Takase, Okita et al. 1987).

1.2.3 The CNS in DM1

The CNS function is disrupted in classic DM1 patients. Some of the most problematic symptoms experienced by patients include CNS deficits. CNS phenotypes are often very debilitating for DM1 patients and can affect quality of life more than symptoms in peripheral tissues (Heatwole, Bode et al. 2012, Heatwole, Bode et al. 2014). These symptoms include profound fatigue (90.8% of patients), hypersomnia (87.9% of patients), and various cognitive challenges (>50% of patients). Affected children have intellectual disability (ID), autism spectrum disorders (ASD) and other neuropsychiatric disorders (Calderon 1966, Ekstrom, Hakenas-Plate et al. 2008, Ho, Cardamone et al. 2015, Seijas-Gomez, Basterra-Jimenez et al. 2015). Although less severe compared with congenital and childhood onset DM1, cognitive impairments and intellectual disabilities are common in adult classic DM1 patients. Progressive cognitive decline and working memory

impairment are also detected in adult patients (Gaul, Schmidt et al. 2006, Fujino, Shingaki et al. 2018). Patients can have abnormal personality, such as avoidant, obsessive–compulsive and passive–aggressive personality (Winblad, Lindberg et al. 2005). Other CNS related symptoms include anxiety, depression, and excessive daytime sleepiness (Antonini, Soscia et al. 2006, Dauvilliers and Laberge 2012). At later stages of disease, neurofibrillary tangles and pathologic tau proteins are present in DM1 brain, although less abundant than in Alzheimer's brain (Vermersch, Sergeant et al. 1996, Weber, Roebling et al. 2010), and DM1 has been characterized as a peculiar tauopathy (Sergeant, Sablonniere et al. 2001).

Imaging studies of myotonic dystrophy provided direct evidence indicating that DM1 is not only a muscular dystrophy, but also a brain disease. Imaging of congenital DM1 patients brain indicated ventricular enlargement, cortical atrophy and small corpus callosum (Mutchnick, Thatikunta et al. 2016). Diffusion Tensor Imaging (DTI) and Magnetic resonance imaging (MRI) of DM1 patients brain showed cortical atrophy, cerebral metabolites abnormalities, and white matter abnormalities (Wozniak, Mueller et al. 2014, Takado, Terajima et al. 2015). Also, gray matter abnormalities have been shown by structural imaging studies (Antonini, Mainero et al. 2004). These detections of the patients' brain features may explain the CNS symptoms of DM1, such as intellectual disability and work memory impairment. Taken together, all of these clinical features indicate that the CNS is affected in DM1 patients with a spectrum that depend on age of onset, suggesting neurodevelopmental and neurodegenerative features.

1.2.4 Clinical features of DM2

DM2 is relatively milder and less common compared with DM1. DM2 typically appears between 30 to 50 years (Day, Ricker et al. 2003). DM2 patients usually have more severe muscle pain and

stiffness compared with DM1 patients. Excessive daytime sleepiness is also common in DM2 patients (Meola 2000). Other clinical features of DM2 include myotonia, muscle weakness, proximal legs, hyperinsulinaemia, and so on (Machuca-Tzili, Brook et al. 2005).

1.3 Molecular mechanism

Myotonic dystrophy is one of over thirty genetic diseases, called microsatellite repeat expansion (MRE) disorders, induced by expansion of tandem repeats (Brook, McCurrach et al. 1992). The molecular basis of DM1 is the expansion of CTG trinucleotide repeats in the 3' untranslated region (UTR) of the *dystrophia myotonica protein kinase* (*DMPK*) gene, whereas DM2 is caused by the expansion of CCTG tetranucleotide repeats in the intron 1 of the *cellular nucleic acid-binding protein* (*CNBP*) gene (also known as *ZNF9*) (Buxton, Shelbourne et al. 1992, Liquori, Ricker et al. 2001). Similar to other tandem repeats caused diseases like fragile X syndrome, the number of repeats correlates with the onset age and severity of myotonic dystrophy. Clinically, the tandem repeats and muscle weakness are considered as diagnostic criteria of myotonic dystrophy (Yum, Wang et al. 2017).

1.3.1 *DMPK* and CTG repeats

The expansion of CTG repeats in the 3'-UTR of *DMPK* is the genetic basis of DM1. *DMPK* gene locates on chromosome 19q13.3 (Brook, McCurrach et al. 1992). The gene product DMPK is a serine/threonine protein kinase sharing homologous structure with the Rho family kinase (Llagostera, Catalucci et al. 2007). In healthy individuals, the 3'-UTR of normal *DMPK* contains about 5 to 37 CTG repeats (Turner and Hilton-Jones 2010). While the upper bound of normal CTG repeats size is about 37, DM1 patients can have expanded repeats size from 50 to several thousand

(T. Ashizawa 2000, Meola and Cardani 2015). Each of DM1 subtypes can have different size of CTG repeats, which is correlated with the onset age and severity of the disease (Table 1.1, Figure 1.1). Mild DM1 patients may have only less than 100 CTG repeats, whereas the more severe congenital DM1 patients can have thousands of repeats.

1.3.2 Instability of CTG repeats

Mutant *DMPK* allele with more than 38 CTG repeats is unstable and the repeats size can change during meiosis and mitosis (Turner and Hilton-Jones 2010). Clinically, CTG repeats size can be used to predict onset age and severity of DM1. However, the prediction is more precise when the patients have lower than 400 repeats, which may be caused by the instability of mutant *DMPK* allele (Hamshere, Harley et al. 1999). Individuals with DM1 may have different repeats size in different tissues (Lavedan, Hofmann-Radvanyi et al. 1993). Therefore, the prediction based on the CTG repeats size of one tissue may be inconsistent with the severity and CTG repeats size of another tissue.

CTG repeat size can change when transmitting mutant *DMPK* allele between generations. *DMPK* with 38 to 50 CTG repeats is recognized as 'pre-mutation' allele, and DM1 patients with this allele can be asymptomatic throughout their whole life. However, patients can have offspring with high risk of having larger repeat size because of the instability of the *DMPK* allele with more than 38 CTG repeats (Martorell, Monckton et al. 2001). Although there is a low probability (only about 6.4%) that the repeat size can decrease when transmitting from parents to children, the DM1 patients' offspring usually have longer CTG repeats than their parents (Ashizawa, Anvret et al. 1994). This phenomenon is known as "anticipation", which refers to the increased severity and younger onset age in subsequent generations of DM1 patients (Pearson, Nichol Edamura et al.

2005). Almost all individuals with congenital DM1 acquired *DMPK* allele with CTG repeats from their mother. Because of anticipation, congenital DM1 affects infants perinatally and can cause neonatal deaths. Survived infants usually have more than a thousand of CTG repeats and more severe symptoms compared with other DM1 subtypes.

1.3.3 Molecular mechanism of DM2

DM2 is caused by the expansion of the CCTG tetranucleotide repeat in the first intron of *CNBP* gene located on chromosome 3 (Liquori, Ricker et al. 2001). The gene product is a zinc finger nucleic acid binding protein. The normal *CNBP* allele in healthy individuals contains about 7 to 24 CCTG repeats, whereas the mutant alleles can have repeats expanded to the range of 75 to 11,000 (LoRusso, Weiner et al. 2018). CCTG repeats in DM2 are considered more unstable and more likely to expand during meiosis and mitosis than CTG repeats in DM1. Therefore, children of DM2 patients are prone to have mutant *CNBP* allele with longer CCTG repeats. However, there is no close relationship between the repeat size in DM2 and onset age or severity of disease (Day, Ricker et al. 2003).

1.3.4 Accumulation of Intranuclear Ribonucleic acid (RNA) foci

In both subtypes of DM, RNAs containing expanded tandem repeats are transcribed and can form toxic nuclear RNA foci (also known as ribonuclear inclusions) and sequestrate RNA-binding proteins (Mooers, Logue et al. 2005, Zhang and Ashizawa 2017). DM1 and DM2 share many clinical symptoms, such as myotonia, muscle weakness, and hyperinsulinemia, but their corresponding genes are on different chromosomes and have no obvious functional relationship (DM1: *DMPK* gene is on chromosome 19 and produces a serine/threonine protein kinase; DM2:

CNBP gene is on chromosome 3 and produces a zinc finger nucleic acid binding protein). Previous research indicated that disrupting or overexpressing DMPK in mice induced only mild DM symptoms (Jansen, Groenen et al. 1996). Researchers also proved that the *CNBP* messenger RNA (mRNA) level and protein level are unaffected in DM2 patients cell line or tissues (Botta, Caldarola et al. 2006, Margolis, Schoser et al. 2006). These evidences implied that the loss of protein products encoded by the affected mutated allele with the repeat expansion are not the main reasons causing the disease. On the other hand, expanded tandem repeats are transcribed and can form intranuclear RNA foci in both DM1 and DM2. In DM1, the nuclear retention of DMPK mRNA in DM1 patient fibroblasts was shown by Singer and Housman (Taneja, McCurrach et al. 1995, Davis, McCurrach et al. 1997). In DM2, RNA foci contain only the CCUG repeats and no other parts of *CNBP* transcript (Margolis, Schoser et al. 2006). By using different animal models of DM, researchers detected that the expression of RNA with expanded repeats can induce RNA toxicity and DM symptoms. For example, transgenic mice expressing an RNA with about 250 CUG repeats inserted into a non-translated RNA sequence also formed intranuclear RNA foci and induced classical features of DM1, such as myotonia and muscle weakness (Mankodi, Logigian et al. 2000, Houseley, Wang et al. 2005, de Haro, Al-Ramahi et al. 2006). Therefore, the expansion of CTG or CCTG repeats in DM1 or DM2 plays a major role in disease pathogenesis.

The mechanism of the expansion of tandem repeats induced myotonic dystrophy is RNA toxic gain-of-function. In DM1, mRNA with CUG repeats can form a stable secondary double-stranded hairpin structure, and the stability of the hairpin has positive correlation with the size of expanded repeats (Tian, White et al. 2000). The hairpin structure is retained in RNA foci and can bind and sequestrate RNA binding proteins within the nucleus, which can induce the disruption of the splicing and transportation of target RNAs (Mulders, van Engelen et al. 2010, Caillet-Boudin,

Fernandez-Gomez et al. 2014). The most important RNA binding proteins sequestrated by the toxic RNA foci in DM include the muscleblind-like (MBNL) family proteins (Miller, Urbinati et al. 2000, Jiang, Mankodi et al. 2004).

1.3.5 Muscleblind-like (MBNL) proteins

MBNL family is a highly conserved, developmentally regulated protein family of RNA-binding factors with nuclear and cytoplasmic enriched isoforms (Figure 1.2). MBNL presents in different species including nematoda, arthropoda, tunicata, and vertebrata (Begemann, Paricio et al. 1997, Pascual, Vicente et al. 2006). All of invertebrate species contain a single copy of *MBNL*. For example, *Drosophila*, where *MBNL* was originally identified, has a single copy of the gene, *mbl*, which regulates muscle and eye development (Begemann, Paricio et al. 1997, Artero, Prokop et al. 1998, Goers, Voelker et al. 2008). On the other hand, most vertebrates contain three developmentally regulated *MBNL* homologs (*MBNL1*, *MBNL2*, and *MBNL3*) (Fardaei, Rogers et al. 2002). These three genes have very similar number of exons and exon size, and their protein products share structural similarities (Fardaei, Rogers et al. 2002, Pascual, Vicente et al. 2006). However, the distribution pattern of these genes is different. In human and mice, MBNL1 and MBNL2 are widely expressed across many different tissues including muscle, CNS, and heart. In brain, MBNL2 is predominantly expressed compared with MBNL1 (Squillace, Chenault et al. 2002, Kanadia, Urbinati et al. 2003). The expression of MBNL3 is more restricted. Although MBNL3 is related to muscle regeneration, it mainly expressed in placenta (Squillace, Chenault et al. 2002, Kanadia, Urbinati et al. 2003, Poulos, Batra et al. 2013). In the cellular level, because of alternative splicing, MBNL1 has two isoforms localizing in the cytoplasm and nucleus respectively.

Previous research indicated that these two isoforms can regulate different intracellular functions (Kanadia, Shin et al. 2006, Wang, Chang et al. 2018).

MBNL family proteins share a common structural feature: tandem zinc finger (ZnF) domains, which can bind to target pre-mRNA to regulate alternative splicing, RNA localization, and mRNA stability (Begemann, Paricio et al. 1997, Pascual, Vicente et al. 2006, Masuda, Andersen et al. 2012, Wang, Cody et al. 2012). In *MBNL1*, exons 1, 2 and 4 encode ZnF domains containing two tandem ZnF pairs (ZnF 1 and 2, and ZnF 3 and 4) with similar structure. These ZnF pairs are composed of three cysteine and one histidine residue (CCCH) (Pascual, Vicente et al. 2006). There are identical number of amino acid residues between cysteine and histidine residues in ZnF 1 and 3 (CX7CX6CX3H), and in ZnF 2 and 4 (CX7CX4CX3H) (Fardaei, Rogers et al. 2002). In spite of the similar structure, these ZnF pairs have different binding affinity with their target RNAs. Compared with ZnF2 and ZnF 3, ZnF 1 and ZnF 4 are more closely related to the interaction of MBNL1 and expanded CTG repeats (Kino, Mori et al. 2004). An exon 3 encoded linker sequence, which separates two ZnF pairs, is also essential for the interaction between MBNL and target RNAs (Tran, Gourrier et al. 2011).

ZnF pairs in MBNL can recognize and bind target RNA with binding motif YGCY (Y stands for pyrimidines, and GC is dinucleotide) (Teplova and Patel 2008, Du, Cline et al. 2010). Through the enrichment analysis of RNA motifs and computational analysis based on splicing-sensitive microarray assays, researchers proved that YGCY motif, especially UGCU, is the most common MBNL binding target for all three MBNL family members (Du, Cline et al. 2010, Goers, Purcell et al. 2010, Charizanis, Lee et al. 2012, Poulos, Batra et al. 2013). Our lab collaborator Eric Wang et al. used the analysis of cross-link-induced mutations and ultraviolet

crosslinking/immunoprecipitation/ sequencing (CLIP-seq) to confirm that UGCU is an essential motif of MBNL binding site (Wang, Cody et al. 2012).

1.3.6 Intracellular function of MBNLs in RNA processing and localization

MBNL can bind target mRNA with YGCY motif in either introns or 3'-UTRs, regulating alternative splicing, alternative polyadenylation, or RNA localization (Figure 1.2). When binding to the introns, MBNL can regulate the alternative splicing of target mRNA in cell nucleus (Osborne, Lin et al. 2009, Wang, Cody et al. 2012, Poulos, Batra et al. 2013). On the other hand, MBNL can regulate alternative polyadenylation and RNA stability and cellular localization through binding to 3'-UTR of target RNAs (Masuda, Andersen et al. 2012, Batra, Charizanis et al. 2014).

There is extensive research about the mechanism of MBNLs regulating alternative splicing. Alternative pre-mRNA splicing is a process through which different mRNAs can be generated from a same pre-mRNA by including or excluding specific exons (Black 2003). Accordingly, the protein products from different mRNAs contain altered amino acid sequences and can participate in various intracellular functions. Because the ZnF pairs in MBNLs are composed of CCCH domain, a domain involved in various aspect of RNA metabolism, MBNL proteins are initially considered as regulators of RNA metabolism (Begemann, Paricio et al. 1997, Blackshear 2002). However, now it has been proven that MBNL proteins also participate in the regulation of RNA alternative splicing. Kanadia et al. used *MBNL1* knockout mouse to detect that the disruption of MBNL1 induced abnormal RNA alternative splicing, which is an evidence showing that MBNL can directly regulate RNA splicing (Kanadia, Johnstone et al. 2003). Corresponding to its involvement in myotonic dystrophy, knockout of MBNL1 induced mis-splicing events of several terminal muscle differentiation related transcripts, including skeletal muscle ion-transporter

chloride channel 1, *insulin receptor* (*IR*), *Bridging integrator 1*, *cardiac troponin T* (*cTNT*), *sodium channel 5a*, and fast skeletal muscle *troponin T* (Philips, Timchenko et al. 1998, Savkur, Philips et al. 2001, Charlet, Savkur et al. 2002, Fugier, Klein et al. 2011, Wahbi, Algalarrondo et al. 2013, Freyermuth, Rau et al. 2016).

In the CNS, MBNLs are involved in the regulation of various RNA alternative splicing events, which are important for normal development and functions of the CNS. Knockout of MBNL2 in mice induced the mis-splicing of many RNAs in the brain, including *Ndrg4*, *Tanc2*, *Ppp1r12a*, *Add1*, *Kcnma1*, *Csnk1d*, *Cacna1d*, *tau, Grin1* subunit of NMDA receptor (NMDAR) and *Gabarg2* subunit of GABA-A receptors, which are collectively hypothesized to caused DM-related CNS features, such as abnormal REM sleep propensity and deficits in spatial memory (Charizanis, Lee et al. 2012). MBNL2 knockout mice and MBNL1 / MBNL2 double knockout mice showed similar disrupted CNS splicing patterns characteristic of DM1 and/or DM2 (Charizanis, Lee et al. 2012, Goodwin, Mohan et al. 2015). MBNL knockout and RNA mis-splicing in those mice induced various DM-associated CNS features including abnormal REM sleep propensity, spatial memory deficits, and disruption of hippocampal synaptic plasticity (Charizanis, Lee et al. 2012, Goodwin, Mohan et al. 2015). In DM1 patients cortical neurons, RNA foci can sequestration MBNL and induce mis-splicing of NMDAR components, amyloid beta precursor protein (APP) and microtubule-associated protein tau (MAPT) (Jiang, Mankodi et al. 2004, Goodwin, Mohan et al. 2015). However, unlike the case in muscle, where mis-splicing of the RNA encoding the chloride channel *Clcn1,* it has yet to be proven that any mis-splicing events in the brain causes CNS symptoms in mouse models or human DM1 patients.

Depending on the spatial relationship between the MBNL binding sites and the regulated exons, MBNLs can promote either exclusion or inclusion of exons during alternative splicing.

Overexpression of MBNL proteins can promote the exclusion of exon 5 in *cTNT*, but facilitate inclusion of exon 11 in *IR* (Savkur, Philips et al. 2001, Ho, Charlet et al. 2004, Du, Cline et al. 2010). This alternative splicing function of MBNLs is achieved by binding to different regions of target RNA. When directly binding to the alternative exon or its upstream intronic motif, MBNLs facilitate the exclusion of the exon. On contrary, MBNLs facilitate inclusion of exons when binding to their downstream intronic regions (Philips, Timchenko et al. 1998, Charlet, Logan et al. 2002, Dansithong, Paul et al. 2005, Goers, Purcell et al. 2010).

MBNLs can affect pre-mRNA processing through regulating alternative polyadenylation (APA). Unlike alternative splicing, which regulates the inclusion and exclusion of exons, APA regulates the maturation of pre-mRNA by cleaving the 3' end of pre-mRNA and adding multiple untemplated adenosines (polyA tail) (Chen, Jia et al. 2017). APA can affect the interaction of RNA with various trans-acting factors (Yeh and Yong 2016). Batra, R. et al. used MBNL knockout mice to detect that MBNL loss-of-function can disrupt APA. By using polyA-seq analysis, they detected APA dysregulation in MBNL knockout mouse embryonic fibroblasts (MEFs). Through high throughput sequencing-crosslinking immunopurification (HITS-CLIP) and minigene reporter analyses, they showed that MBNLs can bind directly to the target RNA and regulate polyA site selection. Disruption of APA was also detected in DM1 mouse model and human DM muscle samples (Batra, Charizanis et al. 2014).

