FMRP AND MOV10 REGULATE LOCAL DICER1 EXPRESSION AND DENDRITE DEVELOPMENT

BY

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DISSERTATION

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Abstract

RNA binding proteins (RBPs) are involved in all aspects of the RNA life cycle and constitute a critical component of maintaining proper transcription and translation. RBPs act as mediators of the critical intermediate between DNA and proteins, messenger RNA (mRNA), which is required for cell survival and growth. RBPs form ribonucleoprotein (RNP) complexes with their target mRNAs. Over 500 genes encode RBPs in human DNA, and although RBPs have a crucial role in post-translational regulation of gene expression, few have been studied systematically. In the nervous system, RBPs and their associated mRNAs, play a key role in normal neuronal development and function and in neurological disease. Fragile X syndrome, a cognitive impairment disorder, results from the loss of expression of the Fragile X Mental Retardation Protein (FMRP). FMRP associates with the RNA helicase Moloney Leukemia Virus 10 (Mov10) in brain and modulates its translational activity through the microRNA (miRNA) pathway. We previously showed that MOV10 is important in developing and maintaining normal brain activity using both murine and *Xenopus* models. The deletion of Mov10 in Neuro2a cells caused abnormally decreased neurite outgrowth on differentiation which was restored upon exogenous expression of MOV10. Furthermore, culturing and staining of hippocampal neurons from MOV10 Heterozygotes (Het) confirmed these results showing markedly short dendrites as seen in the Mov10 knockout Neuro2a cells suggesting impaired neuronal function (Skariah et al., 2017). We were thus interested in investigating the consequences of *Mov10 and Fmr1* reduction on dendritic development. *Mov10* Het and *Fmr1* KO neurons possess an abnormal morphology compared to WT neurons at day in vitro 14

(DIV14). Additionally, *Mov10* Het mice have reduced density of immature dendritic spines compared to WT and a smaller soma. The impaired neurite phenotype, spine maturation and reduced soma size have previously all been found to be associated with impaired miRNA biogenesis, and since MOV10 and FMRP are involved in regulation of the miRNA pathway, we sought to determine whether misregulation of the pathway was contributing to the abnormal neuronal phenotypes we observed in culture. We found a global reduction of Argonaute-2 (AGO2) – associated miRNAs in the absence of FMRP. This is important because AGO2 is the main effector of miRNA-mediated regulation. Furthermore, another component of the miRNA pathway, DICER, a ribonuclease which regulates biogenesis of miRNA and small interfering RNA (siRNA), was significantly decreased in the absence of MOV10 and FMRP. Through a series of knockdown and luciferase reporter experiments, we determined that MOV10 and FMRP modulate expression of the *Dicer1* mRNA via the 3 ’untranslated region (UTR). Overexpression (OE) of MYC-*Dicer1* rescues the impaired neuronal phenotype in Mov10 Het neurons suggesting a mechanism for regulating local DICER expression when MOV10 and FMRP are present. Our work represents a new understanding of how FMRP and MOV10 regulate cobound mRNAs and neuronal development.

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

RNA binding proteins play a major role in proper development and function of the nervous system. RNA binding proteins like FMRP dynamically control protein translation by facilitating mRNA localization, by being locally translated and degraded by stimulation and finally by modulating mRNA stability and translation through the miRNA pathway. Although studies of single RBPs like FMRP can yield insights into cognition, learning, and memory, it has become clear that RBPs seldom act alone, therefore, it is not sufficient to unravel the individual contribution of a single RBP and its consequences but rather to study and understand the tight interplay between different RBPs.

RBPs are typically present within complexes called messenger RNPs (mRNPs) at the synapse and regulate the synaptic mRNAs within this complex in a dynamic activity-dependent manner. A single mRNA in an mRNP can be bound by hundreds of RBPs simultaneously, therefore the expression dynamics of RBPs in post-transcriptional regulatory networks has direct implications in balancing brain function and neurodegenerative disease at both a local and global level. For protein synthesis at the synapse to take place there needs to be a system for organization, delivery and accurate regulation of the many factors involved: from localization of mRNAs, recruitment of RNA-protein-containing granules, accessory RNA binding proteins,

polysome formation and miRNA-mediated regulation. In the brain, one of the most important processes RBPs are involved in is regulation of miRNA expression.

1.1 miRNA biogenesis and mRNA expression

Small ncRNAs represent an important subset of non-coding RNA transcripts. First discovered in 1993, they are loosely defined by their small size (typically less than ~30 nucleotides), association with members of the Argonaute (AGO) family of proteins and their ability to regulate gene expression (Lee et al., 1993). miRNAs are a type of small non-coding RNA transcript that is now estimated to regulate over 50% of all human genes (Friedman et al., 2008).