An increasing number of studies have indicated another important function of MBNLs: regulating RNA localization in early development and/or differentiation of muscle and neurons. RNA localization and local translation are fundamental to the cellular compartmentalized organization and intracellular functions (Bashirullah, Cooperstock et al. 1998). In neurons, RNA localization and local translation regulates asymmetry of neuronal protein distribution at remote sites, which

is important for axon guidance, development of synapse, and synaptic plasticity (Martin and Zukin 2006, Zappulo, van den Bruck et al. 2017). Compared with the extensive research about how MBNLs regulate alternative splicing, the role of MBNLs in RNA localization has not been sufficiently studied. However, previous studies have indicated that the cytoplasmic enriched isoform of MBNLs contribute to the regulation of RNA stabilization and localization in non-neural cells (Adereth, Dammai et al. 2005, Du, Cline et al. 2010). MBNLs can bind to the YGCY motif in 3'UTR of target RNA to stabilize the RNA and facilitate its localization (Wang, Cody et al. 2012, Wang, Ward et al. 2015). In non- neuronal cells, several studies proved that MBNLs can regulate RNA localization. In a few cancer cell lines, MBNLs can bind to the 3'UTR of *integrin alpha3* and regulate its localization and local translation in prominent adhesion plaques, which are required for cell adhesion and migration (Adereth, Dammai et al. 2005). In MBNL1 knockout mouse myoblasts, Eric Wang et al. used RNA-seq to detect that MBNL1 target RNA re-localized toward the insoluble cytoskeletal components and away from the RER membrane, which disrupted the protein secretion process (Wang, Cody et al. 2012).

MBNL is an important regulator of RNA localization and local translation in neurons. CLIP-Seq analysis showed that MBNLs can bind to 3' UTRs of target RNAs in neurons encoding proteins correlated with synaptic functions, such as synaptic vesicle fusion related proteins (e.g. Snap25 and Vamp1), signaling proteins like Camk2α, and the extracellular matrix (ECM) related proteins like integrin β1 and collagens (Wang, Cody et al. 2012). MBNLs facilitates the localization of RNAs to cellular RER membrane and MBNL targets are enriched in signal peptide containing proteins (Wang, Cody et al. 2012). In one report in a neuronal model system, by using CLIP-seq and RNA-seq, Taliaferro, J. M. et al. detected a strong enrichment of conserved MBNL binding motifs in mRNAs isolated from neuritic fractions of cultured cortical neurons. Specifically, MBNL

binding motifs were enriched in 3'UTR isoforms with a distal last exon 3'UTRs compared to proximal last exon. Furthermore, knockout of *MBNL1* and/or *MBNL2* disrupted the localization of many mRNAs to neurites, which indicated that MBNLs are required for the normal mRNA localization to neural projections (Taliaferro, Vidaki et al. 2016). A limitation of these experiments is that they were done on immature cortical neurons that were cultured for two days prior to differentiation of axons and dendrites, and formation of synapses. Therefore, it is still unclear whether this mechanism would be involved in more mature neurons. Tushev, G. et al. also detected the enrichment of *cis*-regulatory binding motifs of MBNL1 in 3'UTR in neuronal processes in vivo of mouse brain (Tushev, Glock et al. 2018). All of these evidences indicated that MBNLs can facilitate the localization of target RNAs to distal compartments of neurons at various developmental stages. Whether MBNL sequestration by RNA foci in DM1 due to CTG repeat expansion is sufficient to affect RNA localization is not known. A major goal of my thesis is to develop a model system that will allow us to answer this question.

In spite of these previous studies, detailed mechanism about MBNL regulating RNA localization remain unclear. For example, it is unclear what factors are involved in the process of MBNL regulated RNA localization. The details of MBNL regulating RNA localization and local translation in fully differentiated tissues like muscle and neurons remain unclear. Although it has been detected that knockout of MBNL can induce mis-localization of target RNAs, detailed mechanism of how MBNL sequestration by RNA foci affecting MBNL regulated RNA localization in DM1 has not been examined. Therefore, further studies are necessary to fully understand the role of MBNL in RNA localization and local translation, and consequences in DM1 models due to CTG expansions on RNA foci.

Because of its critical role in regulating RNA processing and localization, MBNL is required for normal developmental processes of various tissues and organs, and the sequestration of MBNL in myotonic dystrophy disrupted its functions. MBL in *Drosophila* regulates the development of muscle and photoreceptors (Begemann, Paricio et al. 1997). MBNLs in mammals are required for the development of muscle, neurons, blood cells, and so on (Kania, Salzberg et al. 1995, Miller, Urbinati et al. 2000, Phillips, Ernst et al. 2000). Nuclear sequestration of MBNLs by expanded CTG repeats in DM1 or CCTG repeats in DM2 is major driver of myotonic dystrophy. In DM1 patient cortical neurons, RNA foci can sequestrate MBNL1 and MBNL2 in nucleus and reduce their cytoplasmic level, which disrupted MBNL regulated RNA alternative splicing (Jiang, Mankodi et al. 2004). In a DM1 mouse model conditionally expressing RNA with 960 copies of CTG repeats in brain, Wang, P. Y. et al. detected reduced cytoplasmic MBNL1 expression and decreased dendritic length (Wang, Lin et al. 2017). They observed reduction of MBNL levels in dendrites that correlate with dendritic morphologic defects occurred prior to any mis-splicing. The possible role of mRNA localization to account for these morphologic defects has not been studied.

1.3.7 Target RNA of MBNL: *Snap25*

MBNLs can bind to 3'UTRs of target RNAs, regulating their alternative splicing and localization, and affect downstream intracellular activities. One important target of MBNLs is *Snap25*, which expresses synaptosomal nerve-associated protein of 25 kDa (SNAP-25) (Wang, Cody et al. 2012). SNAP-25 is important for both presynaptic and postsynaptic functions in neurons. The disruption of SNAP-25 is linked with many neurological disorders (Fatemi, Earle et al. 2001, Kim, Biederman et al. 2007, Etain, Dumaine et al. 2010, Beeri, Haroutunian et al. 2012).

SNAP-25 is an important component of a group of highly-conserved proteins called soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex, which is required for the calcium depended synaptic vesicular fusion and the exocytosis of neurotransmitters during synaptic transmission (Washbourne, Thompson et al. 2002). The SNARE proteins regulate the fusion of vesicles with their target membrane bound compartments (Ramakrishnan, Drescher et al. 2012). In neurons, SNAP-25, together with VAMP2/synaptobrevin-2 and syntaxin-1, forms the best studied SNARE complex, which regulates docking of synaptic vesicles and releasing of neurotransmitters (Trimble, Cowan et al. 1988, Bennett, Calakos et al. 1992, Kadkova, Radecke et al. 2018). SNAP-25 has two isoforms, SNAP-25a and SNAP-25b, which are resulted from alternative splicing of the exon 5 of *Snap25* (Bark and Wilson 1994). While SNAP-25a is the predominant isoform in embryonic stage, SNAP-25b is upregulated postnatally and becomes dominant isoform in adult brain (Bark and Wilson 1994, Bark, Hahn et al. 1995). SNAP-25 consists of a short N-terminus, two SNARE-motifs, Q_b (SN1) and Q_c (SN2), and a linker region connecting the C-terminal end of Q_b and N-terminal end of Q_c (Fukuda, McNew et al. 2000, Kadkova, Radecke et al. 2018).

SNAP-25 plays an important role in many presynaptic functions in neurons. SNAP-25 is essential for the process of membrane fusion and synaptic vesicles exocytosis during synaptic transmission. In the absence of SNAP-25, presynaptic vesicle docking still exists, but the pool of release-ready (or primed) vesicles is empty and fast calcium-triggered vesicle exocytosis is abolished (Sorensen, Nagy et al. 2003). Similar to SNAP-25 knockout, botulinum neurotoxins (BoNT A, C, and E) can cleave both SNAP-25a and SNAP-25b, and the cleavage of SNAP-25 by botulinum neurotoxins can block synaptic vesicle exocytosis and neurotransmitter release (Ahnert-Hilger, Munster-Wandowski et al. 2013, Pantano and Montecucco 2014). SNAP-25 can interact and regulate

several presynaptic voltage-gated calcium channels (VGCCs), such as N-type, P/Q type, L-type, and T-type calcium channels (Martin-Moutot, Charvin et al. 1996, Sheng, Rettig et al. 1996, Wiser, Bennett et al. 1996, Wiser, Trus et al. 1999, Zamponi 2003, Catterall and Few 2008, Weiss, Hameed et al. 2012). SNAP-25 can bind to the synaptic protein interaction (synprint) site of those channels and inhibit the channel activities (Pozzi, Condliffe et al. 2008). Overexpressing SNAP-25 in GABAergic interneurons reduced calcium response, whereas silencing SNAP-25 in glutamatergic neurons induced higher VGCC activity (Verderio, Pozzi et al. 2004). Moreover, it was also showed that SNAP-25 is involved in clathrin-dependent endocytosis at hippocampal synapses (Zhang, Wang et al. 2013).

One of the most important functions of SNAP-25 is regulating neurite development, which requires normal *Snap25* localization and local translation. SNAP-25 is important for neurites outgrowth. SNAP-25 is expressed in neurites growth cone, a growing tip of neurites regulating extension and guidance (Osen-Sand, Catsicas et al. 1993, Ide 1996). The disruption of SNAP-25 expression by antisense phosphorothioate oligonucleotides inhibited the neurites outgrowth of rat cortical neurons and PC-12 cells (Osen-Sand, Catsicas et al. 1993). Similarly, cleavage of SNAP-25 with BoNT/A inhibited axonal growth and prevented synapse formation (Osen-Sand, Staple et al. 1996). The mechanism of SNAP-25 regulating neurites outgrowth is that it can form SNARE complex with VAMP and syntaxin. SNARE regulated vesicles fusion with the plasma membrane at growth cone is essential for the membrane expansion and neurites outgrowth (Craig, Wyborski et al. 1995, Futerman and Banker 1996, Ulloa, Cotrufo et al. 2018). Different SNARE complex components play different roles in neurites outgrowth. SNAP-25a can stimulate neurite sprouting, while VAMP2 is responsible for neurite elongation (Shirasu, Kimura et al. 2000). There are many other factors involved in SNAP-25 regulated neurite outgrowth. For example, the transcription

factor Brn-3a participates in neurite outgrowth via stimulating *Snap25* expression and type VI adenylyl cyclase can interact with SNAP-25 and Snapin to regulate neurite extension (Lakin, Morris et al. 1995, Wu, Lin et al. 2011). It has been reported that ubiquitination of MBNL1 is required for its cytoplasmic localization and this cytoplasmic isoform can promote neurite outgrowth. Reduced MBNL1 can impair axon outgrowth and dendrite development (Wang, Chang et al. 2018). Since *Snap25* is a target RNA of MBNL (Wang, Cody et al. 2012), it is possible that MBNL is involved in SNAP-25 regulated neurite development.

It has been demonstrated that SNAP-25 can regulate the formation of presynaptic terminals, which requires mRNA localization and local synthesis of SNAP-25 in axon. SNAP-25 is recruited and locally synthesized in synapse, and the inhibition of *Snap25* RNA localization and local translation in axon disrupted the release of synaptic vesicles from presynaptic sites (Batista, Martinez et al. 2017). *Snap25* mRNA has also been identified in the local transcriptome of synaptic neuropil in the hippocampus (Cajigas, Tushev et al. 2012). These studies revealed that normal *Snap25* localization and local translation in distal processes are important for neurites development. Detailed mechanisms of *Snap25* localization remain unclear, but MBNL may play a role in this process since it contains binding motifs in 3'UTR. My thesis will address this important question.

Besides regulating various presynaptic functions, accumulating data indicated that SNAP-25 is involved in postsynaptic activities. SNAP-25 can regulate postsynaptic proteins trafficking, plasticity, and maturation of dendritic spines (Antonucci, Corradini et al. 2016). SNAP-25 is functionally relevant to the trafficking of synaptic kainate receptors (KARs) and NMDAR. SNAP-25 can interact with GluK5 subunit of KARs, reduce the stability of GluK5 on cell membrane, and facilitate the internalization of GluK5-containing KARs (Selak, Paternain et al. 2009). Phosphorylation of serine residue-187 in SNAP-25 by protein kinase C (PKC) is critical for the

delivery of NMDAR to the cell surface through SNARE-dependent exocytosis. Disruption of SNAP-25 function by RNAi, mutation of serine residue-187(S187A), BoNT A/B cleavage, and/or SNAP-25 C-terminal blocking peptide can impair PKC dependent insertion of postsynaptic NMDAR and abolish PKC potentiation of NMDA currents. (Lau, Takayasu et al. 2010). SNAP-25 also participates in synaptic plasticity because of its role in regulating postsynaptic receptors trafficking. NMDAR mediated Ca^{2+} influx is important for synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD) (Hunt and Castillo 2012). Knockdown of SNAP-25 reduced NMDAR level at synapses and impaired LTP induction (Jurado, Goswami et al. 2013). Disruption of the interaction between SNAP-25 and the GluK5 subunit of KARs can prevent LTD (Selak, Paternain et al. 2009). Another postsynaptic role of SNAP-25 is regulating dendritic spine morphogenesis. Reduction of SNAP-25 induced impairment of both maturation and function of dendritic spines, whereas overexpression of SNAP-25 increased the density of mature, Postsynaptic Density protein 95 (PSD-95)-positive spines (Tomasoni, Repetto et al. 2013). It has also been showed that SNAP-25 can regulate PSD-95 clustering. Downregulation of SNAP-25 reduced both dendritic spines densities and the PSD-95 puncta in neurons (Fossati, Morini et al. 2015).

Alterations in SNAP-25 is related to a variety of human neurological disorders. For example, previous studies have suggested that SNAP-25 is related to major depressive disorder (MDD) (Kim, Biederman et al. 2007). Increased SNAP-25 level was detected in the hippocampus of MDD patients (Fatemi, Earle et al. 2001). It has been shown that *Snap25* promoter is correlated with early onset of Bipolar Disorder (BPD), and abnormal SNAP-25 protein level was detected in patients' brains (Etain, Dumaine et al. 2010). Disrupted SNAP-25 protein level is also detected in patients with Alzheimer's disease (Beeri, Haroutunian et al. 2012, Brinkmalm, Brinkmalm et al.

2014). SNAP-25 is associated with schizophrenia in both male and female patients. Increased SNAP-25 level and SNAP-25b / SNAP-25a ratio have been detected in schizophrenia patients' brains (Houenou, Boisgontier et al. 2017). Other SNAP-25 related disorders include autism, seizure, intellectual disability, and attention deficient hyperactivity disorder (ADHD) (Feng, Crosbie et al. 2005, Rohena, Neidich et al. 2013, Braida, Guerini et al. 2015, Fukuda, Imagawa et al. 2018). Although whether SNAP-25 is directly related to myotonic dystrophy is unclear, it is possible that the function of SNAP-25 is affected in DM1 since *Snap25* is a target RNA of MBNL. It will be interesting to study the role of SNAP-25 in DM1.

1.4 Research models for DM1

In order to study the mechanism and develop efficient therapeutic strategies for DM1, researchers have developed various models of this disease. These models can be generally divided into two categories: *in vitro* model, such as human patients derived cells, and *in vivo* model, such as DMSXL mouse model and MBNL knockout mouse model. All of these models have been widely used by researchers as powerful tools to study DM1, but they also possess different limitations and disadvantages.

1.4.1 *In vitro* model

One widely used group of *in vitro* models is cellular models established through introducing exogenous tandem repeats into normal cell lines. To produce DM1 *in vitro* model, the most common strategy is introducing CTG repeats inserted in 3'UTR of a truncated *DMPK* gene into human or murine cell lines, such as HeLa, HEK, or C2C12 cells (Philips, Timchenko et al. 1998, Ho, Charlet et al. 2004, Warf and Berglund 2007). The expression of repeats is usually driven by

a cytomegalovirus (CMV) promoter (Nakamori, Taylor et al. 2016). The effectiveness of the cellular model can be assessed by detecting whether the exogenous CTG repeats can induce various DM1 features, such as RNA foci formation in cell nuclear, sequestration of MBNLs by RNA foci, and disruption of MBNL functions like alternative splicing (Matloka, Klein et al. 2018). Many constructs with different number of repeats have been used to produce *in vitro* model of DM1. For example, a construct with 960 CTG repeats has been widely used in studying the molecular mechanism and developing therapeutic strategies for DM (Konieczny, Selma-Soriano et al. 2017). One problem of these constructs with large size of repeats is that they may be unstable. To increase the stability of the repeats, researchers can insert short nucleotide sequences into CTG repeats. For example, after every 20 CTG repeats, a TCGA sequence can be inserted to stabilize the repeats in construct (Philips, Timchenko et al. 1998). However, whether this interruption of CTG repeats can affect the validity of cellular models is unclear. There are some constructs expressing large size of pure CTG repeats (800 to 914) without interruption, which may be more suitable to producing DM1 cellular model (Huichalaf, Sakai et al. 2010, Nakamori, Pearson et al. 2011, Sobczak, Wheeler et al. 2013).

Another method to establish *in vitro* DM1 model is to use cells acquired from human patients. There are many cell models produced form different types of cells derived from patients, such as primary myoblasts, immortalized fibroblasts, and stem cells (Furling, Doucet et al. 2003, Arandel, Polay Espinoza et al. 2017, Ueki, Nakamori et al. 2017). Because these cell models contain genuine mutations and canonical DM1 features of patients, they can be powerful tools for studying the mechanism and therapeutics development of DM1.

An important type of human patients derived cell model is generated from human primary cells, such as skeletal muscle cells (myoblasts) and skin fibroblasts (Timchenko, Timchenko et al. 1996,

Furling, Lemieux et al. 2001). Those cells are obtained directly from patients' tissue and therefore can represent DM1 features well. Primary myoblasts from DM1 patients can be used to study the process of differentiation of DM1 myoblasts and the features of DM1 muscles. Researchers have used DM1 primary myoblasts to differentiate muscles and detected DM1 features, such as RNA foci, MBNLs sequestration, and mis-splicing of muscle-specific transcripts, such as dystrophin and syntrophin (Nakamori, Kimura et al. 2007, Nakamori, Kimura et al. 2008). Compared with myoblasts, fibroblasts are easier to acquire from patients and manipulate during culture, making them popular cells for DM1 research. Moreover, when myoblasts are not available, researches can express the myogenic regulator factor MYOD1 in immortalized skin fibroblasts to activate the myogenic programming process (Lattanzi, Salvatori et al. 1998, Cooper, Kizana et al. 2007, Chaouch, Mouly et al. 2009). This process can produce muscle-like cells with DM1 features such as mis-splicing (Savkur, Philips et al. 2001, Ravel-Chapuis, Belanger et al. 2012, Arandel, Polay Espinoza et al. 2017).