Once fully processed, miRNAs are typically between 22-26 nucleotides. They form canonically when the primary transcript of a miRNA gene (pri-miRNA), a larger RNA precursor, is cleaved in the nucleus by the microprocessing complex DROSHA/DGCR8 into an approximately 70 nucleotide local hairpin structure called a precursor-miRNA (pre-miRNA) (Lee et al., 2002; Lee et al., 2003; Denli et al., 2004; Gregory et al., 2004; Han, 2004). After this cleavage, pre-miRNAs are exported into the cytoplasm and further processed by the RNAse III DICER to generate the final length miRNA, which is bound by AGO2 (Lee et al., 2002; Chendrimada et al., 2005; Lee et al., 2006).

miRNAs regulate gene expression through binding of the seed sequence to the complementary target sequence in the mRNA called the miRNA Recognition Element (MRE). MREs are primarily in the 3 ’UTR but have also been reported in 5’UTRs and in coding regions (reviewed in Brodersen and Voinnet, 2009). In *Drosophila* and many

plants, miRNA base pairing to target mRNAs is perfectly complementary whereas in other eukaryotes, the presence of a shorter seed sequence still allows for mRNA regulation upon miRNA binding (Meister and Tuschl, 2004; Bartel, 2009). Furthermore, although miRNAs can bind to any portion of the mRNA sequence that possesses a degree of complementarity, mRNAs with longer 3 ’UTRs have been shown to contain a greater number of miRNA binding sites, and therefore have a higher potential for post-transcriptional regulation (Keebara et al., 2009; Fang et al., 2011; Brummer and Hauser, 2014).

The most common mode of regulation is at the level of transcript stability when complete base pairing between the miRNA and its target mRNA leads to cleavage of the transcript by AGO2. The mRNA transcript itself can be degraded at differential rates, thereby reducing the amount of template available for protein translation (Hutvagner, 2002; Bagga et al., 2005; Nottrodd et al., 2006). In the case of incomplete base pairing, translational suppression can take place as an mRNA is transported to a distinct location in the cell and not be relieved until a particular signal such as synapse stimulation is received, resulting in local protein synthesis. (Okamura, 2004; Miyoshi et al., 2009; Pratt and Macrae 2009; Mikl et al., 2010; Muddashetty et al., 2011).

1.2 miRNA biogenesis factors

The importance of the miRNA pathway is underscored through the characterization of the knockout mice made of the individual components. Ablation of DGCR8, part of the nuclear microprocessor complex which generates pre-miRNAs (Fig. 1), results in embryonic lethality at E6.5 (Wang et al., 2010), establishing the importance of miRNA

function in normal development. DROSHA, the ribonuclease III that forms the other half of the microprocessing complex, also leads to embryonic lethality when ablated in mice (Fan et al., 2013). DICER deficient mice are embryonic lethal at day E8.5 (Krill et al., 2013). Functional DICER is also crucial for maintaining neuronal integrity, as absence of DICER in brain leads to neurodegeneration, most notably cell shrinkage (Hebert et al., 2010).

Although AGO2 is one of four main Argonaute proteins in cells, when individual Argonautes are knocked out in mice, only loss of AGO2 causes embryonic lethality (Liu et al., 2004). The other AGO proteins are dispensable for animal development once again highlighting the importance of proper miRNA regulation of target mRNAs in embryonic tissue and organ development (Liu et al., 2004; Morita et al., 2007). AGO2 is also the only family member with mRNA slicing activity (Liu et al., 2004; Meister et al., 2004) making it the main effector of miRNA-mediated regulation.

The embryonic lethal phenotypes due to loss of miRNA biogenesis and effector components are not limited to this core group of proteins. Genetic studies have demonstrated that alterations in the levels of RISC accessory proteins and in the miRNAs, themselves can lead to a wide variety of aberrant phenotypes both during development and postembryonically (Schratt et al., 2006; Bicker and Schratt, 2008, Edbauer et al., 2010; Skariah et al., 2017). One example of this is AGO2-associated protein MOV10. Although more than one type of helicase can be found in the brain, MOV10 is particularly highly expressed embryonically and during early postnatal stages in mice, decreasing around P14, with a further drop in adulthood (Skariah et al., 2017). MOV10 ablation results in embryonic lethality before E9.5 (Skariah et al., 2017), and in

Xenopus embryos it has been shown to be essential for completion of gastrulation (Skariah et al., 2018).

1.3 Role of RNA binding proteins in miRNA-mediated regulation

The main mechanism of regulation of miRNAs takes place at the level of biogenesis by altering the rates of cleavage or downstream processing, as well as through alterations to the downstream functions of miRNAs. In addition, a growing body of research suggests that one of the keyways that the mRNA-miRNA interaction is regulated is through association of RNA binding proteins (RBPs) with the mRNA in the proximity of the MRE (Connerty et al., 2015; Loffreda et al., 2015; Kenny and Ceman, 2016).

Hundreds of RBPs have been discovered to play a key role in post-transcriptional regulation (Mata et al., 2005; Keene, 2007; Glisovic et al., 2008; Collins and Penny, 2009) due to their unique ability to interact with RNA while forming protein-protein interactions with key players in the cell such as AGO2. RBPs are generally conserved between yeast and humans (Gerstberger et al., 2014; Beckman et al., 2015) yet can be functionally diverse and while many do not possess canonical RNA binding sequences, they still exert a great deal of control over their targets. We will focus now on RBPs that facilitate miRNA-mediated regulation.