Immortalized human fibroblasts and myoblasts can also be used as *in vitro* models of DM1. One constraint of primary cells from human is that they can only be used for a few generations. To circumvent the limitation, researches can produce immortalized cells form primary cells. Expression of the human telomerase (hTERT), an enzyme subunit that maintains telomere ends, can induce the immortalization of human fibroblasts, whereas the co-transduction of hTERT and CDK4, which blocks the p16-mediated cellular stress pathway, can induce the immortalization of myoblasts (Vaziri and Benchimol 1998, Zhu, Mouly et al. 2007, Mamchaoui, Trollet et al. 2011). The immortalized cell models have several advantages in comparison with primary cells, such as easy to culture and manipulate, cost effectively, and unlimited number of proliferations. The immortalized DM1 models can still represent similar DM1 features as primary cell models, such

as RNA foci and MBNL target RNAs mis-splicing (Arandel, Polay Espinoza et al. 2017, Konieczny, Selma-Soriano et al. 2017).

Another powerful group of *in vitro* DM1 models are derived from human pluripotent stem cells (hPSCs). Sometimes the patient biopsies can be limited and the primary human cells, such as neurons, are difficult to obtain (Han, Williams et al. 2011). Also, it is hard to acquire primary cells from DM1 patients at early stage. One solution for these problems is to establish DM1 cell models using hPSCs, which include human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) (Dakhore, Nayer et al. 2018). hESCs are obtained from the inner mass of the blastocyst (Matloka, Klein et al. 2018). They are powerful tools in DM1 research because of their potential to differentiate to various type of cells, including cells that are difficult to obtain directly from patients, such as neurons (Vazin and Freed 2010). They can also be used to study DM1 features at early stage (Seriola, Spits et al. 2011). Researchers have used hESCs as *in vitro* model to detect DM1 features, such as intra cellular signaling pathway defect and neuropathological phenotypes (Marteyn, Maury et al. 2011, Denis, Gauthier et al. 2013). Although hESCs provide potential for understanding mechanism of development and disorders, there are ethical and political controversies of using human stem cells in scientific research (Lo and Parham 2009). In recent years, hiPSCs have become increasingly popular in research as an effective replacement for hESCs. Human somatic cells can be introduced with transcription factors, such as Oct3/4, Sox2, c-Myc, and Klf4, and be 'reprogrammed' to stem cells with multiple differentiation potential (Takahashi and Yamanaka 2006). Several DM1 models have been established using hiPSCs derived from human patient fibroblasts (Xia, Santostefano et al. 2013, Gao, Guo et al. 2016, Ueki, Nakamori et al. 2017).

1.4.2 *In vivo* model

In vivo DM1 models include non-mammal animal models, such as *Caenorhabditis elegans* (*C. elegans*), *Danio rerio* (zebrafish), and *Drosophila melanogaster* (fruit fly), and mammal models like mouse. Animal models for DM1 provide unique resources to study the molecular mechanisms of DM1 and to develop novel therapeutic strategies. They have been widely used in various DM1 studies.

Several invertebrate DM1 models have been developed for scientific research and drug discovery. The *C. elegans* DM1 model have mutation in *mbl-1*, a *C. elegans* homolog of *MBNL*, and this mutation can not only induce defects in muscle, but also disrupt the neuronal functions, such as synapse formation (Spilker, Wang et al. 2012). In zebrafish, knockdown of *MBNL2* induced major features of DM, such as morphological and functional abnormalities at brain and muscle (Machuca-Tzili, Buxton et al. 2011). *Drosophila* has been used as *in vivo* model for many neurodegenerative and neuromuscular diseases. *Drosophila* DM1 model with 60 pure and 480 interrupted CUG repeats reproduced major DM1 pathology, including formation of RNA foci, muscle degeneration, and abnormal neural functions (Garcia-Lopez, Monferrer et al. 2008, Koon and Chan 2017). Because of the relatively low maintenance cost and short generation time, non-mammal animal models are useful for drug screen at large scale (Garcia-Alcover, Lopez Castel et al. 2013).

Transgenic mouse model is one of the most important tools for studying the molecular mechanism of human disorders like DM1. There are generally two main methods to develop DM1 mouse model, corresponding to different factors causing the disease. The first method to develop DM1 mouse model is to introduce toxic CTG repeats into mouse. *HSA*^{LR} mouse model is produced by overexpressing a fragment of human skeletal actin (*HSA*) gene with about 250 CTG repeats in

mice muscle, which can induce functional and histological abnormalities in skeletal muscle. Disrupted RNA alternative splicing was also detected in this mouse model at late stage (Mankodi, Logigian et al. 2000). Overexpressing DMPK transcripts fragment with various size of expanded repeats in different tissues of mice can reproduce multisystemic DM1 features (Gourdon, Radvanyi et al. 1997, Seznec, Lia-Baldini et al. 2000). DM300 transgenic mice carrying over 300 CTG repeats display abnormalities in different tissues, such as muscle, heart, and CNS. Myotonia, RNA foci, and RNA mis-splicing were detected in this mouse model (Seznec, Agbulut et al. 2001, Vignaud, Ferry et al. 2010). Because of the intergenerational instability of CTG, another transgenic mouse model called DMSXL can be generated from DM300 mice. DMSXL mice carry about 1000–1800 CTG repeats together with human exons from DMPK gene and display more severe phenotypes compare with DM300, such as high mortality, severe growth retardation, and splicing abnormalities in the CNS and muscle (Gomes-Pereira, Foiry et al. 2007). DMSXL mice have impairments in synaptic protein expression, impairments in long term potentiation (LTP) and altered learning and memory (Hernandez-Hernandez, Guiraud-Dogan et al. 2013). In contrast to these transgenic mice that express repeats in multiple tissues, inducible tissue-specific expression of CTG repeats in mice can be more useful in research to distinguish CNS from muscle effects. By using the Cre-lox system, researchers have expressed CTG repeats in specific tissues and studied the effect of expanded CTG in those tissues. For example, using CaMKII-Cre to express 960 CTG repeats in mouse forebrain induced impairment in dendrite development that correlated with loss of MBNL from cytoplasm, which was followed by mis-splicing and neurodegeneration at later stages (Wang, Lin et al. 2017).

Another method to develop DM1 mouse model is to disrupt the function of MBNL. *MBNL1* knockout mice display typical DM1 features, such as myotonia, disfunction of muscle and heart,

and RNA mis-splicing (Kanadia, Johnstone et al. 2003, Lueck, Mankodi et al. 2007, Osborne, Lin et al. 2009). Previous research showed that knockout *MBNL2* in mice can induced DM1 features, but it has also been reported that mice with disrupted *MBNL2* are relatively normal with regard to muscle function, consistent with the predominant role of MBNL2 in brain (Lin, Miller et al. 2006, Hao, Akrami et al. 2008). MBNL2 knockout mice have mis-splicing, reduced LTP, increased REM sleep and impaired learning and memory (Charizanis, Lee et al. 2012). Whether mis-splicing causes these phenotypes in MBNL2 knockout mice is not known, nor is whether mRNA localization is impaired.

1.4.3 Limitations of DM1 models

Although the existing DM1 models are useful for DM1 research, they have different limitations and constraints. The *in vitro* model produced by expressing exogenous CTG repeats in established cell lines have limitation in studying DM1 features and molecular mechanisms in specific cell type or tissues. Primary human cells have limited biopsies from patients and will enter replicative senescence after limited times of divisions. Depends on the status of the patients, such as the age of the patients and severity of the disease, the primary cell behavior may be inconsistent. Immortalized cells can have more consistent status, but the immortalization process may affect their cell behavior. It is also unclear if the unlimited number of divisions of immortalized cells can affect the reproduction of DM1 features. There are ethical and political controversies of using hESCs in research, and the reprogramming process of hiPSCs may affect their cell behaviors. Producing animal models, especially mouse models, for DM1 is time consuming and the maintenance costs are relatively high. Mouse models are not suitable for large-scale therapy

screening. Therefore, establishing novel and effective DM1 disease model is always a worthwhile task.

1.5 Treatment for DM1

There is currently no cure for myotonic dystrophy. However, the symptomatic treatments and supportive care can improve the quality of life and relieve the symptom of DM patients, and novel therapeutic strategies targeting molecular intermediates of the disease, such as toxic RNA foci, are currently being pursued.

1.5.1 Symptomatic treatments for DM1

Traditional treatments for DM1 focus on relieving the symptoms. For example, mecasermin rinfabate (SomatoKine/iPLEX) can improve insulin insensitivity and increase muscle mass in DM1 patients (Furling, Marette et al. 1999). Various sodium channel blockers and calcium antagonists can be used to treat myotonia (Desaphy, Carbonara et al. 2014). Cholestyramine, norfloxacin, and erythromycin can be used to relieve diarrhea or other gastrointestinal symptoms in DM1 patients (Ronnblom, Forsberg et al. 1996, Turner and Hilton-Jones 2010). These symptomatic treatments cannot cure myotonic dystrophy, but they are indispensable because they can improve the life quality of patients.

1.5.2 Antisense oligonucleotides (ASOs)

Previous studies have revealed various molecular mechanisms of DM1, and numerous therapeutic approaches targeting these mechanisms have been developed. Because the major pathogenesis of DM1 is toxic RNA gain-of-function, many therapeutic strategies are designed to target the mutant

RNAs. Among those strategies, ASOs treatment has been proved as a powerful tool to relieve RNA toxicity in both *in vitro* and *in vivo* DM1 models, therefore possessing potential for DM1 treatment in patients.

Generally, ASOs can function through two main mechanisms: blockage of binding sites and degradation of target RNA (Gao and Cooper 2013). ASOs bind to RNA targets with complementary nucleotide sequences. After binding to target RNA, ASOs can block the interaction between RNA and proteins or the translation process without promoting RNA degradation. On the other hand, ASOs can facilitate RNase-H endonucleases mediated cleavage, inducing the degradation of its binding RNA (Bennett and Swayze 2010). ASOs for DM1 treatment can target mutant *DMPK* mRNA and function mainly through RNA degradation. Previous studies have showed that an antisense 25-mer morpholino oligonucleotide CAG25 can directly bind to CUG repeats in *DMPK* RNA and preclude MBNL sequestration. Injection of CAG25 into the muscle of *HSA*^{LR} mice can eliminate RNA foci and restore the MBNL regulated alternative splicing. Although the CAG25 is not designed to active RNase-H, these studies showed that ASOs with CAG repeats can effectively reduce the intracellular mutant *DMPK* mRNA level (Mulders, van den Broek et al. 2009, Wheeler, Sobczak et al. 2009, Gonzalez-Barriga, Mulders et al. 2013). As a more effective strategy, many ASOs are designed to target the sequence outside of the CUG repeat tract and facilitate RNase-H activity and mutant RNA degradation. Treatment of this kind of ASOs in *HSA*^{LR} mice can reduce the toxic RNA in skeleton muscle, release MBNL from RNA foci, rescue alternative splicing, and eliminate myotonia (Wheeler, Leger et al. 2012).

It has been proved that ASOs can be used to treat DM1 features in both *in vitro* and *in vivo* DM1 models. In various DM1 cell models, such as mouse myoblasts expressing exogenous CTG repeats and myoblasts or fibroblasts from DM1 patients, ASOs can effectively reduce mutant *DMPK*,

preclude the MBNL sequestration by RNA foci, relieve RNA toxicity, and correct RNA alternative splicing (Mulders, van den Broek et al. 2009, Gonzalez-Barriga, Mulders et al. 2013, Wojtkowiak-Szlachcic, Taylor et al. 2015). In DMSXL mice, ASOs can significantly reduce intracellular level of mutant *DMPK* mRNA and RNA foci in different skeletal muscles. ASOs treatment also improved body weight, muscle strength, and muscle histology in DMSXL mice (Jauvin, Chretien et al. 2017). Similar results were detected in HSA^{LR} mice. In these experiments, ASOs were well tolerated, and the inhibition of *DMPK* mRNA levels in muscles can last for weeks after treatment. These results indicated that ASOs are promising for the treatment of DM1 patients.

In order to apply ASOs treatment to DM1 patients, there are still many problems need to be solved. For example, the pure ASOs are vulnerable to intracellular nucleases digestion, and therefore not suitable for DM1 patient treatment. Numerous chemical modifications can be used to increase the intracellular stability and binding affinity of ASOs. Introducing phosphorothioate backbone is a basic modification to increase ASOs stability. 2′-O-methyl (2′-OMe) and 2′-O-methoxyethyl (2′-MOE) ribonucleoside sugar modifications can improve the stability and binding affinity of ASOs. Adding a gap between the 2′-OMe or 2′-MOE can facilitate RNase-H activity. Introducing phosphorodiamidate morpholino can neutralize charges and increase ASOs stability (Kole, Krainer et al. 2012, Overby, Cerro-Herreros et al. 2018). Another challenge of ASOs treatment for patients is to improve multi-systemic tissue delivery efficiency. It has been indicated that the cell membrane in DM1 patients tissue is integrated and could be a barrier for drug delivery (Gonzalez-Barriga, Kranzen et al. 2015). Some chemical modification of ASOs could improve delivery efficiency. For example, peptide-linked morpholino (PPMO) ASOs can be systematically delivered to all tissues of HSA^{LR} mice (Leger, Mosquea et al. 2013). Many research groups and

pharmaceutical companies are studying modification of ASOs to improve cell uptake and systematic delivery efficiency in patients (Geary, Norris et al. 2015, Gourdon and Meola 2017). Despite many challenges, ASOs treatment is one of the most promising therapeutic strategy for DM1. Ionis Pharmaceuticals Inc. is a leading company in antisense therapy field, which has developed several US Food and Drug Administration (FDA) approved antisense medicines, such as SPINRAZA for the treatment of spinal muscular atrophy (SMA) and TEGSEDI for the polyneuropathy caused by hereditary transthyretin-mediated amyloidosis (hATTR) for adults. The company has performed several placebo-controlled clinical trials to test safety and tolerability of ASOs in adult DM1 patients (Overby, Cerro-Herreros et al. 2018, Wurster and Ludolph 2018).

1.5.3 Other therapeutic strategies

To circumvent the systematic delivery challenge of ASOs, many research groups and companies are looking for small molecules as an alternative therapeutic strategy for DM1. Through rational design and high-throughput screens, various small molecules that can reduce RNA foci, disrupt the interaction between MBNL and foci, or increase MBNL level have been discovered (Konieczny, Selma-Soriano et al. 2017). Previous studies have shown that some intracellular signaling pathways have been disrupted in DM1mouse model and patients, such as protein kinase C (PKC), glycogen synthase kinase 3 beta (GSK3beta), and AMP-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR). Small molecules or drugs that can correct those abnormal pathways can be used to treat DM1 (Thornton, Wang et al. 2017). Gene editing is another potential strategy to treat DM1. By using CRISPR/Cas9 system, researchers can remove the CTG repeats from DM1 patient fibroblasts, prevent RNA foci formation, and rescue RNA alternative splicing (Provenzano, Cappella et al. 2017).

1.6 Dissertation rationale and objectives

1.6.1 Rationale

DM1 is a multisystemic genetic disease affecting not only skeletal and smooth muscle, but also other tissues and systems including the CNS. It has been proven that DM1 is caused by the expansion of CTG repeats in the 3'UTR of *DMPK* gene, which can form RNA foci and sequestrate MBNLs. The sequestration of MBNLs disrupts their functions in regulating target RNA processing. Previous studies have made significant progress in understanding the mechanism of RNA mis-processing induced DM1 symptoms in muscle. However, a major gap exists in our knowledge of how altered RNA processing or localization causes CNS deficits in DM1.

An effective *in vitro* disease model is indispensable to study the mechanisms of CNS dysfunction in DM1. There are currently no effective cell models for studying neural function deficit in DM1. Cell models expressing exogenous CTG repeats are usually driven by CMV promoter and established from non-neuronal cells. Primary DM1 patient neurons are difficult to obtain. Although hiPSCs can be used to generate neural cells, the differentiation process is time consuming and the maintenance cost of hiPSCs model is relatively high (Xia, Santostefano et al. 2013). *In vivo* mouse models are powerful for studying the molecular mechanisms of DM1 including the CNS deficit mechanism. However, maintaining mouse models is also costly and takes long time, thus it is helpful to test hypothesis and study mechanisms using *in vitro* models before or along with *in vivo* experiments.

In my thesis project, I used developing mouse cortical neurons to develop an *in vitro* neuronal model for DM1 by expressing CTG repeats selectively controlled by a neuron specific human

synapsin promoter. This model will be effective to study neuronal deficits caused by CTG repeats in DM1 and test potential therapeutic strategies.

MBNL can regulate pre-mRNA alternative splicing and RNA localization. While extensive of studies have been performed to understand the mechanism of MBNL regulating alternative splicing, the detailed mechanism of how MBNL regulates RNA localization remains unclear. Previous study has indicated that knockdown of MBNL can induce mRNAs mis-localization in cultured cortical neurons (Taliaferro, Vidaki et al. 2016). However, no study has investigated whether mRNAs are mis-localized in neurons expressing CTG repeats that sequester MBNLs as a DM1 disease model. Previous research detected reduced dendritic length and cytoplasmic MBNL1 expression in cortical neurons in EpA960/CaMKII-Cre DM1 mouse model, but no changes in alternative splicing of MBNL targets were detected at early stages (Figure 1.3) (Wang, Lin et al. 2017). This implied that the dysregulation of MBNL-mediated RNA localization may affect neural development and function independent of mis-splicing at early developmental stage. SNAP-25 is involved in both presynaptic and postsynaptic function of neuron and can regulate the neurites outgrowth and presynaptic formation. It has been proved that *Snap25* 3'UTR is a target RNA of MBNL (Wang, Cody et al. 2012). I used my novel *in vitro* DM1 model to investigate whether MBNL sequestration can induce *Snap25* RNA mis-localization and neuronal function deficit independent of mis-splicing.

Previous studies have shown that neurites outgrowth is disrupted in both *in vitro* and *in vivo* DM1 models. Neurite and synapse formation defects were detected in hESCs from DM1 patients (Marteyn, Maury et al. 2011). Reduced dendritic length was also detected in a conditional DM1 mouse model mouse model expressing 960 CTG repeats under CaMKII promoter (Wang, Lin et al. 2017). Besides these previous studies, Dr. Kathryn Moss in our lab transfected mouse cortical

neurons with a construct expressing 480 or 960 CTG repeats driven by a CMV promoter and detected reduced dendritic arbor complexity (Figure 1.4). Since the cortical neurons also contain glia cells, we do not know whether the dendritic phenotypes are entirely due to CTG repeat expression in neurons. I have sought to detect whether neurite outgrowth is disrupted in my *in vitro* neuronal DM1 model.

ASOs can effectively rescue DM1 features in both *in vitro* and *in vivo* DM1 models and have enormous potential to treat DM1 patients. Ionis Pharmaceuticals Inc. as a leading company in antisense therapy has rich experience in ASOs drug development. Our lab collaborates with Ionis and in my thesis research, I have applied their ASOs to my *in vitro* model to test whether ASOs can rescue any potential DM1 phenotype.