1.4 FMRP

One RNA binding protein that has emerged as a key interactor with the miRNA-mediated silencing pathway is the Fragile X Mental Retardation Protein (FMRP)

(Ashley et al., 1993). FMRP is the protein product of the *FMR1* gene, whose loss leads to Fragile X syndrome (FXS), a triplet-repeat expansion disease which is the leading cause of inherited cognitive impairment affecting 1:4000 males and 1:8000 females (Rajaratnam et al., 2017). Loss of FMRP leads to defects in synaptic plasticity and cognition (Bolduc et al., 2008; Kelleher and Bear, 2008; Liu-Yesucevitz et al., 2010).

FMRP is primarily a cytoplasmic protein although it does possess a nuclear localization signal (NLS) (Devys et al., 1993; Feng et al., 1997; Willemsen et al., 2004; Vanderklish and Edelman, 2005; Kim et al., 2009). In mammals FMRP possesses conserved functional domains, three of which are RNA binding domains (Siomi et al., 1993). Two of the RNA-binding motifs are homology to hnRNP K (KH) domains and the third is an arginine-glycine-glycine (RGG) box, which is thought to bind G-rich secondary structures, such as G-Quadruplexes (GQs) (Darnell et al., 2001; Schaeffer et al., 2001; Phan et al., 2011). FMRP primarily binds within the coding sequence of its target mRNAs (Darnell et al., 2011) although it has also been shown to associate with the 3'UTR (Ashley, 1993, Caudy, 2002; Ascano et al., 2012; Kenny et al., 2014).

Over 800 mRNA targets of FMRP have been identified, primarily in brain (Brown et al., 2001; Miyashiro et al., 2003, Darnell et al., 2011). FMRP functions in translational regulation and silencing of its mRNA targets, although recent research has shown in a small subset of mRNAs, along with other RNA binding proteins, FMRP promotes the opposite effect, leading to enhanced expression (Kenny et al., 2014).

FMRP was first implicated in miRNA-mediated regulation of transcript expression in two independent studies using its *Drosophila* homolog *dFMR1* (Caudy, 2002; Ishizuka et al., 2002). dFMR1 bound and repressed translation of the mRNA

encoding FUTSCH (mammalian MAP1b) at the neuromuscular junction (NMJ) (Zhang et al., 2001; Ishizuka et al., 2002). FMRP is believed to bind *Map1b* in a G-quartet-dependent manner (Brown et al., 2001; Darnell et al. 2001) and reduce the solubility and mobility of the mRNA in association with other RNA binding proteins like TAR DNA-binding protein 43 (TDP-43), keeping it in a translationally suppressed RNP until signal-induced polysome association takes place (Coyne et al., 2015).

Furthermore, in these early studies dFMR1 was found in a complex with ribosomal proteins, DICER, AGO2 as well as miRNAs in vivo, which was later confirmed in mammalian cells (Jin et al., 2004).

1.5 MOV10

The RNA helicase MOV10 was first implicated in the miRNA pathway in a mass spectrometry screen to identify proteins associated with AGO2. Importantly, knockdown of MOV10 eliminated miRNA-mediated suppression of a reporter construct, suggesting that this helicase played an important role in the miRNA pathway (Meister, 2005).

MOV10 possesses 5’ to 3’ helicase activity (Gregersen et al., 2014). The majority of MOV10 is localized to the cytoplasm and AGO2-containing cytoplasmic foci (Messaoudi-Aubert et al., 2010; Goodier et al., 2012), although MOV10 has been described in the nucleus in some cultured cell lines (Messaoudi-Aubert et al., 2010) and in early mouse hippocampal neurons, where it has been implicated in protection against retroviral elements such as LINE-1 (Skariah et al., 2017).

MOV10 is highly expressed in the murine brain, primarily in embryonic and early postnatal stages (Skariah et al., 2017). MOV10 is also required for the completion of

gastrulation and for neural tube formation (Skariah et al., 2018). In the 3'UTR where MOV10 primarily binds its mRNA targets, it is found in G-C rich regions that include stable secondary structures like G-quadruplexes (Kenny et al., 2014). MOV10 has been implicated in the miRNA pathway as its activity-stimulated degradation leads to upregulation of bound targets (Banerjee et al., 2009) MOV10 also colocalizes with FMRP and AGO2 *in vivo* in cultured neurons (Wulczyn et al., 2007; Liu-Yesucevitz et al., 2011). FMRP and MOV10 associate in brain and in cell lines in a partially RNA-dependent manner, while possessing a protein-protein interaction, as well, based on experiments with purified recombinant MOV10 and FMRP (Kenny et al., 2014).

In addition to its role in facilitating AGO2-mediated silencing, MOV10 is also able to block miRNA-mediated translational suppression in some mRNAs by directly interacting with FMRP on the same site in the mRNA. This is a significant observation because it suggests that the protein complex that assembles at an MRE can control AGO2 association (Kenny et al., 2014; Kenny and Ceman, 2016). In the absence of FMRP, MOV10 had reduced association with the subset of mRNAs that are shared between the two proteins (Kenny et al., 2014), suggesting that FMRP binds the mRNAs first and then recruits MOV10 to the mRNA. However, whether MOV10 serves as an agonist or antagonist in the miRNA pathway (Kenny et al., 2014) is directly related to its binding with FMRP in the 3'UTR. If FMRP and MOV10 bind the same 3'UTR at different sites, those mRNA targets are suppressed by AGO2. We hypothesize that this miRNA-mediated translational suppression occurs when MOV10 recruited by FMRP proceeds to remodel the 3'UTR landscape by unwinding secondary structures, potentially revealing embedded or previously inaccessible MREs for AGO2 recognition and binding.

However, if FMRP and MOV10 bind the same site in the 3' UTR, the direct proximity of these proteins leads to a protein-protein interaction that results in the MRE being protected from AGO2 association—likely by blocking AGO2 recognition of the MRE (Kenny et al., 2014; Kenny and Ceman, 2016).

In brain, a 50% reduction in MOV10 such as in heterozygous mice has been shown to have a dramatic impact on proper neuronal morphology, with MOV10-deficient hippocampal neurons exhibiting reduced dendritic arborization (Skariah et al., 2017). How this reduction in Mov10 results in impaired dendritic arborization is the subject of this thesis.

1.6 Conclusion

MOV10 and FMRP are two RBPs that depending on where they are bound either facilitate or decrease access to miRNA binding sites by AGO2 (Kenny et al., 2014). By controlling the accessibility to an MRE, a particular mRNA can either be stabilized, or degraded at a faster rate. This process can be dynamic and in response to intra- or extracellular signals ranging from phosphorylation of one or more RBPs (Ceman et al., 2003) to activation of signaling cascades like β-catenin induced regulation of c-Myc mRNA by CDB-BP (Ioannidis et al., 2003; Noubissi et al., 2006).

The work presented here outlines a role for MOV10 and FMRP in miRNA regulation of the endonuclease DICER which processes longer precursor RNAs into 22-26 nucleotides long (Lee et al., 1993) (Lee et al., 2002; Lee et al., 2003b; Denli et al., 2004; Gregory et al., 2004; Han et al., 2006; Adams-Cioaba et al., 2010). Once processed, miRNAs complex with AGO2, forming what is referred to as the RNA

induced Silencing Complex (RISC). Local activation of DICER activity in neurons has been demonstrated before (Sambandan et al., 2017). Dynamically altering local production of miRNAs by changing expression levels of DICER is one way in which neurons could respond to a wide range of temporal and environmental signals (Pilotte et al., 2011; Daugaard and Hansen, 2017; O'Brien et al., 2018). Here we show that FMRP and MOV10 affect local expression of DICER in dendrites and this perturbation results in impaired neuronal architecture.