1.6.2 Objectives

The overall objective of my project was to establish a novel *in vitro* neuronal model of DM1 caused by CTG repeat expression to investigate potential MBNL regulated RNA mis-localization and DM1 associated neuronal phenotypes. I established this model through transducing cultured primary cortical neurons with adeno-associated virus serotype 9 (AAV9) to deliver CTG480 or CTG960 repeats driven by a synapsin promoter. To assess the validity of the model, I characterized DM1 like features, including RNA foci and the subsequent MBNL1 and MBNL2 sequestration. Our collaborator Dr. Eric Wang and his colleagues has performed RNA-seq to detect RNA alternative splicing in our model. I detected and quantitated *Snap25* mRNA localization in neural processes to investigate the potential RNA mis-localization in DM1. To characterize the neural deficit in DM1, I detected the morphological defects in dendritic branching and neuronal

outgrowth in my model. Finally, I tested ASOs provided by Ionis Pharmaceuticals and investigated whether ASOs can rescue potential phenotypes of our *in vitro* DM1 model.

1.6.3 Hypotheses

AAV9 with synapsin promoter will specifically express CTG repeats in neurons. AAV9 expressing 480 CTG repeats (AAV-CTG480) and AAV9 expressing 960 CTG repeats (AAV-CTG960) transduced cortical neurons will form intranuclear RNA foci and sequestrate MBNL1 and MBNL2 in nucleus. RNA mis-splicing will not be detected in my DM1 model. Sequestration of MBNL in DM1 model neurons will impair *Snap25* mRNA localization in neural processes and neurites outgrowth. ASOs can degrade RNA foci, release MBNL, and rescue impairments in dendritic branching and neuronal outgrowth.

1.7 Table and Figures

Table 1.1. Clinical features of myotonic dystrophy type 1

DM1 subtype	Clinical Features	CTG repeat size	Age of onset (years)	Age of death (years)
Mild / asymptomatic	Mild myotonia Cataracts	50 - 100	20 - 70	60 or normal life span
Congenital	Hypotonia Respiratory distress Intellectual disability Tented upper lip	>1000	Birth	About 45 (30% - 40% neonatal death rate)
Childhood	Facial weakness Intellectual disability Psychosocial problems myotonia	50 - 1000	1 - 10	About 60
Classical	Myotonia Muscle weakness Cataracts Conduction defects Insulin resistance Respiratory failure Cardiac arrhythmia	50 - 1000	10 - 30	48 - 60

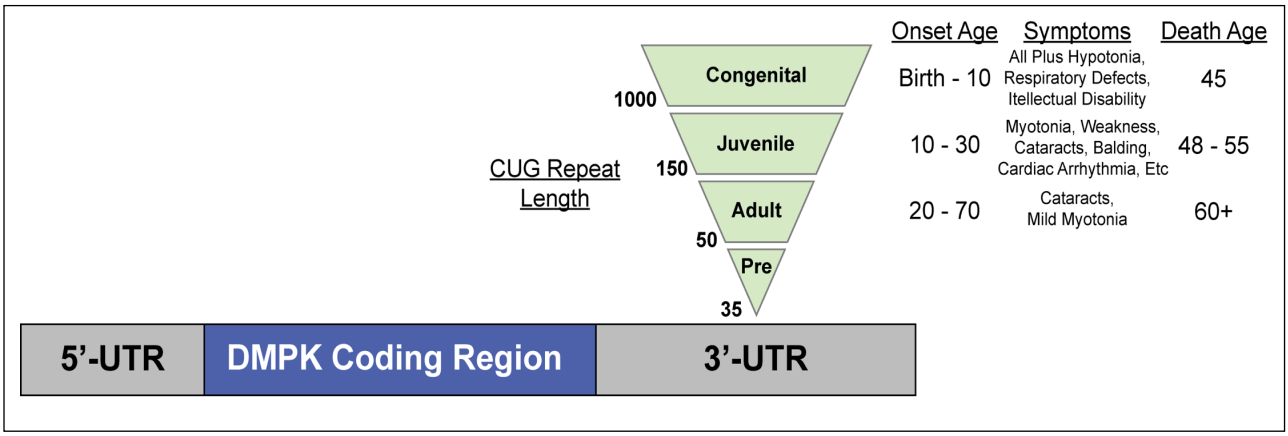


Figure 1.1 Relationship between CUG repeat size in *DMPK* 3'TUR and DM1 phenotype. 20-70 repeats can induce the mild adult-onset form of myotonic dystrophy. 100-1000 repeats are consistent with the classic adult or childhood onset DM1. Over 1000 repeats result in congenital form DM1. Adapted from Bird, GeneReviews 1993.

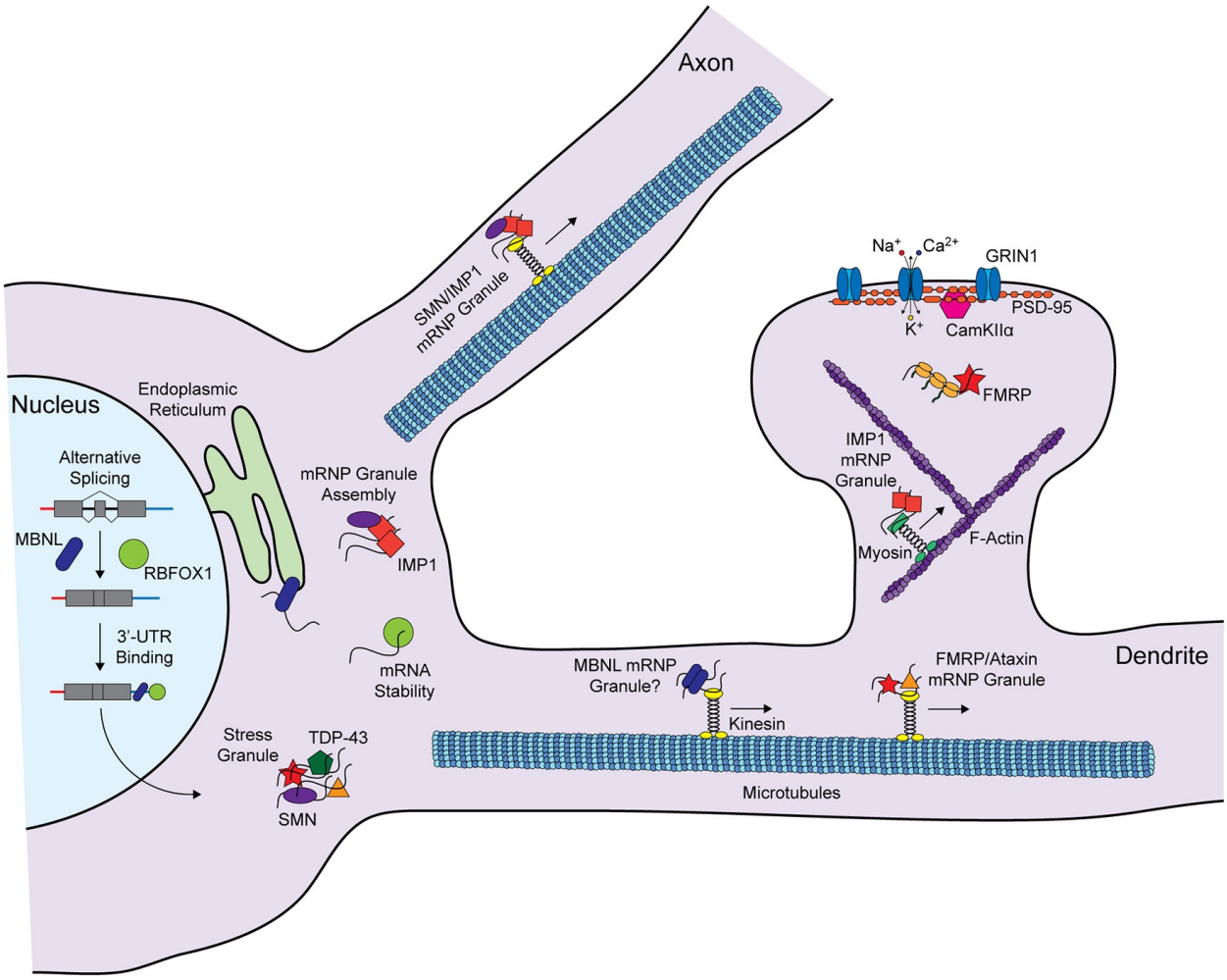


Figure 1.2 MBNL can regulate RNA alternative splicing and localization in neurons. Two isoforms of MBNL localize in the nucleus and cytoplasm. In the nucleus, MBNL can bind to 3'UTR of pre-mRNA and regulate alternative splicing. In the cytoplasm, MBNL associates with the cytoskeleton to regulate mRNA localization. Adapted from Wang, E. T. et al. J Neurosci 2016.

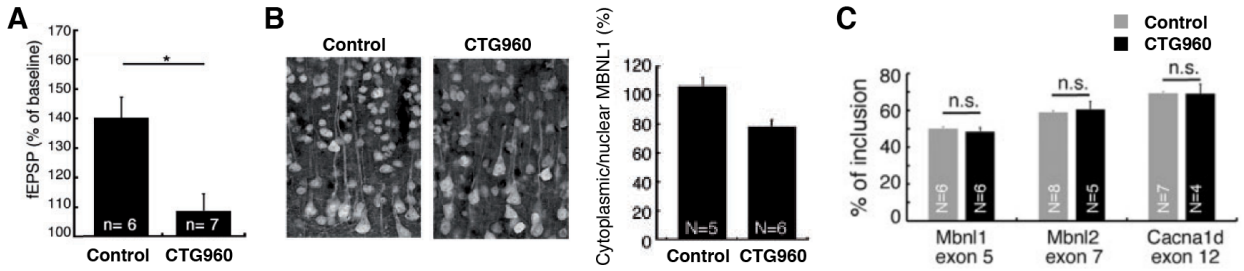


Figure 1.3 Central nervous system defects in Myotonic Dystrophy may be mediated by defects in MBNL-dependent RNA localization. A) A published CNS mouse model, EpA960/CaMKII-Cre, shows impaired LTP as measured by fEPSP. B) MBNL1 level reduced in the cytoplasm of neurons. C) No significant MBNL-dependent splicing alternation was detected in this mouse model. "Control" indicates the CaMKII-Cre mouse, and "CTG960" indicates the mouse expressing 960 CTG driven by CaMKII-Cre activation. All panels adapted from Wang et al, HMG 2017.

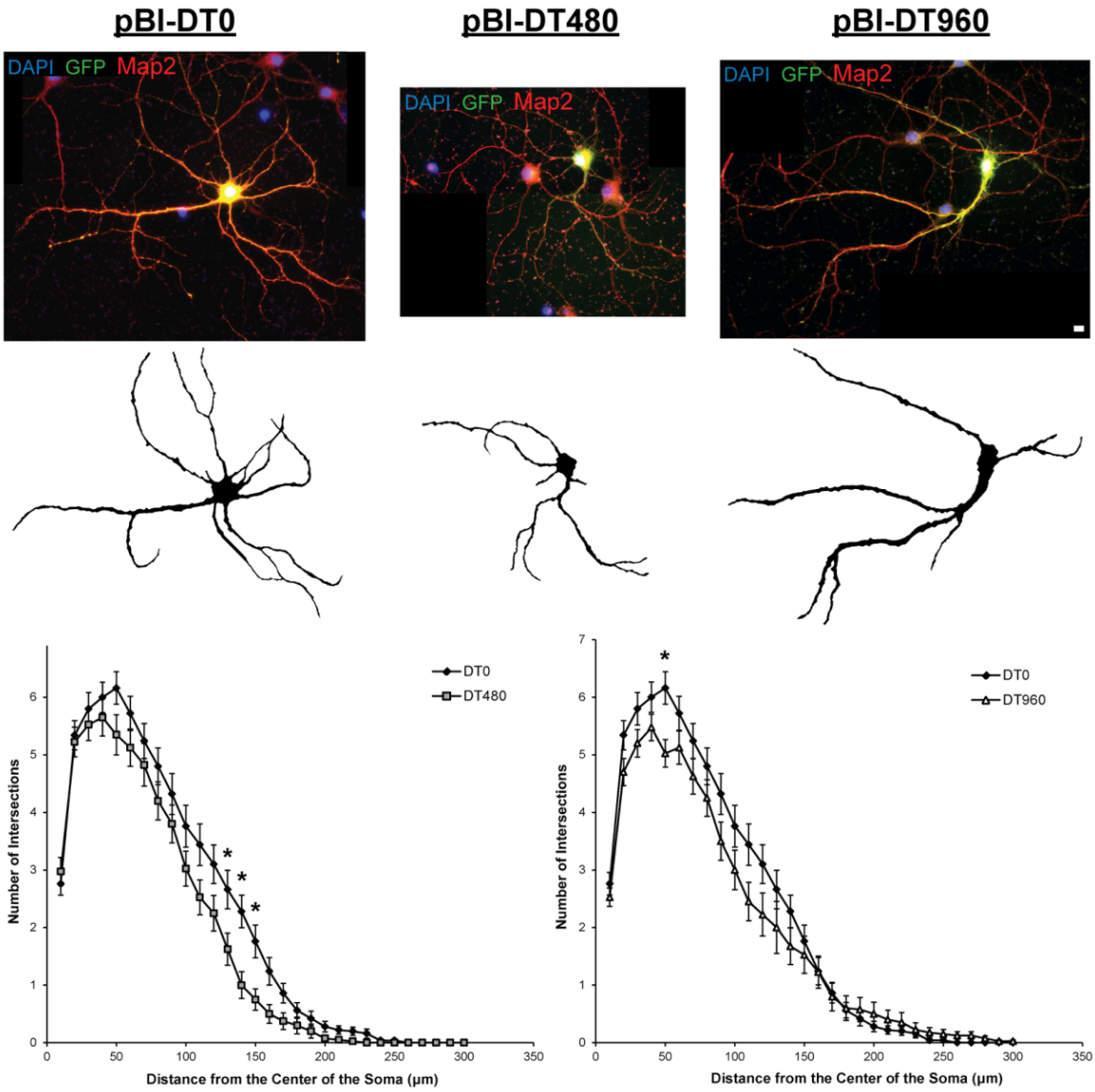


Figure 1.4 Reduced dendritic arbor complexity in DM1 CTG Model cultured cortical neurons. Mouse primary cortical neurons were magnetofected with constructs expressing 0, 480, or 960 CTG repeats driven by CMV promoter. Immunofluorescence with MAP2 antibody was performed to identify dendrites of neurons. Sholl analysis was used to quantify the dendritic arbor complexity. In transfected cortical neurons, dendritic arbor complexity was significantly reduced. Student's t-test. *$p < 0.05$. All panels produced by Dr. Kathryn Moss.

Chapter 2. Materials and Methods

2.1 Acquire and culture primary cortical neurons

Cerebral cortices were dissected from embryonic day 17 (E17) mouse embryos (C57BL/6J, Jackson Laboratories). The dissection was performed in cold HBSS (Fisher, Catalog number: 21-022-CV). Primary cortical neurons were dissociated from cerebral cortices with 0.25% Trypsin in HBSS without Calcium and Magnesium (Corning, Catalog number: 45000-658). Neurons were planted on Poly-L-Lysine (Sigma-Aldrich, Catalog number: P2636-1G, 0.02mg/ml)-coated acid-pretreated glass coverslips or Poly-L-Lysine coated Corning 96-Well Clear Bottom Plates (VWR, Catalog number: 29444-008) and incubated in minimal essential medium (MEM) (Fisher, Catalog number: 10-010-CV) with 10% Fetal Bovine Serum (FBS) (Hyclone, Catalog number: SH30070.03) for about two hours in 37°C cell incubator. Then, Neurons on coverslips were inverted into 6-well cell culture plate (BD Falcon, Catalog number: 353046) with pre-planted secondary glia cells in Neurobasal medium (Fisher, Catalog number: 21103049) with 1x B27 Supplements (Invitrogen, Catalog number: 17504001) and GlutaMAX supplement (Fisher, Catalog number: 35050061). For neurons in 96 well plate, MEM was changed to the same glia-conditioned Neurobasal medium with B27 and GlutaMAX. The culture medium was changed to fresh conditioned Neurobasal medium once a week.

2.2 Magnetofection

Neurons on coverslips were moved face up into a 12 well tissue culture plate (BD Falcon, Catalog number: 353043) with glia-conditioned Neurobasal medium with B27 and Glutamax (1ml / well). For each coverslip, the transfection mix solution was prepared as follow: every 200ng plasmids were mixed with 100ng pcDNA3.1 and 1µl Neuromag (OZ Biosciences, Catalog number: NM50500) into 100µl Neurobasal medium with GlutaMAX. The transfection mix solution was

incubated at room temperature for 20 minutes then added directly on top of the neurons. Neurons were incubated at 37°C on top of a magnetic plate for about 20 minutes. The magnetic plate was then removed.

2.3 AAV9 construct and virus transduction

The AAV9 vector was produced from a construct with a bidirectional human synapsin promotor (developed and provided by Dr. Eric Wang) (Figure 3.1A). The synapsin promoter drives expression of 0, 480, or 960 CTG repeats in the context of human DMPK exon 15. In both AAV-CTG0 and AAV-CTG480, the bidirectional synapsin promoter drives expression of maxGFP in the opposite direction of DMPK exon 15.

During transduction, neurons on coverslips were moved face up into a 12 well tissue culture plate at days *in vitro* (DIV) 1. AAV9 virus was mixed with fresh conditioned Neurobasal medium (1μl AAV9 / ml) then added into the 12 well plate containing neurons (1 ml / well). Neurons were then cultured at 37°C.

2.4 Immunofluorescence (IF)

Neurons were rinsed briefly with 1x PBS (Biotium, Catalog number: 22020) to get rid of culture medium. Neurons were fixed with 4% paraformaldehyde (PFA) (Sigma-Aldrich, Catalog number: P6148) for 15 minutes in the chemical hood. Then neurons were washed in 1x PBS for three times (5 minutes each time). Neurons were permeabilized through incubating in 0.2% Triton X-100 (Fisher Scientific, Catalog number: AC327372500) in PBS for 10 minutes. Neurons were then blocked for 1 hour at room temperature in blocking buffer, which is composed of 1x PBS, 5% normal donkey serum (Jackson Labs, Catalog number: 017-000-121), 0.1% BSA (Invitrogen,

Catalog number: AM2616), and 0.1% Triton X-100. Neurons were incubated with primary antibodies in blocking buffer overnight at 4°C. Then, neurons were washed three time with 1x PBS (10 minutes each time). Neurons were incubated with secondary antibodies in blocking buffer for about 1 hour at room temperature in darkness. Neurons were then washed three time with 1x PBS (10 minutes each time). Neurons on coverslips were mounted on a slide using ProLong™ Gold Antifade Mountant with DAPI (Invitrogen, Catalog number: P36935). Neurons in 96 well plate were kept in 1x PBS.

Primary antibodies used in IF: Anti-TurboGFP(d) (1:1000, Evrogen), NeuN (1:500, GeneTex), GFAP (1:1000, Millipore Sigma), MAP2 (1:200, Synaptic Systems), MBNL1 (1:1000, Millipore), MBNL2 (3B4) (1:50, DSHB).