1.7 Figure

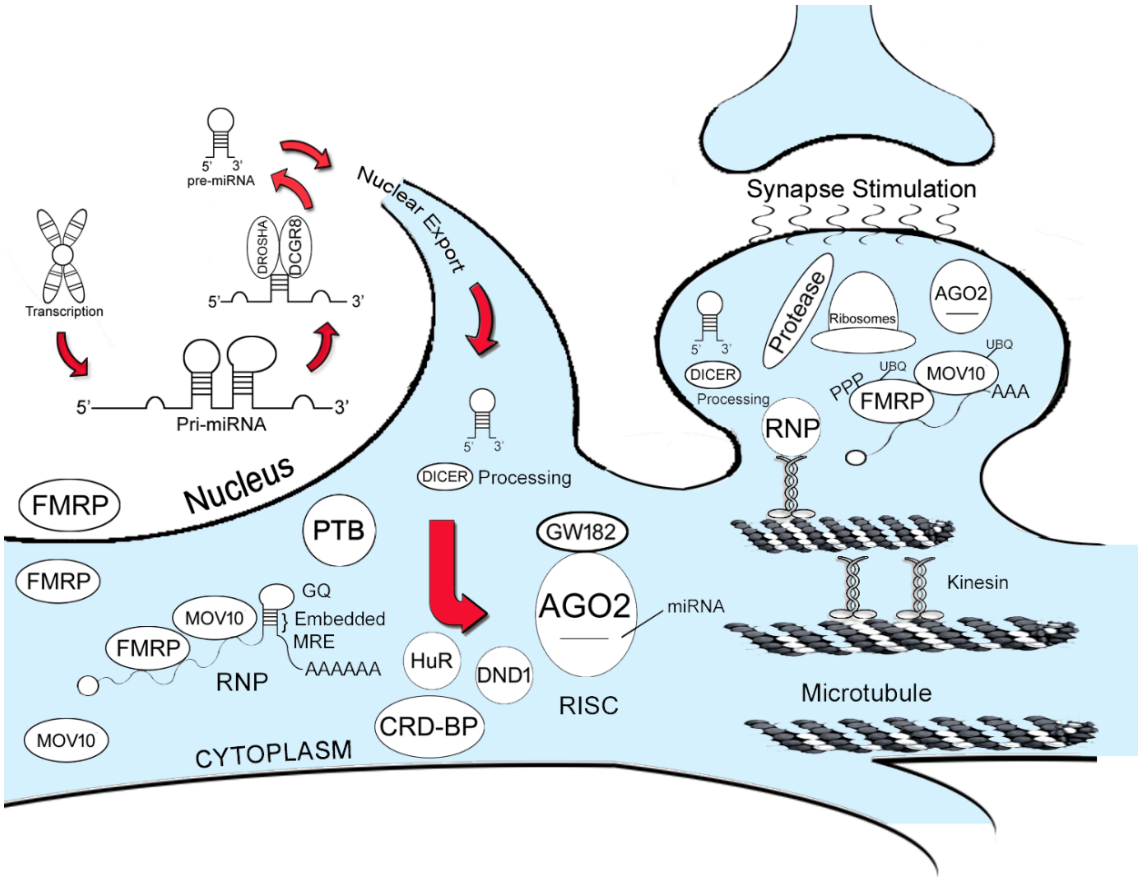

Fig 1.1 miRNA biogenesis and neuronal protein translation regulation of RNP-associated mRNAs. Abbreviations: GQ: G-quadruplex; P- phosphorylation; UBQ-ubiquitination.

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CHAPTER 2
LOSS OF MOV10 AND FMRP RESULTS IN IMPAIRED NEURONAL PHENOTYPE

2.1 Abstract

Fragile X syndrome results from the loss of expression of the Fragile X Mental Retardation Protein (FMRP). FMRP and RNA helicase Moloney Leukemia virus 10 (MOV10) are important Argonaute (AGO) cofactors for miRNA-mediated translation regulation. We previously showed that MOV10 functionally associates with FMRP. Here we quantify the effect of reduced MOV10 and FMRP expression on dendritic morphology. Murine neurons with reduced MOV10 and FMRP phenocopied *Dicer1* KO neurons which exhibit impaired dendritic maturation (Hong et al., 2013), leading us to hypothesize that MOV10 and FMRP regulate DICER expression. In cells and tissues expressing reduced MOV10 or no FMRP, DICER expression was significantly reduced. Moreover, the *Dicer1* mRNA is a Cross-Linking Immunoprecipitation (CLIP) target of FMRP (Darnell et al., 2011b), MOV10 (Skariah et al., 2017) and AGO2 (Kenny et al., 2020). MOV10 and FMRP modulate expression of *DICER1* mRNA through its 3'untranslated region (UTR) and introduction of a *DICER1* transgene restores normal neurite outgrowth in the *Mov10* KO neuroblastoma Neuro2A cell line and branching in MOV10 heterozygote neurons. Moreover, we observe a global reduction in AGO2-associated microRNAs isolated from *Fmr1* KO brain. We conclude that the MOV10-FMRP-AGO2 complex regulates DICER expression, revealing a novel mechanism for regulation of miRNA production required for normal neuronal morphology.

2.2 Introduction

Neuronal architecture is affected in many neurodevelopmental disorders. Fragile X syndrome (FXS) is caused by loss of the RNA binding protein (RBP) FMRP (Ashley et al., 1993). Extensive characterization of FMRP loss in *Drosophila*, mice and humans has led to robust observations revealing the role of FMRP in the development of abnormal dendritic spines (Irwin et al., 2005) . FMRP has also been shown to play an important role in neuronal maturation. FXS patient-derived neurons from induced pluripotent stem cells (iPSCs) and hippocampal neurons from neonatal *Fmr1* knockout (KO) mice and adult *Fmr1* knockdown (KD) exhibit defects in neurite extension and dendritic maturation (Doers et al., 2014; Guo et al., 2015; Telias et al., 2015; Telias et al., 2016; Shen et al., 2019).

FMRP binds to both the coding region of mRNAs and the 3 ’UTR (Ashley et al., 1993; Darnell et al., 2011b; Ascano et al., 2012; Tran et al., 2019). It is still poorly understood how loss of a single protein can lead to cognitive impairment although it is known that RBPs seldom act alone, existing in complexes with other RBPs and with the intermediary carrier of information, the mRNA, can enact widespread translational changes depending on their target (Brown et al., 2001; Miyashiro et al., 2003; Darnell et al., 2011a).

FMRP functionally associates with the RNA helicase MOV10 (Kenny et al., 2014), a component of the microRNA (miRNA) pathway and cofactor of Argonaute family members AGO1 and AGO2 (Meister et al., 2005; Banerjee et al., 2009). Through Cross-Linking ImmunoPrecipitation (CLIP) experiments, it has been established that FMRP and MOV10 share a common RNA interactome. Unlike FMRP,

the *Mov10* knockout (KO) is embryonic lethal (Skariah et al., 2017; Skariah et al., 2018); however the *Mov10* heterozygous (Het) mouse has increased anxiety and hyperactivity, which are features shared with Fragile X syndrome (Zafarullah and Tassone, 2019) and suggest impaired neuronal function (Skariah et al., 2017). We were thus interested in investigating the consequences of *Mov10 and Fmr1* reduction on dendritic development. Here we show that loss of FMRP and MOV10 leads to impaired dendrite maturation.

MOV10 and FMRP work dynamically to regulate expression of the *Dicer1* mRNA. DICER, a type III endonuclease, generates the final functional miRNA from pre-miRNAs, and is highly regulated at every stage of transcription and translation from primary transcript processing to enzyme activity (Kurzynska-Kokorniak et al., 2015). DICER associates with AGO2 to facilitate the transfer of the mature miRNA. It is unknown what other RBPs participate in this process. Many groups using different organisms have shown that both FMRP and MOV10 associate with DICER and AGO2 (Chendrimada et al., 2007, Darnell et al., 2011b; Ascano et al., 2012; DeMarco et al., 2019; Tran et al., 2019;). In addition, a recent paper provides compelling evidence that FMRP binds some miRNAs in regions outside of the seed sequence (DeMarco et al., 2019). This would be an intriguing mechanism for FMRP to recruit specific AGO-miRNA complexes to its bound mRNAs. Here, we provide evidence for local miRNA production through regulation of *DICER1* expression by FMRP and MOV10.

2.3 Results

Both *Mov10* Heterozygote (Het) and *Fmr1* Knockout (KO) cultured hippocampal neurons show abnormal morphology

We cultured hippocampal neurons from *Mov10* Het mice and showed that they have significantly reduced dendritic arborization compared to wild type (WT) [($p < .0001$, Fig 1A, E) and (Skariah et al., 2017)]. Because MOV10 and FMRP bind a common set of mRNAs (Skariah et al., 2017) and colocalize in dendrites (Wulczyn et al., 2007), it was logical that FMRP would also be required for normal dendritic arborization of hippocampal neurons, as reported by others (Guo et al., 2011; Doers et al., 2014; Guo et al., 2015; Telias et al., 2015; Telias et al., 2016; Shen et al., 2019).

We characterized the neurons by measuring average dendrite length, average dendritic branch points, called nodes, and the number of primary branches. Neurons from the *Mov10* Het and the *Fmr1* KO had significantly shorter dendrites than those of WT; however, dendrite length was not significantly different between the *Mov10* Het and the *Fmr1* KO neurons ($p < .001$, Fig 1B). In contrast, the average number of dendritic nodes and the number of primary branches in the *Mov10* Het neurons were significantly reduced compared to WT ($p < 0.05$); furthermore, these same features in *Fmr1* KO neurons were significantly reduced compared to both WT and *Mov10* Het ($p < .0001$, Fig 1C, D), suggesting that the reduction in both the number of nodes and the number of primary branches may underlie the results illustrated in Fig 1A. Concordantly, we observed significantly reduced dendritic branching in the *Fmr1* KO neurons compared to WT within 190 micrometers from the cell body. The amount of branching of the *Fmr1* KO neurons was also significantly reduced compared to the

Mov10 Het neurons within 50 micrometers of the cell body, suggesting that complete loss of FMRP was more detrimental to normal dendritic arborization than a 50% reduction in MOV10 ($p < .0001$, Fig 1E).

Our results agree with independent studies of neurons in *Fmr1* KO mice, which had significant reductions in dendritic complexity, total dendritic length, number of branching points and number of dendritic ends compared to WT neurons in the dentate gyrus (Guo et al., 2012; Guo et al., 2015; Shen et al., 2019). A similar result was observed with FXS human neurons which also exhibited impaired dendritic maturation (Shen et al., 2019).

It was previously shown that loss of MOV10 and FMRP results in shorter neurites in a murine neuroblastoma cell line (Neuro2A) compared to WT (Skariah et al., 2017). When we measured the length and width of neurites in *Mov10* KO Neuro2A, we confirmed that in the absence of MOV10, neurites are shorter than in the WT cells independent of the amount of retinoic acid (RA) used to differentiate the Neuro2A cells ($p < 0.001$, Supplemental Fig 1 A-C). Furthermore, we found that the neurites have a larger width in the absence of MOV10 ($p < 0.001$, Supplemental Fig 1D).

Reduced expression of *Mov10* leads to smaller soma size

When we further analyzed our neurons in culture, one individual characteristic stood out: a reduction in soma size between WT and *Mov10* Het (Fig 2). A reduction in cell body size is a feature observed in various X-linked disorders, including human FXS neurons (Telias et al., 2015) and Rett syndrome (Rangasamy S, 2016). It has also been observed in schizophrenia (Rajkowska G, 1998; Marchetto MC, 2010;

Chailangkarn T, 2012; Rangasamy S, 2016). Accordingly, we quantified the cell soma of the *Mov10* Het neurons compared to WT neurons and discovered a significant reduction in total soma area in *Mov10* Het neurons ($p < .05$, Fig 2A and 2B). We also measured the soma size of the *Fmr1* ko cultured hippocampal neurons and although it, too, was smaller, it was not significantly different than WT (NS, $p < .05$, Supplemental Fig 2 A-B).