2.5 Fluorescent in situ hybridization (FISH)

Neurons were rinsed briefly with 1x PBS prepared with DEPC-treated water (DEPC-PBS) (Thermo Fisher Scientific, Catalog number: 4387937) to get rid of culture medium. Neurons were fixed with 4% PFA (Electron Microscopy Sciences, Catalog number: 15710) prepared with DEPC-treated water for 10 minutes and then washed in 1x DEPC-PBS for three times (5 minutes each time). Neurons were washed in 2x SSC (Thermo Fisher Scientific, Catalog number: AM9763) buffer for 10 minutes then in pre-warmed 2x SSC with 10% formamide (Fisher Scientific, Catalog number: BP227-100) for 5 minutes. Pre-hybridization solution was prepared as follow: for each coverslip, hybridization buffer was prepared: 14µl dextran sulfate (Sigma-Aldrich, Catalog number: D8906) 14µl 20mg/ml RNase-Free BSA (sigma, Catalog number: 10711454001), 14µl 20x SSC, 7µl ribonucleoside vanadyl complexes (Sigma-Aldrich, Catalog number: R3380) 0.7µl 10x PBS, and 20.3µl DEPC-treated water; Carrier buffer was prepared: 4µl UltraPure™ Salmon

Sperm DNA Solution (Thermo Fisher Scientific, Catalog number: 15632011), 4µl tRNA, 6µl formamide, 16 DMPC- treated water. Then the hybridization buffer and carrier buffer were combined 1:1 to produce pre-hybridization solution. Neurons were incubated with pre-hybridization solution (about 58µl / coverslip) on parafilm at 37°C for 1.5 hours in a moisture chamber. Then, neurons were incubated with pre-hybridization solution (about 58µl / coverslip) and probes (about 1µl / coverslip) on parafilm at 37°C overnight in a moisture chamber. Neurons were washed twice with pre-warmed 2x SSC with 10% formamide for 20 minutes at 37°C. Neurons then were washed briefly three times with 2x SSC, followed by two 5 minutes washes in 2x SSC. Neurons on coverslips were mounted on a slide using ProLong™ Gold Antifade Mountant with DAPI. Probe used to detect RNA foci: Cy3 CAG10 probe (IDT, IA).

2.6 FISH combined with IF

IF can be performed after FISH. After finishing washes with 2x SSC, neurons were rinsed with 1x DEPC-PBS and then permeabilized through incubating in 0.2% Triton X-100 in 1x DEPC-PBS for 10 minutes. Blocking buffer was prepared with DEPC-PBS, and ribonucleoside vanadyl complexes were added (1:20). Neurons were incubated with blocking buffer, primary antibodies, secondary antibodies as described above.

2.6 ASO treatment

At DIV 8, neurons on coverslips were moved face up into a 12 well tissue culture plate, or discard culture medium of neurons in 96 well plate. 5µM ASOs (Ionis Pharmaceuticals) were mixed with fresh conditioned Neurobasal medium then added into the 12 well plate or 96 well plate containing neurons. Neurons were then cultured at 37°C.

ASOs were developed and generously provided by Ionis Pharmaceuticals. ASO-control (ISIS 676630) sequence: CCTATAGGACTATCCAGGAA. ASO-DMPK (ISIS 1052867) sequence: CAGACAATAAATACCGAGGA. Both ASO-control and ASO-DMPK are 2'-MOE modified gapmer ASOs with mixed backbone. Concentration: 100 mg/ml.

2.7 Microscopy and Imaging

The images of dendritic defects and ASO treatment rescue were taken and analyzed using Molecular Devices ImageXpress Micro XLS widefield high-Content analysis system. All other images were taken through Nikon eclipse ti. Images were processes and analyzed using ImageJ. For all the FISH imaging and dendritic phenotype imaging, neurons were selected through GFP signal without knowledge of RNA foci. All samples for imaging were acquired from at least three experiments. About 50 to 100 neurons were imaged for RNA foci or MBNL sequestration. Over 60 neurons were imaged for dendritic defects analysis or ASO treatment analysis.

2.8 Statistics

Student's t-test was used to analyze data as indicated in each figure. All statistical analyses were performed in Prism 8.

Chapter 3. Development of an *In* Vitro Neuronal Model of DM1

3.1 Establishment of novel *in vitro* neuronal DM1 model

As a multisystemic genetic disorder, DM1 affects both muscle and the CNS in patients. While extensive studies about muscle symptoms have been performed, the detailed molecular mechanism of how DM1 affects the CNS remains unclear. The development of an *in vitro* neuronal model could be used to characterize cellular and molecular phenotypes that may have important implications for understanding the impact of DM1 on the CNS. However, there is currently no effective cellular model for studying the neuronal defects and related molecular mechanisms in DM1 specifically. In order to study the mechanism of DM1 neuronal defects, our lab first used a construct with a CMV promoter to express 480 or 960 CTG repeats in primary mouse cortical neurons and detected reduced dendritic arbor complexity in transfected neurons (Figure 1.4 by Dr. Kathryn Moss). Although this construct can produce RNA foci in neurons effectively, the CMV promoter can non-selectively drive CTG repeats expression in both neurons and non-neuronal cells, like glial cells, which makes this method ineffective in producing a pure neuronal model and limits its usage in studying cell autonomous neuronal defects in DM1. My objective was to develop a novel *in vitro* model of DM1 expressing CTG repeats exclusively in cultured primary mouse cortical neurons.

To produce the DM1 neuronal model, I used AAV9 under control of synapsin promoter to deliver 480 or 960 CTG repeats into primary cortical neurons. AAV9 is a non-pathogenic parvovirus that can infect both dividing and non-dividing cells (Baba, Satoh et al. 2012). Because it has no obvious toxicity, high transduction efficiency, and long-term gene expression, AAV9 has been widely used in *in vitro* and *in vivo* scientific research to deliver exogenous genes. It is also considered as a promising delivery tool for gene therapy in patients (Chen, He et al. 2015, Lukashchuk, Lewis et al. 2016, Chandler, Williams et al. 2017, Lykken, Shyng et al. 2018).

The AAV9 vector used in my thesis was produced from constructs developed and provided by our collaborator Dr. Eric Wang (Figure 3.1A). These constructs have a human synapsin promotor. The synapsins are a family of neuronal phosphoproteins that express exclusively in neurons regulating neurotransmitter release at synapses (Schoch, Cibelli et al. 1996). The synapsin promoter in our construct drives 480 or 960 CTG repeats in the context of human DMPK exon 15. An AAV expressing no CTG repeats (AAV-CTG0) was used as a negative control. Both AAV-CTG0 and AAV-CTG480 have a bidirectional synapsin promoter that drives maxGFP in the opposite direction, which facilitates our recognition of transduced neurons. By using AAV-CTG480 and AAV- CTG960, I have been able to establish this novel *in vitro* model of DM1.

3.1.1 AAV9 with human synapsin promoter can exclusively transduce primary cortical neurons

The first step of establishing the *in vitro* DM1 model was to verify the effectiveness of our virus and constructs. The human synapsin promoter of our constructs should target AAV9 to neurons exclusively. To test this, I dissected cerebral cortices from embryonic day 17 (E17) mouse embryos, dissociated and cultured cortical neurons, and transduced the neurons with AAV-CTG0 or AAV-CTG480 at one day *in vitro* (DIV 1). At DIV 18, I fixed the neurons with 4% paraformaldehyde (PFA) and performed immunofluorescence (IF). NeuN antibody and GFAP antibody were used to label cortical neurons and glial cells respectively.

As shown in Figure 3.1B, the AAV-CTG0 and AAV-CTG480 transduced cells effectively expressed GFP. The GFP expression was only detected in cortical neurons (NeuN+), and no GFP signal was detected in astrocytes (GFAP+). These data indicated that our constructs can express in neurons specifically.

To quantify the transduction efficiency of our AAV9, I performed cell counting on the transduced neurons. In over 60 scored cortical neurons, more than 90% of neurons were transduced by AAV-CTG480. Similar ratio was detected in AAV-CTG0 transduced neurons. Therefore, the AAV9 used in my thesis can exclusively transduce primary mouse cortical neurons with high efficiency.

3.1.2 RNA foci formed in AAV9 transduced primary cortical neurons

I verified the validity of our *in vitro* model through detecting whether the model can reproduce DM1 features. One of the most well characterized DM1 molecular features is the formation of RNA foci in the nucleus. To detect RNA foci in our *in vitro* DM1 model, I cultured the primary cortical neurons from E17 mouse embryos and transduced them with AAV-CTG0 or AAV-CTG480 at DIV 1. At DIV 18, the neurons were fixed and the fluorescent *in situ* hybridization (FISH) was performed to detect RNA foci using a Cy3 CAG10 probe.

As shown in Figure 3.2, RNA foci were detected in nuclei of most AAV-CTG480 transduced mouse cortical neurons (GFP+). On the other hand, no RNA foci were detected in AAV-CTG0 transduced neurons. To quantify the efficiency of AAV-CTG480 to produce RNA foci, I scored transduced neurons. In over 40 AAV-CTG480 transduced cortical neurons, RNA foci were detected in more than 80% of neurons. RNA foci were also detected in the nucleus of AAV-CTG960 transduced neurons (Figure 3.3, 3.4). These data indicated that AAV-CTG480 and AAV-CTG960 can effectively express CTG repeats in cortical neurons and induce the formation of RNA foci in the nucleus.

3.1.3 Sequestration of MBNLs by RNA foci in DM1 model

To further prove the validity of our *in vitro* model, I detected another significant DM1 molecular feature in the DM1 model: the sequestration of MBNLs. I used AAV-CTG0, AAV-CTG480, or AAV-CTG960 to transduce the cultured primary mouse cortical neurons from E17 mouse embryos at DIV 1. I then performed FISH to detect RNA foci and IF after FISH to detect MBNL1 or MBNL2 localization at DIV 18.

As data shown in Figure 3.3 and 3.4, MBNL1 and MBNL2 were detected in both nucleus and cytoplasm of AAV-CTG0 transduced neurons. RNA foci formed in nuclei of both AAV-CTG480 and AAV-CTG960 transduced neurons. The RNA foci can effectively sequestrate both MBNL1 and MBNL2, which reduced levels of MBNLs from the cytoplasm. Taken together, AAV-CTG480 or AAV-CTG960 transduction can induce the formation of RNA foci and the sequestration of MBNL1 and MBNL2 in primary mouse cortical neurons, which further verifies the effectiveness of our novel *in vitro* model of DM1.

3.2 RNA alternative splicing and localization in DM1 model

MBNLs can bind to their target RNAs and regulate various RNA processing steps. Nuclear and cytoplasmic enriched isoforms of MBNLs can regulate RNA alternative splicing and localization respectively. Compared to the broad research about how MBNLs regulate alternative splicing, the mechanism of MBNLs regulating RNA localization has not been sufficiently studied. Previous research showed impairment in dendritic outgrowth and associated depletion of cytoplasmic MBNL1 in cortical neurons from a transgenic (EpA960/CaMKII-Cre) DM1 mouse model *in vivo*. However, no obvious mis-splicing of MBNL target RNAs was detected within the first few months of CTG repeat expression (Wang, Lin et al. 2017). Mis-splicing of MBNL targets was only

observed after six months, a few months after reduction in dendritic levels of MBNL and impairments in dendritic outgrowth. These results implied that MBNL regulated RNA mis-localization in DM1 may possibly affect dendritic outgrowth independent of alternative splicing at earlier developmental stages.

Previous studies have used different genetic methods to deplete MBNL to study MBNL-dependent RNA localization (Adereth, Dammai et al. 2005, Wang, Cody et al. 2012). However, knockdown or knockout of MBNL *in vivo* will disrupt all MBNL intracellular functions, including both alternative splicing and RNA localization. Therefore, it will be difficult to distinguish the impact of RNA mis-splicing and mis-localization on neuronal functions in DM1. This is less of a concern in cultured neurons. Even at DIV 18, cultured neurons do not mature sufficiently to express all the splicing factors required for the regulation of many MBNL-dependent exons. Thus, our *in vitro* DM1 model is a useful tool to study MBNL-dependent RNA localization in the absence of RNA mis-splicing.

3.2.1 Alternative splicing is normal in DM1 model

In order to study whether MBNL-dependent RNA alternative splicing is disrupted in our *in vitro* DM1 model, our lab collaborate with Dr Eric Wang's lab at University of Florida to perform RNA-seq on AAV-CTG0 or AAV-CTG960 transduced cortical neurons. Neurons were transduced with AAV at DIV 1 and RNA collected using trizol at DIV 18. Dr. Eric Wang and his lab members made a cDNA library, performed RNA-seq and detected the Percent Spliced In (PSI), also known as exon-inclusion ratio, which is a popular quantitative method for measuring alternative splicing. PSI is defined as the percentage of a gene's mRNA transcripts that include a specific exon or splice cite (Park, Pan et al. 2018). It has been proven that MBNL can regulate the alternative splicing of

Cacna1d, a gene expressing a component of calcium channel (Charizanis, Lee et al. 2012, Goodwin, Mohan et al. 2015, Wang, Lin et al. 2017). PSI values of exon 12 of *Cacna1d* and exon 5 of *MBNL1* and *MBNL2* were calculated. Also, the MBNL-regulated alternative splicing was analyzed through a transcriptome-wide RNA-seq.

In AAV-CTG0 or AAV-CTG960 transduced cortical neurons, PSI values for all three selected MBNL-dependent exons were at similar level, indicating that there is no mis-splicing event in AAV-CTG960 transduced neurons (Figure 3.5). Little to no MBNL-dependent mis-splicing events were detected across the transcriptome in these cultured cortical neurons at this early developmental stage (Data not shown). These data showed that the MBNL regulated RNA alternative splicing is relatively unaffected in our DM1 model.

3.2.2 Mis-localization of *Snap25* in DM1 model

Since both MBNL1 and MBNL2 were sequestrated by RNA foci in our *in vitro* model of DM1 but no mis-splicing was detected, I then tested whether MBNL-regulated RNA localization was disrupted in AAV-CTG480 and AAV-CTG960 transduced neurons. *Snap25* was used as a candidate RNA to test potential mis-localization in our DM1 model. The normal localization and local translation of *Snap25* in neural processes are important for both presynaptic and postsynaptic function of neurons. It has been proven that *Snap25* is a target of MBNL (Wang, Cody et al. 2012). Therefore, it is possible that the sequestration of MBNL by RNA foci could disrupt the localization of *Snap25* in neural processes. The role of MBNL in *Snap25* mRNA localization has not been previously studied.

To detect the localization of *Snap25*, I dissociated cortical neurons from E17 mouse embryos and transduced them with AAV-CTG0, AAV-CTG480, or AAV-CTG960 at DIV 1. At DIV 18, FISH was performed to detect *Snap25* localization in neural processes of transduced neurons.

Although no obvious difference was detected in nucleus, *Snap25* level was reduced in neural processes of both AAV-CTG480 and AAV-CTG960 transduced neurons compared with AAV-CTG0 transduced neurons. (Figure 3.6A, 3.7A). Through tracking single neural process and quantification of the *Snap25* signal intensity, I detected that the *Snap25* level was relatively normal in neural processes adjacent to cell soma but decreased in distal segments of processes in AAV-CTG480 and AAV-CTG960 transduced neurons (Figure 3.6B, 3.7B). The overall *Snap25* level quantification in neural processes indicated a significant decrease in both AAV-CTG480 and AAV-CTG960 transduced neurons (Figure 3.6C, 3.7C). Results from RNA-seq analysis also showed that there was no change in the steady state level of *Snap25* mRNA or any change in alternative splicing of *Snap25* in our AAV models (data not shown, from Eric Wang). Taken together, these data showed that the sequestration of MBNL by RNA foci can induce mis-localization of target RNA in early stage cultured cortical neurons independent of mis-splicing.

3.3 Morphological phenotype of DM1 model: reduced dendritic arbor complexity

Next, I detected whether the RNA foci and MBNL sequestration can induce any morphological phenotype in our *in vitro* model of DM1. Previous studies have detected the disruption of neurite outgrowth in both *in vitro* and *in vivo* DM1 models, such as hESCs from DM1 patients and EpA960/CaMKII-Cre DM1 mouse model *in vivo* (Marteyn, Maury et al. 2011, Wang, Lin et al. 2017). Dr. Kathryn Moss in our lab also detected reduced dendritic arbor complexity in mouse cortical neurons expressing 480 or 960 CTG repeats driven by a CMV promoter (Figure 1.4).

Moreover, SNAP-25 is involved in both presynaptic and postsynaptic function of neurons and can regulate the neurite outgrowth. Our data suggest that the mis-localization of *Snap25* in neural processes may contribute to the dendritic defects in our DM1 model.

3.3.1 Dendritic defects in DM1 model

To detect the dendritic phenotype of our *in vitro* DM1 model, I dissociated neurons from the cerebral cortices of E17 mouse embryos and cultured them on Corning 96-well clear bottom plates. Neurons were transduced with AAV-CTG0 or AAV-CTG480 at DIV 1, and IF was performed at DIV 18. Anti-TurboGFP antibody was used to increase native GFP signal in transduced cortical neurons. MAP2 antibody was used to identify dendrites of cortical neurons. Neurons were then imaged and analyzed through Molecular Devices ImageXpress Micro XLS widefield high-Content analysis system.

The dendritic tacking analysis indicated that the dendritic arbor complexity was reduced in AAV-CTG480 transduced neurons compared with AAV-CTG0 transduced neurons (Figure 3.8A). To quantify the dendritic defects, the length of total neurites outgrowth, the number of neurite branches, mean neural process length, and the number of neural processes of neurons were analyzed (Figure 3.8B). While no significant difference of the number of neural processes was detected, the length of total outgrowth, the number of branches, and mean neural process length were decreased in AAV-CTG480 transduced neurons. Thus, the dendritic arbor complexity was significantly reduced in our *in vitro* DM1 model.

3.4 ASO treatment

The last part of my thesis project was to assess possible rescue of the dendritic defects in our *in vitro* model of DM1 with ASO treatment. ASO treatment has been proven as a powerful tool to relieve RNA toxicity in both *in vitro* and *in vivo* DM1 models. In many DM1 cell models like myoblasts and fibroblasts from DM1 patients and mouse models like DMSXL mice and HSA^{LR} mice, ASOs can effectively reduce mutant *DMPK* and RNA foci and relieve RNA toxicity (Mulders, van den Broek et al. 2009, Gonzalez-Barriga, Mulders et al. 2013, Jauvin, Chretien et al. 2017). It has been proven that ASO treatment has low toxicity and long-last inhibition of *DMPK* mRNA levels in those models (Mulders, van den Broek et al. 2009, Gonzalez-Barriga, Mulders et al. 2013, Wojtkowiak-Szlachcic, Taylor et al. 2015, Jauvin, Chretien et al. 2017). These results indicated that ASO has tremendous potential to be used as a treatment for DM1 patients. The effectiveness of ASOs to restore neuronal defects in any DM1 model has not been shown.

In order to rescue the dendritic outgrowth defects, I treated our *in vitro* model of DM1 with a 2′-MOE modified ASO targeted to the 3'-UTR of the *DMPK* gene (ASO-DMPK), which is produced and generously provided by our collaborator Ionis Pharmaceuticals. Previous research used this ASO to reduce RNA foci in skeletal muscles and heart of DMSXL DM1 mouse model and detected increased body weight, muscle strength, and improved skeletal muscle maturation (Jauvin, Chretien et al. 2017).

3.4.1 ASO treatment eliminated RNA foci in DM1 model

To evaluate the efficiency of the Ionis ASO, I first detected whether ASO treatment can eliminate RNA foci in our DM1 model. Neurons form E17 mouse embryo cerebral cortices were transduced

with AAV-CTG480 at DIV1. The transduced neurons were treated with ASOs at DIV 8, and FISH was performed to detect RNA foci at DIV 18.

While control ASOs did not affect the formation of RNA foci in nucleus of AAV-CTG480 transduced neurons, the ASO-DMPK can effectively eliminate RNA foci in neurons (Figure 3.9). Little to no RNA foci were detected in over 90% of AAV-CTG480 transduced neurons after ASO-DMPK treatment, which proved the efficiency of the Ionis ASO. Moreover, Ionis Pharmaceuticals applied ASOs on non-neuronal cells (N2A cells) expressing CTG repeats and detected reduced mutant *DMPK*. We will test whether mutant *DMPK* mRNA level reduced in our *in vitro* model in future.

3.4.2 ASO treatment increased dendritic outgrowth in DM1 model

After confirming the efficiency of ASO, I investigated whether the dendritic defects of our *in vitro* DM1 model can be rescued by ASO treatment. Neurons from the cerebral cortices of E17 mouse embryos were cultured on Corning 96-well clear bottom plates and transduced with AAV-CTG480 at DIV 1. ASO-control or ASO-DMPK treatment were applied to neurons at DIV 8. IF was performed at DIV 18 with Anti-TurboGFP antibody and MAP2 antibody. Neurons were then imaged and analyzed through Molecular Devices ImageXpress Micro XLS widefield high-Content analysis system.

Compared with the control ASOs treated CTG480 neurons, the dendritic arbor complexity increased in the ASO-DMPK treated CTG480 neurons as indicated by the dendritic tracing (Figure 3.10A). The length of total neurites outgrowth, the number of neurite branches, mean neural process length, and the number of neural processes of neurons after ASO treatment were analyzed (Figure 3.10B). The ASO-DMPK treatment significantly increased the length of total outgrowth,

the number of branches, and mean neural process length of AAV-CTG480 transduced neurons. These data indicated that the ASO treatment can effectively increase the dendritic outgrowth in our *in vitro* DM1 model.

3.5 Figures

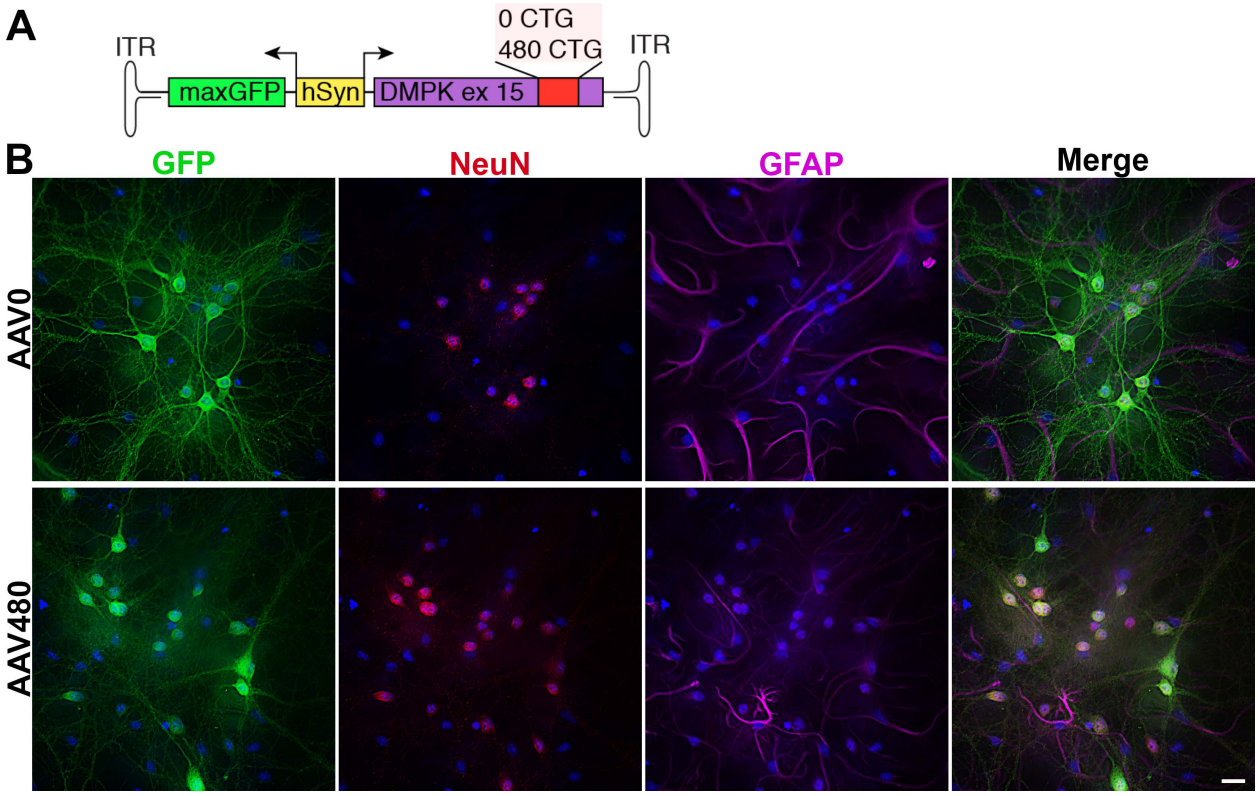


Figure 3.1 AAV-hSyn-GFP-CTG can exclusively transduce primary mouse cortical neurons. A) Schematic of AAV cargoes used to infect cortical neurons. B) Native GFP and immunofluorescence of NeuN and GFAP in cortical neurons transduced by AAV-CTG0 and AAV-CTG480 viruses. GFP expresses exclusively in neurons (NeuN+). Scale bar = 20 μm.

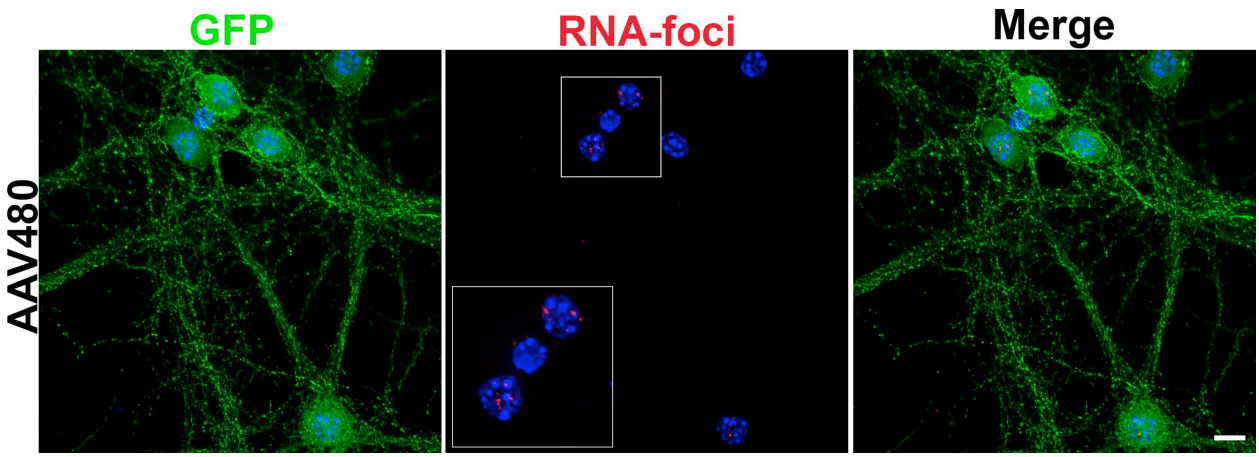


Figure 3.2 **RNA foci are detected in AAV-CTG480 transduced primary mouse cortical neurons.** AAV-CTG480 induced RNA foci are detected in nucleus of transduced neuron through FISH using a Cy3 CAG10 probe. Scale bar = 10 μm.

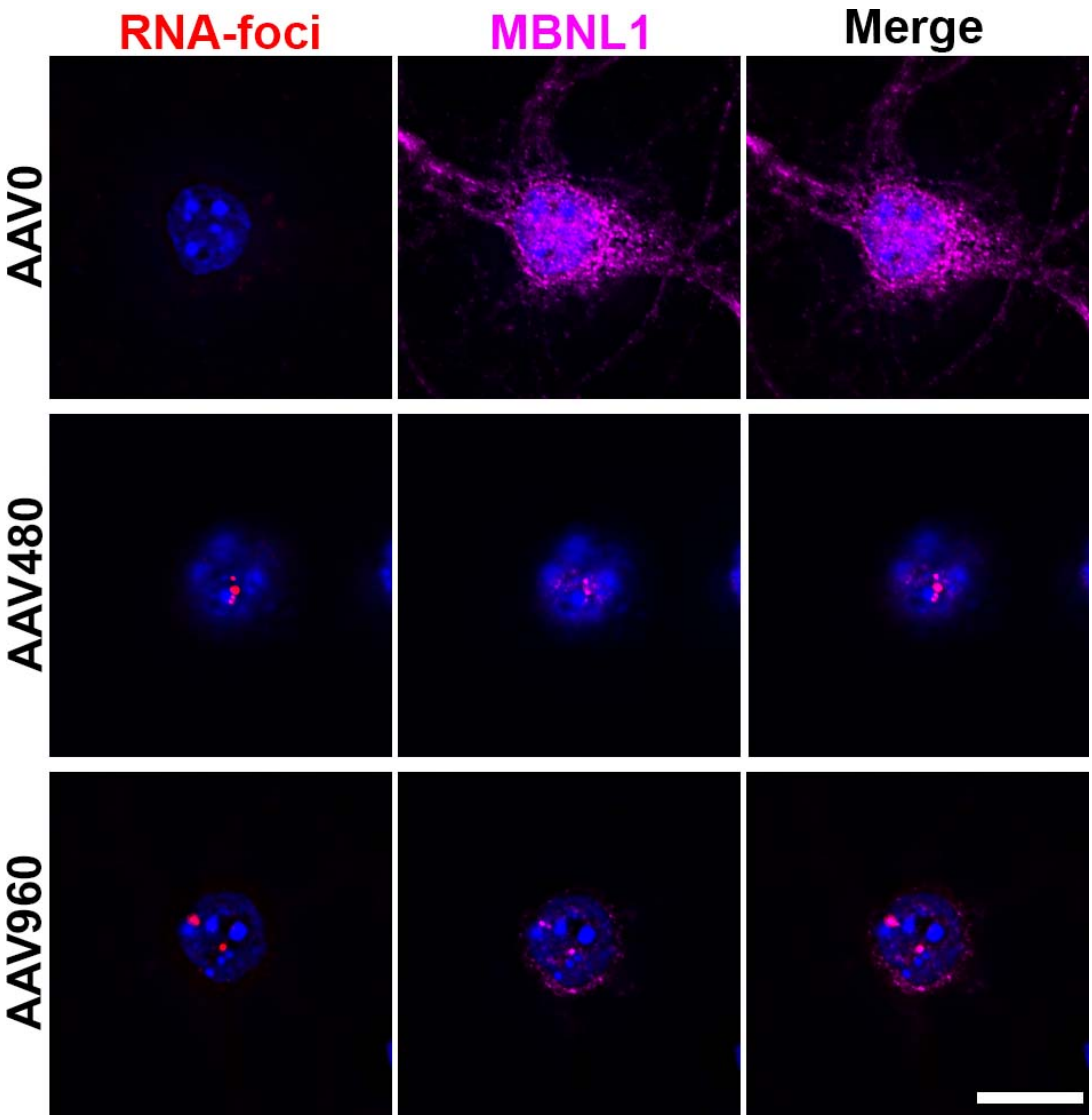


Figure 3.3 RNA foci sequestrate MBNL1 in nucleus of AAV virus transduced neurons. RNA foci are detected by FISH and MBNL1 is detected by IF in DIV 18 cortical neurons transduced by AAV-CTG0, AAV-CTG480, or AAV-CTG960. MBNL1 is sequestered to RNA foci in nucleus of AAV-CTG480 and AAV-CTG960 transduced neurons. Scale bar = 10 μm.

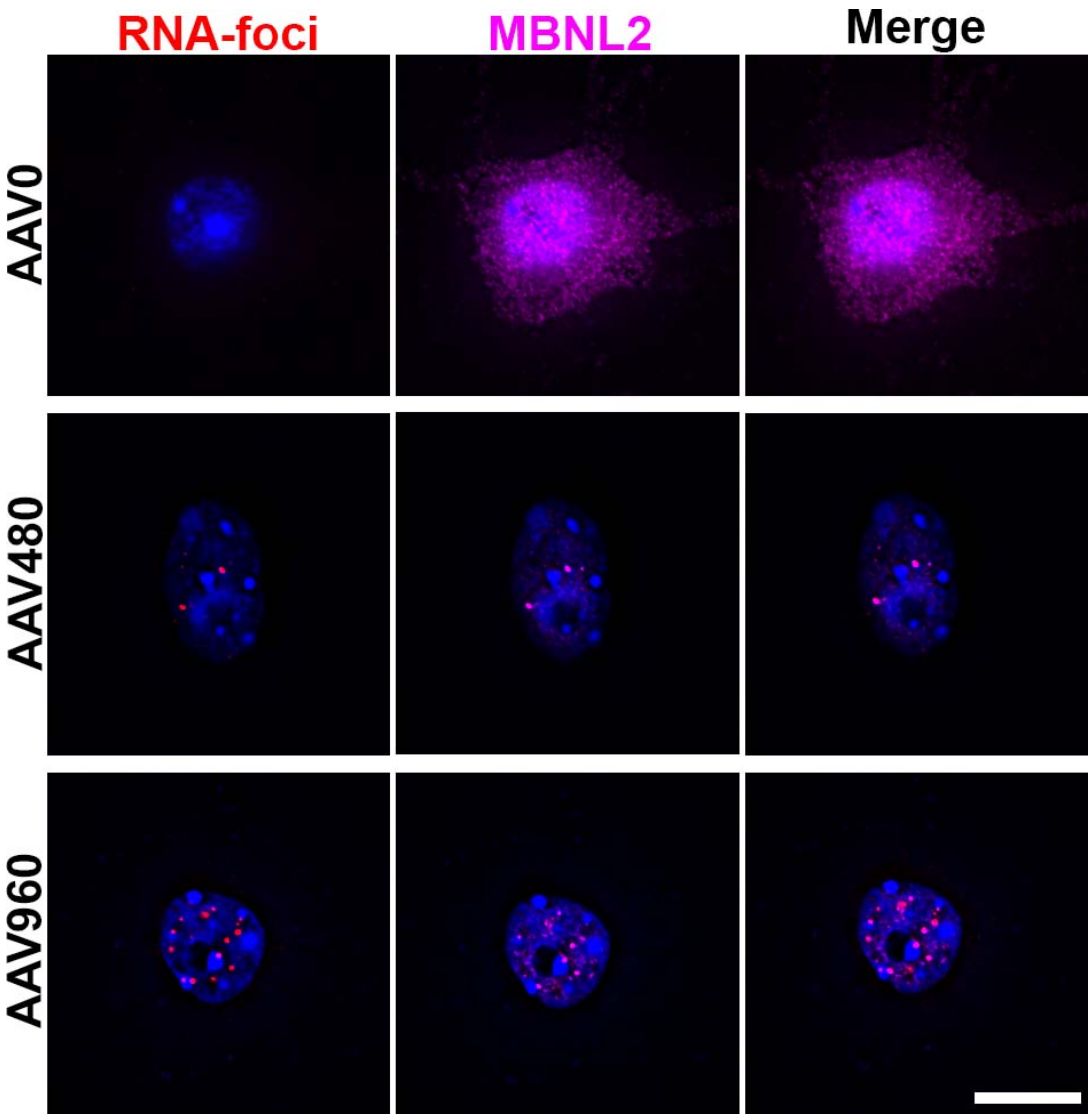


Figure 3.4 RNA foci sequestrate MBNL2 in nucleus of AAV virus transduced neurons. RNA foci are detected by FISH and MBNL2 is detected by IF in DIV 18 cortical neurons transduced by AAV-CTG0, AAV-CTG480, or AAV-CTG960. MBNL2 is sequestered to RNA foci in nucleus of AAV-CTG480 and AAV-CTG960 transduced neurons. Scale bar = 10 μm.

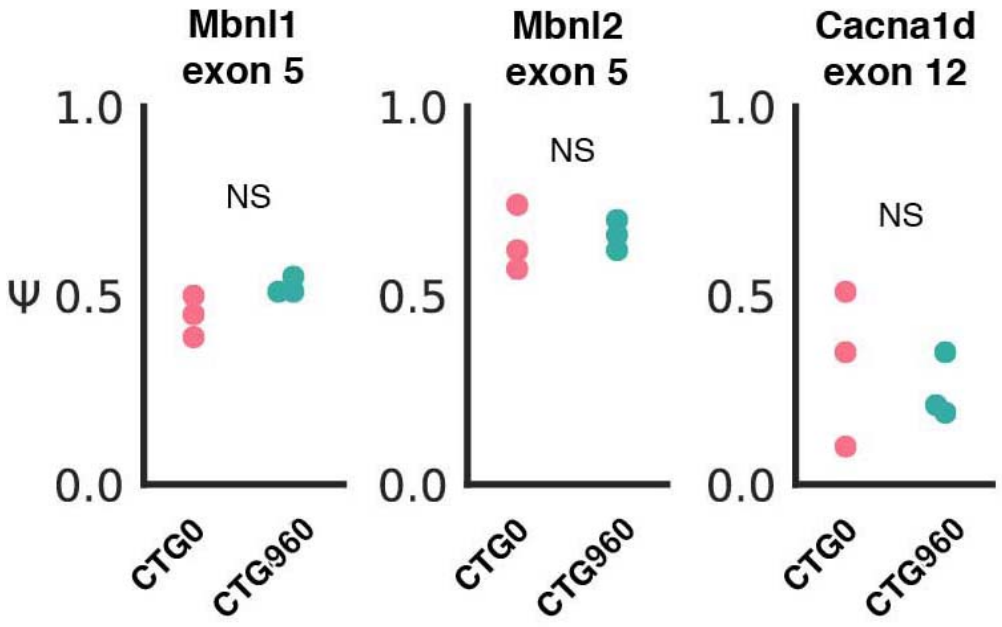


Figure 3.5 No disrupted alternative splicing is detected in DIV 18 AAV-CTG960 transduced neurons. PSI values for 3 selected MBNL-dependent exons are shown, as assessed by RNA-seq for DIV 18 neurons expressing AAV-CTG0 or AAV-CTG960. (Data provided and done by collaboration with Dr. Eric Wang)