Mov10 Het mice have reduced density of immature dendritic spines compared to WT

Because we observed a dendritic phenotype in the hippocampal neurons cultured for 14 days from the three genotypes, we examined dendritic spines in brain sections from animals aged postnatal day 14 (P14) (Fig 2A; (Galvez and Greenough, 2005)). In WT brains, we observed a significantly increased density of the immature spines compared to mature spines ($p < .0001$, Fig 2B, C), which was expected at this particular time point, when rapid pruning is taking place (Greenough et al., 2001; Galvez and Greenough, 2005). In contrast, in the *Mov10* Het, the mature spine density was the same as the immature spine density, suggesting an increase in the rate of immature spine elimination when MOV10 is reduced (NS, Fig 2B, C). Thus, MOV10 appears to stabilize immature spines (Fig 2C). This is a novel role for MOV10 because in *Fmr1 KO* animals, the ratio of immature to mature spines is skewed towards thinner, immature spines in four-week-old and adult mice (Comery et al., 1997; Nimchinsky et al., 2001; Irwin et al., 2002; Galvez and Greenough, 2005; McKinney et al., 2005;

Grossman et al., 2006; Levenga et al., 2011). We conclude that FMRP and MOV10 participate in dendritic arborization but have distinct roles in spine maturation.

We observed no difference in the width of the apical dendritic branches between WT and *Mov10* Het at P14. However, the widths of the apical oblique and basilar dendritic branches were significantly decreased when MOV10 was reduced (NS, $p < .05$, $p < .0001$, Fig 2D). Recent work shows that dendritic widths may be shaped by intracellular transport and forces from the cytoskeleton and the area proportionality accords with a requirement for microtubules to transport materials and nutrients for dendrite tip growth (Liao et al., 2021) once again suggesting altered dendritic maturation when Mov10 is reduced.

The impaired neurite phenotype, reduced soma size, and spine maturation have previously all been found to be associated with impaired miRNA biogenesis (Hong et al., 2013; Rakheja et al., 2014; Thomas Treiber, 2017; Michlewski G, 2019; Ramirez-Moya et al., 2019). miRNAs are 22-26 nucleotides long (Lee et al., 1993) and are produced upon processing from a longer precursor RNA by the endonuclease DICER (Lee et al., 2002; Lee et al., 2003b; Denli et al., 2004; Gregory et al., 2004; Han et al., 2006; Adams-Cioaba et al., 2010). Once processed, miRNAs complex with AGO2, forming what is referred to as the RNA induced Silencing Complex (RISC). Since both MOV10 and FMRP are known interactors of AGO2 and are involved in miRNA-associated regulation (Caudy et al., 2002; Ishizuka et al., 2002; Jin et al., 2004; Meister et al., 2005; Wulczyn et al., 2007; Edbauer et al., 2010; Lee et al., 2010; Kenny et al., 2014; Kenny and Ceman, 2016), we asked whether miRNA biogenesis could possibly be affected by the loss of MOV10 and FMRP?

Global reduction of AGO2-associated miRNAs in the absence of FMRP

We were interested in the FMRP dependence of AGO2-association with RNAs, thus, queried miRNA association with AGO2 in WT and *Fmr1* KO P0 brain using enhanced CLIP (eCLIP) (Tran et al., 2019). The advantage of eCLIP over traditional cross-linking immunoprecipitation (IP) experiments is that it includes the amount of input RNA in the calculation of the RNA enrichment in the IP (Van Nostrand et al., 2016). Using this method, we found that the significantly enriched peaks fell within 279 miRNAs ($p < .001$, Fig 4A), which is a large subset of the 454 miRNAs identified in an earlier study of P13 brain in association with AGO (Chi et al., 2009). The miRNAs are highly correlated between *Fmr1* KO and WT but show an average two-fold depletion in the AGO2 IP from *Fmr1* KO compared to WT. Thus, FMRP is required for normal AGO2-miRNA complex formation. Previous work (Kenny et al., 2020) showed that the level of AGO2 protein is the same in WT and *Fmr1* KO mouse brain thus allowing us to rule out any indirect effects. Other investigators have examined global miRNA levels in *Fmr1* KO brains and found differences (Liu et al, 2015; Zhang et al., 2020) although the age of the mice and methodologies may account for differences in miRNA expression.

Because there is a global reduction in AGO2-associated miRNAs in the absence of FMRP, we hypothesized that one of the miRNA processing proteins could be compromised—either decreased or potentially mislocalized in the absence of FMRP. Examination of the original FMRP brain iCLIP list published by Darnell and colleagues revealed *Dicer1* as a target among the 842 genes identified (Darnell et al., 2011b). *Dicer1* is a MOV10 iCLIP target in brain (Skariah et al., 2017) and *Dicer1* is also a target of AGO2 in mouse brain eCLIP (Kenny et al., 2020). Thus, *Dicer1* is one of the

29 genes in the intersection of these gene sets (Fig 4B). *DICER1* expression was significantly reduced in *MOV10*-knockdown HEK293 cells (Kenny et al., 2014), suggesting that MOV10 protects both murine *Dicer1* and human *DICER1* mRNAs from AGO2-mediated degradation.