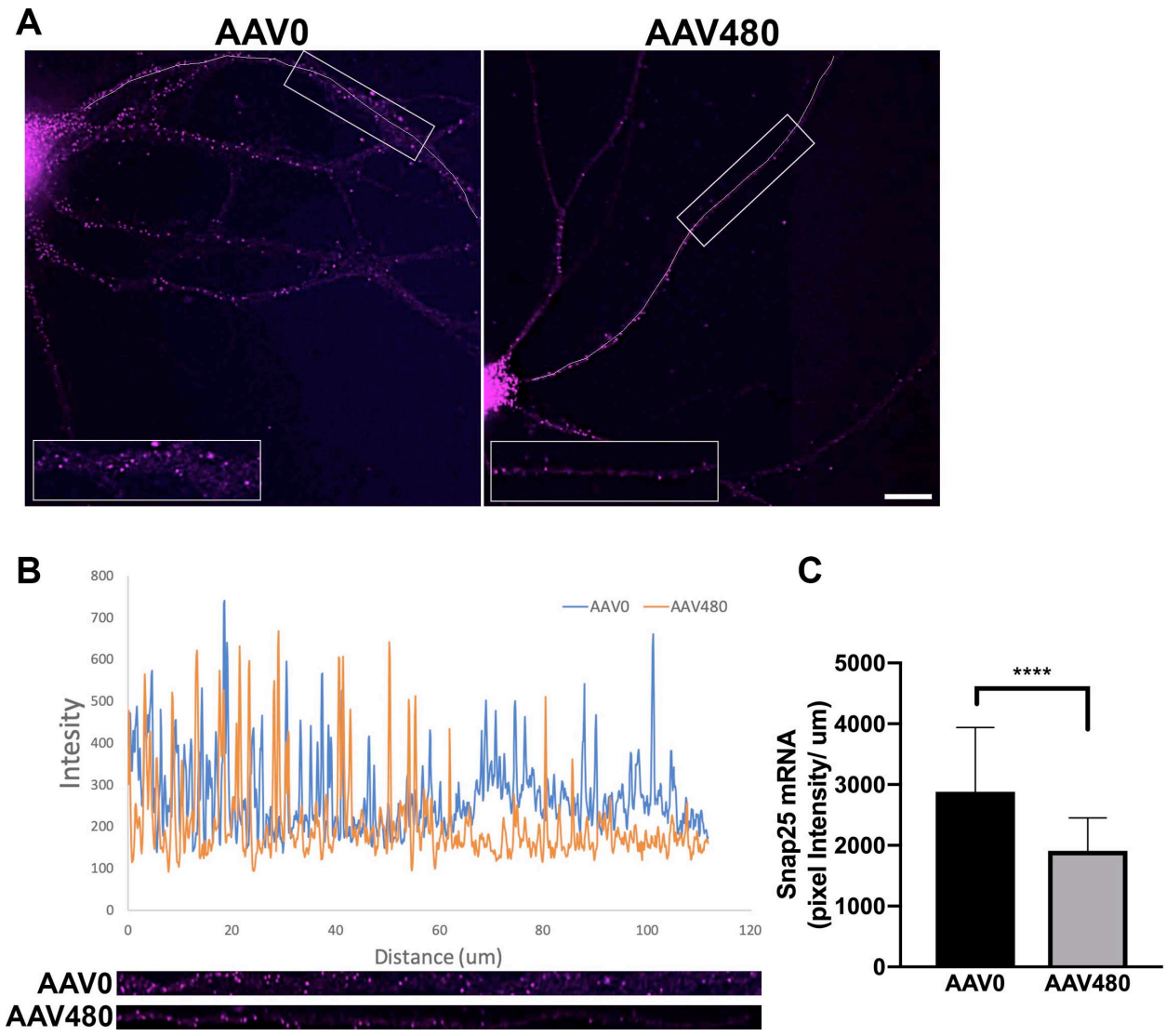


Figure 3.6 *Snap25* mRNA localization is disrupted in the neural process of AAV-CTG480 transduced neurons. A) *Snap25* mRNA as assessed by FISH is depleted from neuronal processes of DIV 18 neurons expressing 480 CTG repeats. B) Tracking of single neural process shows decreased *Snap25* level at distal segment of the process in AAV-CTG480 transduced neurons. C) Statistics of overall *Snap25* level in neural processes. Student's t-test. ****P < 0.0001. Scale bar = 10μm.

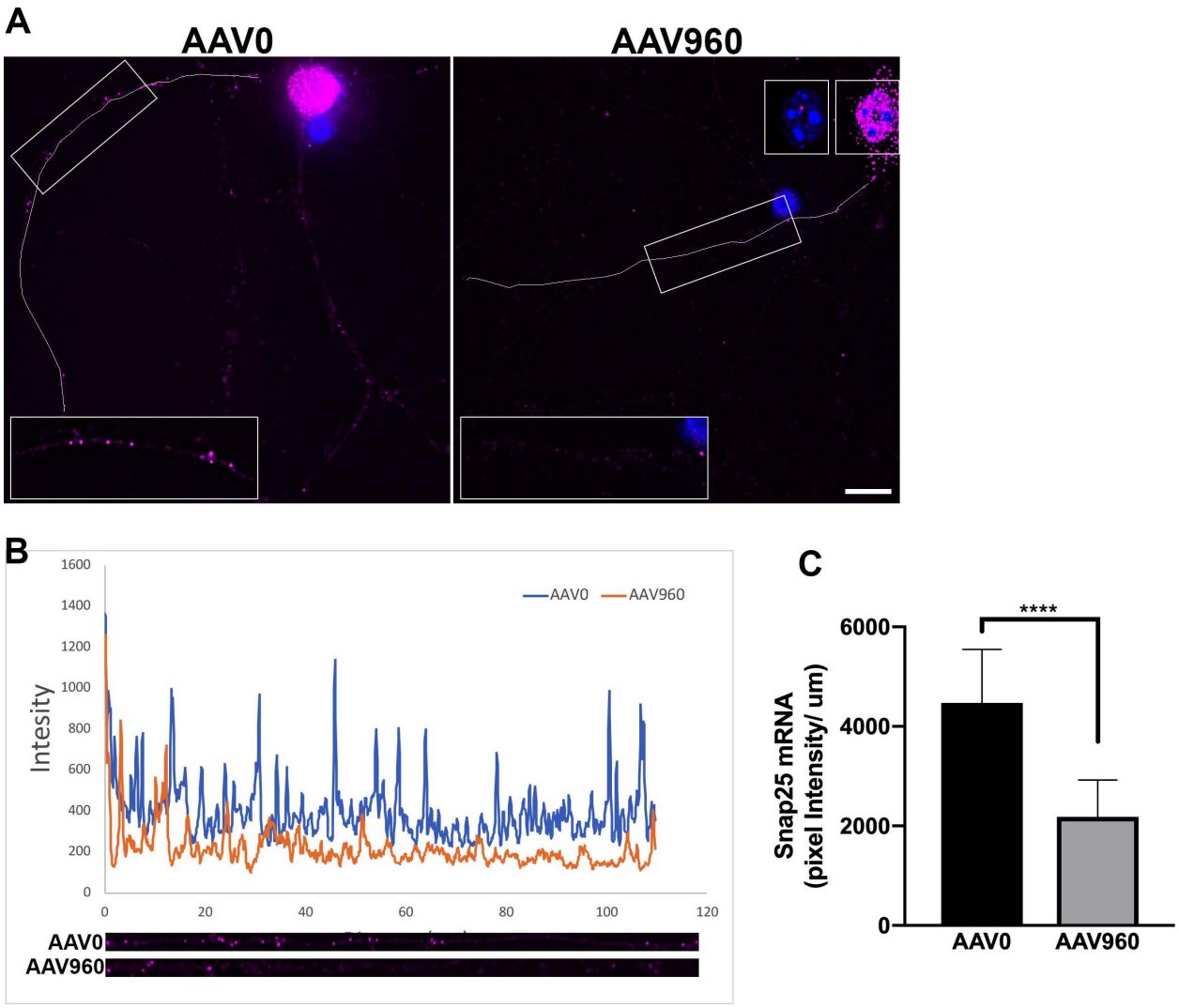
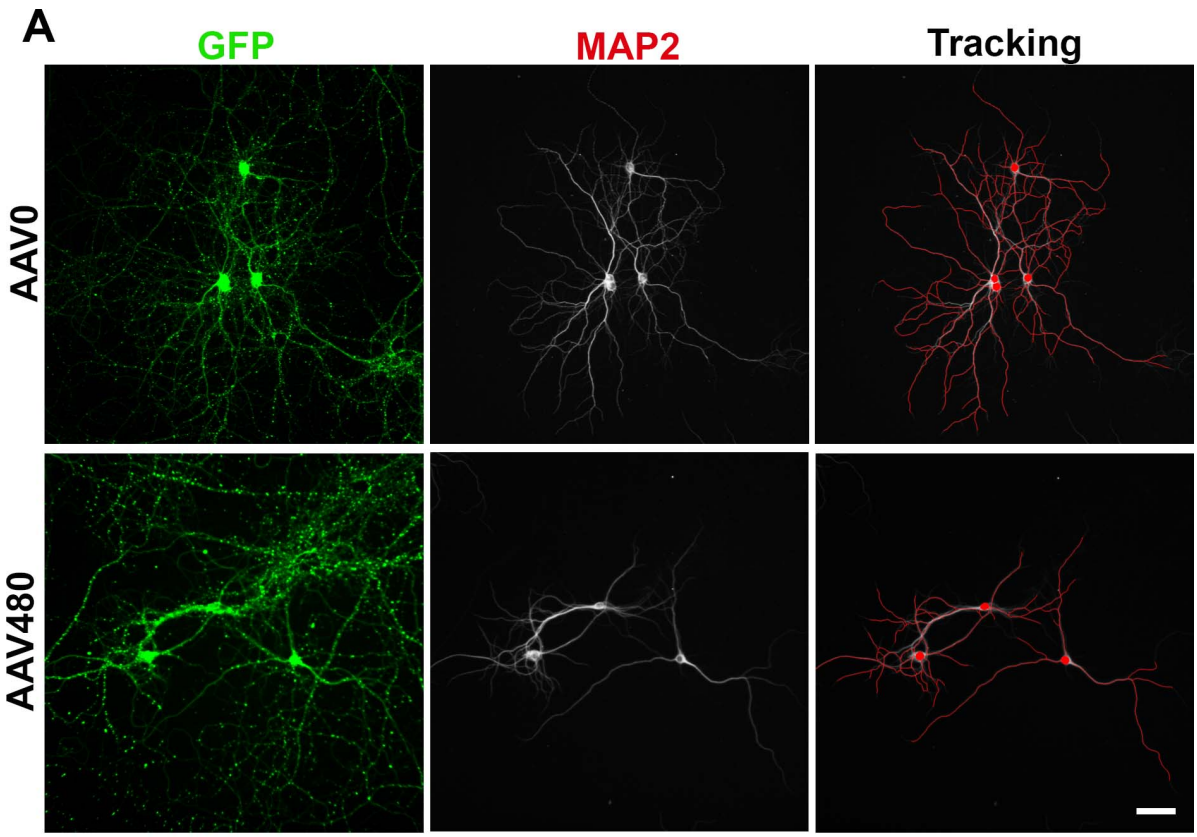
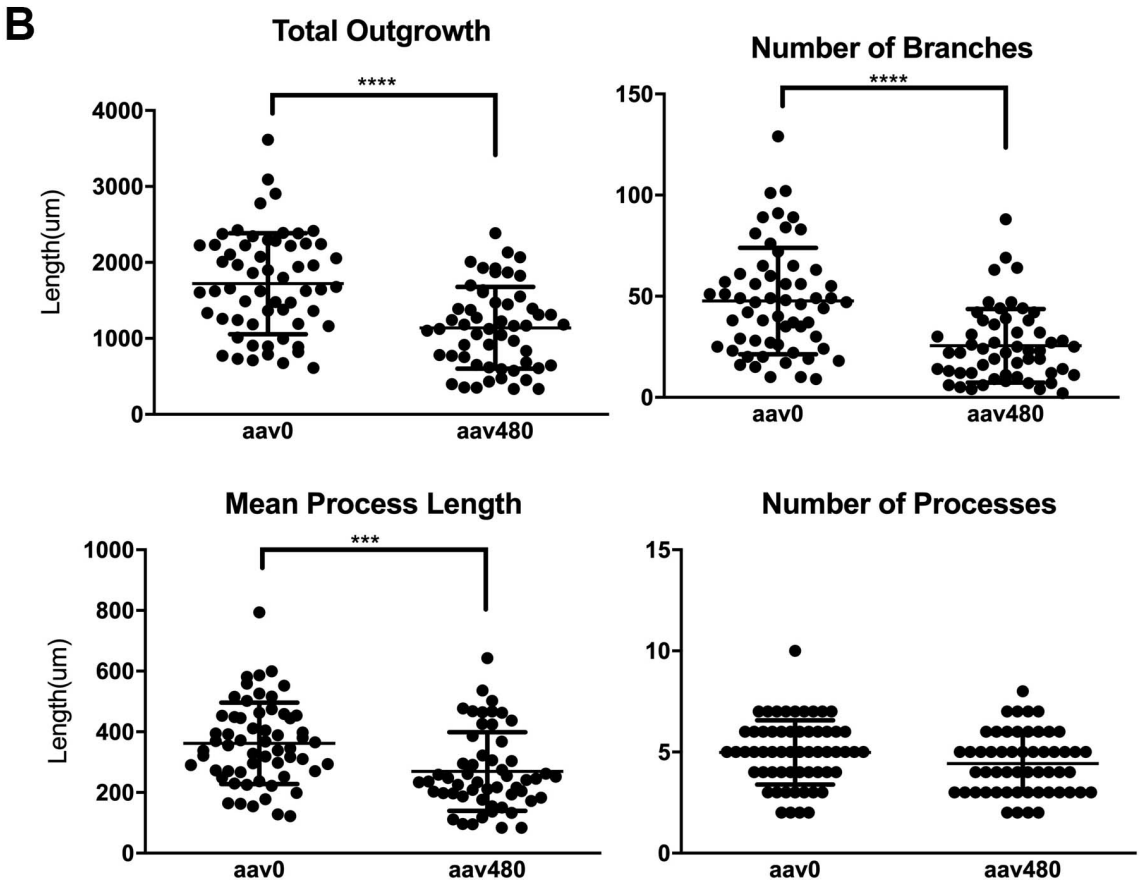


Figure 3.7 *Snap25* mRNA localization is disrupted in the neural process of AAV-CTG960 transduced neurons. A) *Snap25* mRNA as assessed by FISH is depleted from neuronal processes of DIV 18 neurons expressing 960 CTG repeats. RNA foci are detected by FISH. B) Tracking of single neural process shows decreased *Snap25* level at distal segment of the process in AAV-CTG960 transduced neurons. C) Statistics of overall *Snap25* mRNA level in neural processes. Student's t-test. ****P < 0.0001. Scale bar = 10μm.


A
GFP
MAP2
Tracking
AAV0
AAV480


B
Total Outgrowth
****
Length(um)
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3000
2000
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Number of Branches
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aav480
Number of Processes
15
10
5
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aav0
aav480

Figure 3.8 Reduced dendritic arbor complexity in AAV-CTG480 transduced cortical neurons. A) IF of MAP2 on AAV-CTG480 transduced neurons (DIV 18) indicates reduced dendritic arbor complexity. Scale bar = 20μm. B) Statistics indicates reduced total outgrowth of neural processes, number of branches, and mean process length in AAV-CTG480 transduced neurons, but unaffected number of process. Student's t-test. **** $p < 0.0001$, ***$p = 0.0003$. Each data point represents a single neuron. n = 60 - 65 cells per condition from three biological replicates.

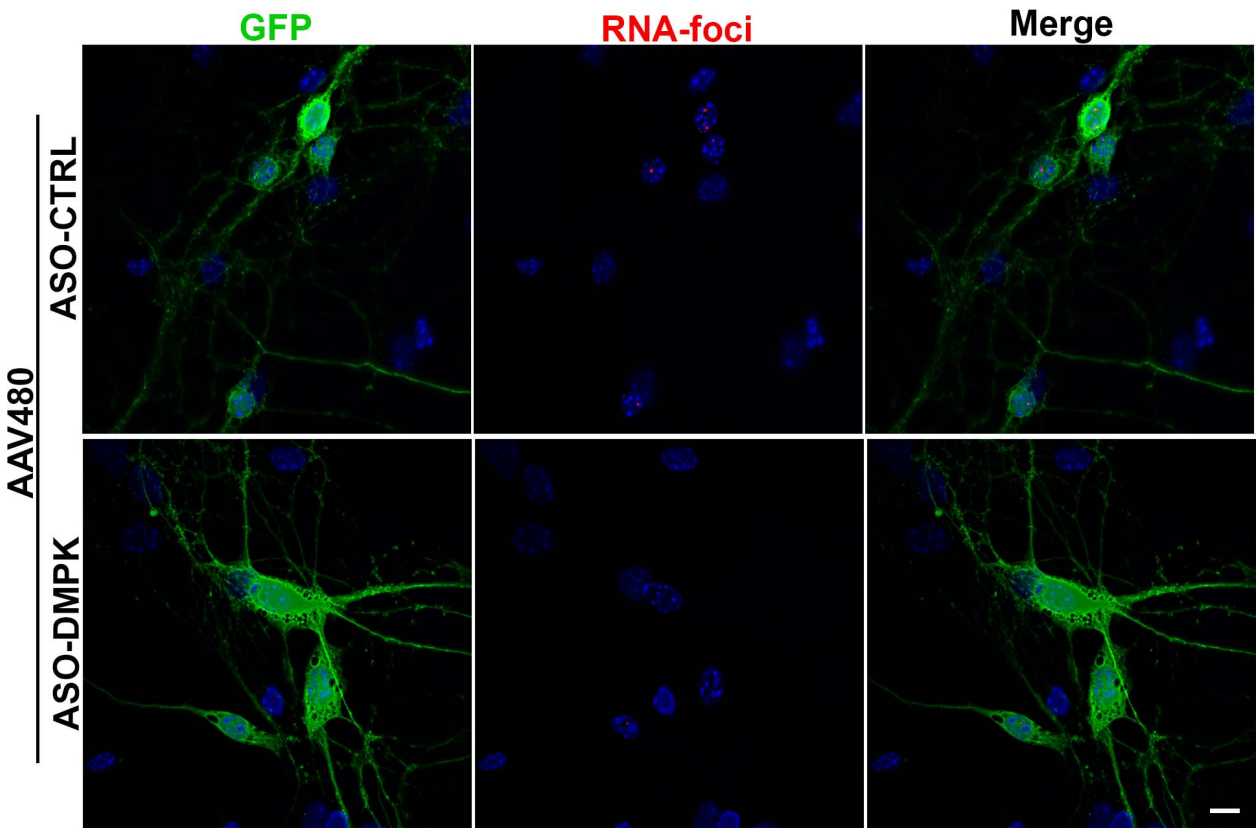
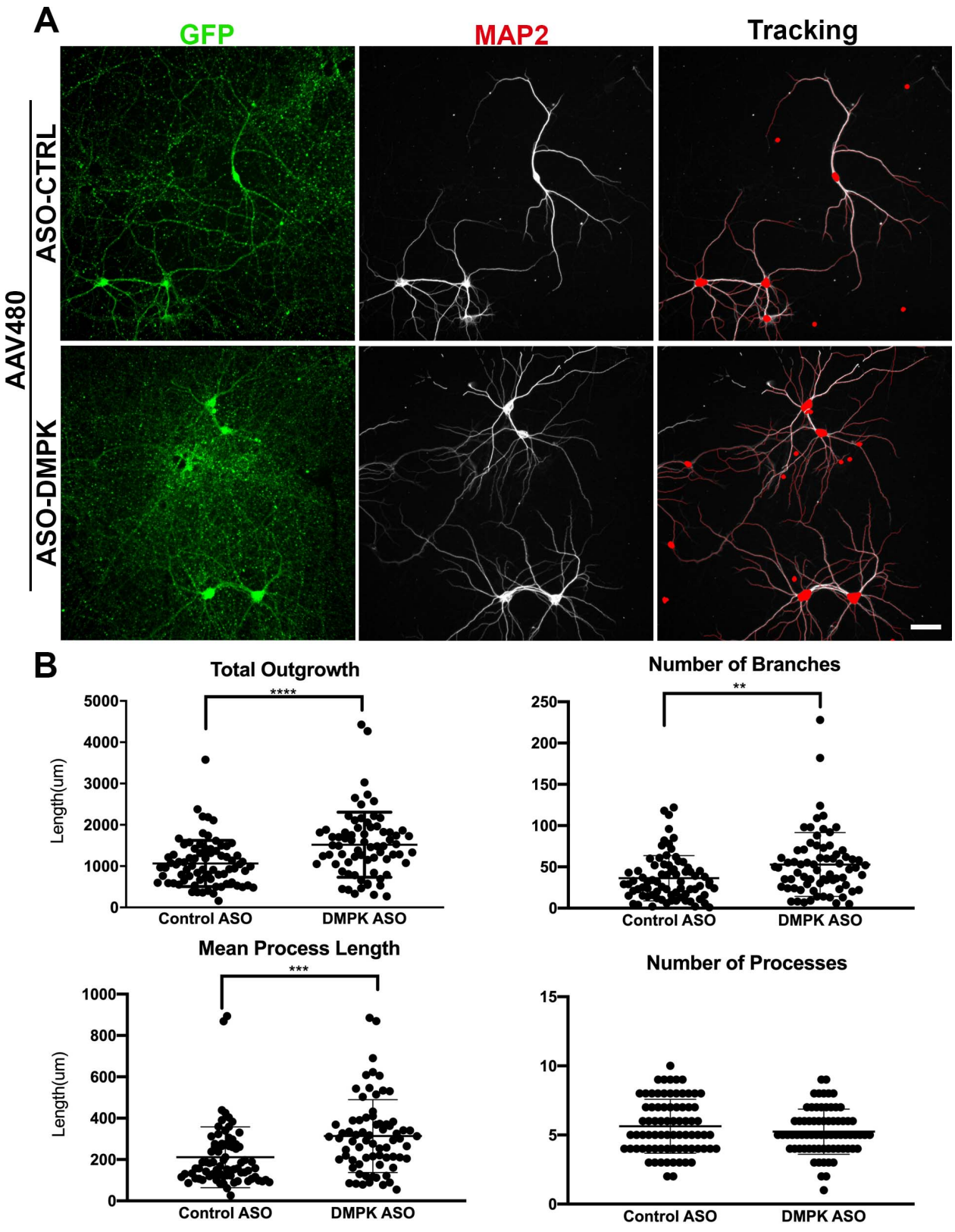


Figure 3.9 ASOs targeting the 3'-UTR of the *DMPK* gene eliminate most of the RNA foci in AAV-CTG480 transduced cortical neurons. ASO treatment at an early timepoint (DIV 8) can prevent nuclear RNA-foci accumulation at DIV18. Scale bar = 10μm.


A
GFP
MAP2
Tracking
AAV480
ASO-CTRL
ASO-DMPK
B
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2000
1000
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Control ASO
DMPK ASO
Number of Branches
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Control ASO
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Mean Process Length
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1000
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400
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Control ASO
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Number of Processes
15
10
5
0
Control ASO
DMPK ASO

Figure 3.10 ASOs targeting the 3'-UTR of the *DMPK* gene rescue the dendritic arbor complexity defect in AAV-CTG480 transduced cortical neurons. A) ASO treatment at DIV 8 increases the dendritic arbor complexity in AAV-CTG480 transduced neurons. Scale bar = 20μm. B) ASO-DMPK treatment can increase total outgrowth of neural processes, number of branches, and mean process length in AAV-CTG480 transduced neurons. Student's t-test. **** $p < 0.0001$, ***$p = 0.0002$, **$p = 0.0029$. Each data point represents a single neuron. n = 65 - 70 cells per condition from three biological replicates.

Chapter 4. Conclusion and Discussion

4.1 Summary

In my thesis project, I successfully developed a novel *in vitro* neuronal model of DM1 using AAV9 with human synapsin promoter. This model express 480 or 960 CTG repeats exclusively in cortical neurons and can reproduce classic DM1 features, including RNA foci formation and MBNL1 and MBNL2 sequestration in nucleus of neurons.

Using this model, I investigated how MBNL sequestration affected its function in RNA alternative splicing and localization during neuronal development. RNA-seq results indicated little to no mis-splicing events in our *in vitro* DM1 model. However, despite no reduction in total levels of *Snap25* mRNA, mRNA levels were reduced in neural processes of AAV-CTG480 and AAV-CTG960 transduced cortical neurons. These findings indicated that the MBNL-dependent RNA localization is disrupted in our *in vitro* model. These discoveries provided evidence suggesting that the MBNL sequestration can induce RNA mis-localization of MBNL target mRNAs in neural processes independent of the disruption of RNA alternative splicing.

Reduced dendritic arbor complexity was detected in our *in vitro* DM1 model. The quantitative dendritic tracing and statistical results showed that the length of total neurite outgrowth, the number of neurite branches, and mean neural process length were decreased in AAV-CTG480 transduced cortical neurons. This dendritic morphological phenotype is consistent with previous research from a transgenic mouse model *in vivo* that expresses 960 CTG (Wang, Lin et al. 2017) and the preliminary data from our lab using CMV promoter to express CTG repeats (Figure 1.4). It will be interesting to analyze whether axonal morphology and outgrowth is also affected, and whether synapse density is compromised.

The dendritic defects in our *in vitro* model of DM1 were rescued by the treatment of IONIS ASOs targeting the 3'-UTR of exon 15 of the human *DMPK* gene sequence encoded by our AAV vector.

Most of the RNA foci in the AAV-CTG480 transduced cortical neurons could be eliminated by 10 days of ASO treatment (from DIV 8 to DIV 18). Along with the elimination of RNA foci, the ASO treatment also increased the dendritic arbor complexity in those neurons. These results suggest that the dendritic defects in our DM1 model are caused by RNA foci and can be effectively rescued by ASO treatment, likely due to liberation of MBNLs.

Taken together, my results suggest that MBNL sequestration by RNA foci can impair neuronal development and the mis-localization of MBNL target mRNAs, especially *Snap25*, is a potential contributing mechanism. My results provide evidence suggesting that RNA mis-localization is independent of RNA mis-splicing at the early neurodevelopmental stage of DM1. I first detected that *Snap25* mRNA is mis-localized in neural processes in DM1 cellular model. If future studies (discussed below) show that Snap25 protein localization and function is altered at synapses, this would imply that *Snap25* may be a potential therapeutic target of DM1. It will be interesting if future work in this *in vitro* model shows that ASO treatment can rescue mRNA localization and restore synaptic function. Such results have implications for identifying assays to test in future preclinical development of novel antisense medicine for potential use in human DM1.

4.2 Advantage of our *in vitro* model of DM1

Our *in vitro* model of DM1 is a convenient and powerful tool for studying neuronal deficits and related molecular mechanisms in DM1. Most of the existing neuronal DM1 models are derived from hESCs or iPSCs. The differentiation processes of those models are time consuming and the maintenance costs are relatively high. To establish our DM1 model, I dissected cerebral cortices from mouse embryos, dissociated and cultured cortical neurons, and applied AAV9 directly to the

neurons. The whole establishment process is inexpensive and only takes about two to three weeks, which makes our DM1 model more convenient and practical compared with other neuronal models. Our *in vitro* model is effective as a neuronal DM1 model. The AAV9 with human synapsin promoter can transduce cortical neurons exclusively. Over 80% of AAV-CTG480 transduced neurons can form RNA foci in nuclei, which sequestrate both MBNL1 and MBNL2. The MBNL sequestration impaired the localization of *Snap25* mRNA and induced dendritic defects. Therefore, our model can reproduce classic DM1 neuronal features due to sequestration of MBNLs.

Our *in vitro* model of DM1 can be used to study DM1 features and molecular mechanism at early stage. In my project, I used this model to study the MBNL-regulated RNA alternative splicing and RNA localization. Because cultured neurons are at early neural developmental stage and do not express all the factors for MBNL-dependent splicing, I can study MBNL-regulated RNA localization independent of mis-splicing. This *in vitro* neuronal model of DM1 can also be used as a supplement of *in vivo* mouse models to test hypothesis and study molecular mechanisms.

4.3 MBNL-regulated RNA localization in DM1

As shown by FISH results, *Snap25* mRNA level in neuronal processes of AAV-CTG480 or AAV-CTG960 transduced cortical neurons is decreased. On the other hand, the RNA-seq data in my project indicated that MBNL-regulated RNA alternative splicing is relatively normal in our DM1 model, including normal splicing of *Snap25*. These results provided direct evidence showing that MBNL-dependent RNA localization can regulate neural development independent of alternative splicing at early stages, which is consistent with the results of previous *in vivo* research that showed dendritic defects yet lack of mis-splicing in brain at early postnatal stages (Wang, Lin et al. 2017). Moreover, these investigators showed loss of MBNL from dendrites that correlated with dendritic

morphologic defects. It will be interesting if future work can determine a role for mRNA mis-localization in this transgenic mouse model *in vivo*.

As a component of SNARE complex, SNAP-25 participates in both presynaptic and postsynaptic functions in neurons. For example, in pre-synapse, SNAP-25 is important for neurites development, the synaptic vesicle exocytosis during synaptic transmission, presynaptic calcium channels activities, and the formation of presynaptic terminals. In post-synapse, SNAP-25 can regulate postsynaptic proteins trafficking, NMDAR mediated synaptic plasticity, and maturation of dendritic spines. The mis-localization of *Snap25* mRNA can disrupt the local translation of SNAP-25 in neuronal processes and affect various synaptic functions. In cultured neurons, axonal localization and translational of *Snap25* mRNA was shown to be dependent on the 3'UTR and necessary for SNARE complex assembly and presynaptic function (Batista, Martinez et al. 2017). Our results suggest a potential role for MBNL binding. Future work could, for example, make a mutant mouse that lacked MBNL binding motifs in *Snap25* 3'UTR. I would predict loss of SNAP-25 protein enrichment at synapses and impaired synaptic function. It is relevant that MBNL2 knockout mice have impaired paired pulse facilitation the formation of pre-synapses, which suggests impaired presynaptic vesicle release (Hernandez-Hernandez, Guiraud-Dogan et al. 2013). Mis-localization of *Snap25* could be investigated as a contributing mechanism. Future work is needed to understand how *Snap25* mRNA mis-localization may contribute to the reduced dendritic arbor complexity in our DM1 model. Further study is necessary to provide evidence that will prove a direct connection between *Snap25* mis-localization and dendritic defects in DM1. One possibility is that the dendritic defects may be secondary to impaired synapse innervation.

Besides the neurite development, it is intriguing to investigate whether other SNAP-25 dependent intracellular functions are disrupted in DM1. For example, SNAP-25 can regulate NMDAR

dependent synaptic plasticity. Inhibition of SNAP-25 function can reduce NMDAR level at synapses and impair LTP induction (Jurado, Goswami et al. 2013). It will be interesting to study the mechanism for impaired NMDAR's function and related synaptic transmission in DM1. Moreover, it has been shown that RNAs of three major subunits of the NMDAR, GRIN1, GRIN2A, and GRIN2B, are the targets of MBNL (Wang, Cody et al. 2012). Reduced NMDAR-mediated excitatory postsynaptic current (EPSC) and impaired LTP in CA1 hippocampal neurons were detected in MBNL2 knockout mice (Charizanis, Lee et al. 2012). In EpA960/CaMKII-Cre DM1 mouse model, hippocampal neurons showed reduced EPSC but no mis-splicing of GRIN1 exon 5 (Wang, Lin et al. 2017). DMSXL transgenic mice expressing expanded CTG repeats also have reduced EPSC (Hernandez-Hernandez, Guiraud-Dogan et al. 2013), although the potential role for mis-splicing or localization has not been studied. It will be interesting to investigate whether MBNL is involved in NMDAR activity and synaptic plasticity through directly regulating the localization of RNAs of NMDAR components or indirectly through regulating *Snap25* mRNA localization.

Beyond *Snap25* and RNAs of NMDAR subunits, MBNL is hypothesized to regulate the localization of many other target RNAs (Figure 4.1). Recent work from Erin Schuman's lab has used RNA-seq and bioinformatics to show that mRNAs localized to neuropil in the hippocampus *in vivo* are enriched for MBNL binding motifs in 3'UTRs of localized RNAs (Tushev, Glock et al. 2018). It has been reported that MBNL is involved in the localization of RNAs encoding proteins within synaptophysin (Syp), synaptotagmin (Syt1, Syt12) and synaptobrevin (vesicle-associated membrane protein 5, VAMP5) families. Furthermore, MBNL depletion in cortical neurons *in vitro* disrupted the localization of complexin-2 (Cplx2) mRNA, which produces a syntaxin associated protein binding to the SNARE complex (Taliaferro, Vidaki et al. 2016). In post-synapse, MBNL

may regulate the localization of the mRNA of ECM-interacting membrane protein integrin β1 (Itgb1), which is important for dendritic and synaptic development (Kerrisk, Greer et al. 2013). MBNL can also target RNAs encoding signaling proteins like Camk2α and secreted ECM proteins such as collagens (Wang, Cody et al. 2012). Therefore, MBNL sequestration by RNA foci in DM1 could induce mis-localization of many different RNAs disrupting relative cellular functions, such as neurotransmitter releasing. Our *in vitro* model of DM1 can be used in future studies about the mis-localization of MBNL target RNAs and related DM1 features.

4.4 ASO treatment

I treated AAV-CTG480 transduced cortical neurons with Ionis ASOs at DIV 8 and detected that ASOs can effectively eliminate RNA foci and rescue the dendritic defects in our *in vitro* model. My preliminary data showed that human synapsin promoter in our constructs began to drive the expression of CTG repeats in cortical neurons around DIV 7. Thus, my results suggest that ASO treatment can prevent the dendritic defects of DM1 when applied at early stage when RNA foci start to form. It will be interesting to test if ASOs can rescue DM1 features at late stage of the disease.

Although ASO treatment can effectively reduce RNA foci and rescue DM1 features in both *in vitro* and *in vivo* DM1 models, there are unsolved problems for using ASO as a treatment for DM1 patient. One of the most critical problems is the delivery efficiency of ASOs in patients. Future studies are necessary to investigate how different ASO modifications and delivery methods can improve the delivery efficiency.

4.5 Future works

As discussed above, it will be worthwhile to identify the role of other MBNL target mRNAs to mediate dendritic defects and disruption of other neuronal functions in our DM1 model in future research. Also, our lab has expressed CTG repeats in mouse brain using the same AAV virus I used to produce the *in vitro* DM1 model. Preliminary data in our lab indicated that these mice with CTG repeats exhibit disease-relevant behavioral phenotypes, such as impaired consolidation of contextual fear memory, known to be dependent on new protein synthesis. In future studies, we can test and characterize the neurophysiological and behavioral phenotypes in these mice that would be consistent with impaired regulation of the synaptic protein composition.

ASO treatment at DIV 8 can eliminate RNA foci and prevent dendritic defects in our DM1 model. In the future, we can apply ASOs around DIV 14 in our DM1 model to investigate whether the dendritic defects can be rescued. Besides the dendritic defects, we can also investigate the effect of ASOs in rescuing the mis-localization of *Snap25* and other MBNL target RNAs in our *in vitro* model of DM1. We can apply ASOs in our DM1 mouse model and investigate whether ASO treatment can rescue physiology and behavior phenotypes *in vivo*.

My results suggest that MBNL can regulate the localization of *Snap25* in neuronal processes. However, the detailed mechanisms of how MBNL regulates RNA localization remain unclear. MBNL may facilitate directed transportation of RNA via cytoskeleton or through simple diffusion. Preliminary data from our lab detected anterograde and retrograde MBNL-containing ribonucleoprotein complexes (RNPs) transportations similar to microtubule-mediated cargo transport. We also detected that the kinesin superfamily proteins KIF1Bα and KIF5A can recruit MBNL1 with different affinities. It will be interesting to investigate the role of KIF1Bα and KIF5A in regulating MBNL-dependent RNA localization in future. Lastly, we will need to investigate to

what extent the phenotypes in our AAV model is entirely due to MBNL sequestration. Perhaps other splicing factors or RNA binding proteins are also sequestered. It does appear that MBNL is the major sequestered factor. I had sought to try to rescue phenotypes in this model of AAV-CTG expression by overexpression of MBNL, but there were technical issues with the constructs. It will be important for future work to show that the phenotypes can be rescued by MBNL. Perhaps we can design MBNL mutants that are selectively impaired in splicing versus localization, which will allow further dissection of the dual roles of MBNLs and their contribution to DM1. Validation of the proposed model that early neurodevelopmental phenotypes (impaired synapse assembly) are due to RNA mis-localization whereas later neurodegeneration is caused by mis-splicing (synapse loss) awaits further study. Nonetheless, my thesis has provided the motive, method and rationale to explore mRNA mis-localization as a contributing factor to mediate neurodevelopmental impairments in DM1.

4.6 Figure

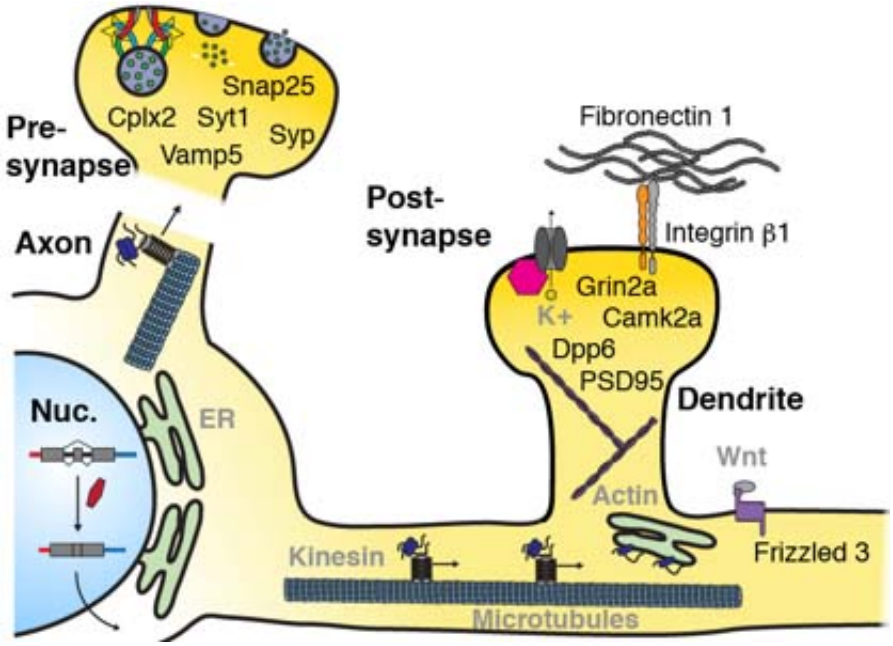

Figure 4.1 Schematic of RNAs bound by MBNL at pre and the post-synapse. In pre-synapse, MBNL regulates the localization of mRNAs including Snap25, Syt1, VAMP5, and complexin-2 (Cplx2). In post-synapse, MBNL can regulate RNAs of NMDAR subunits. Local translation of these and other MBNL targets is hypothesized to contribute to synaptic function, which is impaired in DM1 due to nuclear sequestration of MBNLs.

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