Finally, because of the impaired dendrite phenotype in Fig 1, we used the DAVID Gene Functional Classification tool on the significantly changed AGO2-associated mRNAs between WT and *Fmr1* KO brain from an eCLIP experiment (Kenny et al., 2020) and found the mRNAs encode proteins involved in neuron projection (Fig 4C), as previously shown (Skariah et al., 2017).

Reduced DICER expression in the absence of FMRP or MOV10

When MOV10 and FMRP bind in the 3'UTR of their mRNA targets, depending on where they bind, the fate of the mRNA changes. When FMRP and MOV10 bind in proximity to each other on the mRNA, it appears that FMRP binds first and recruits MOV10 to unwind miRNA Recognition Elements (MREs) to facilitate AGO2 association (Kenny et al., 2020). In contrast, when a G-quadruplex is present in the 3'UTR, FMRP binds it first and binding of FMRP to MOV10 through its KH1 domain stabilizes association of FMRP to the G-quadruplex through its RGG box (Kenny et al., 2020). If an MRE is present in proximity to the G-quadruplex, association with AGO2 is blocked by the FMRP-MOV10 complex and is temporarily protected from degradation (Kenny et al., 2014; Kenny et al., 2020). We hypothesized that the FMRP/MOV10/AGO2 complex regulates *Dicer1* expression by "protecting" the *Dicer1* mRNA, such that in the absence of FMRP and/or MOV10, DICER protein levels would be reduced. When we

examined DICER expression in WT and *Mov10* heterozygote brains, we saw no significant difference (NS, $p < .05$, Supplemental Fig 3). Because MOV10 is a helicase, we hypothesized that we would need a complete knockout to see an effect. Thus, we examined DICER expression in cells in which *Mov10* and *Fmr1*, respectively, were knocked out. We observed reduced DICER expression in the *Mov10* Neuro2A KO and in brain extracts from the *Fmr1* KO mouse compared to WT ($p < .001$, Fig 5A and 5B). It's worth noting that the levels of Dicer1 mRNA do not change in N2A cells in the absence of Mov10 (Skariah et al., 2017) nor in the absence of Fmrp (Darnell et al., 2011b; Korb et al., 2017) but *Dicer1* mRNA is highly expressed in brain, similarly to *Mov10* and *Fmr1* mRNA (Supplemental Fig 3), suggesting an important role there.

Changes in the levels of DICER in the absence of MOV10 and FMRP could potentially lead to a defect in miRNA biogenesis, thus a significantly decreased pool of mature miRNAs. To test this hypothesis, we performed miRNA-seq in P0 WT and *Fmr1* KO brains. Weighted counts to mature miRNA, hairpin miRNA and tRNAs ranged from 58.6 to 64.8% (Supplemental Fig 4A). The mapped reads overwhelmingly came from mature miRNA ranging from 93.5 to 95.8%, with no obvious difference between WT and *Fmr1* KO brains (Supplemental Fig 4B). The tRNA reads made up between 4.2 to 6.5% and did not differ between groups (Supplemental Fig 4B). The precursor/hairpin only accounted for a tiny of fraction of reads, ranging from 0.03 to 0.05%. We had potentially expected to see an increase in hairpin RNA and a decrease in mature in the *Fmr1* KO samples since FMRP regulates *Dicer1* and DICER processes hairpin to mature miRNAs. However, we did not observe any consistent percentage changes between WT and *Fmr1* KO with the exception of two miRNAs, (mmu-miR-144-5p and mmu-miR-

3473c) (Supplemental Fig 5B). Therefore, the regulation of DICER expression by FMRP and MOV10 does not lead to a global reduction in miRNA levels, despite observing significantly reduced AGO2-miRNA complexes isolated from the *Fmr1* ko brain.

Dicer1 3'UTR regulation by MOV10 and FMRP

From the CLIP-seq data we know that MOV10 and FMRP bind murine *Dicer1* mRNA and human *DICER1* mRNA in the 3'UTR, respectively. To further dissect the role of MOV10 and FMRP on the 3'UTR of the *DICER1* mRNA, we obtained two human *DICER1* 3'UTR luciferase constructs (Mayr et al., 2009). One contains the entire 3'UTR (referred to as "long") and the second has the 3'UTR truncated (referred to as "short"), essentially removing any possibility for miRNA-mediated regulation (Fig 6A). In the absence of MOV10, luciferase expression of the full length *DICER1* 3'UTR is significantly decreased compared to WT. We also knocked down FMRP in HEK293T cells ($p < .001$, Fig 6B, C) and observed a similar result, suggesting that MOV10 and FMRP modulate expression of *DICER1* via the 3'UTR.

Next we wanted to identify the specific sites in the 3'UTR of *DICER1* through which MOV10 and FMRP exert their effect. To determine this, we re-aligned the binding sites from previously published CLIP-seq experiments (Gregersen et al., 2014; Kenny et al., 2014) to map all sequence sets to the same updated transcriptome. Using these data, we used TargetScan software (Agarwal et al., 2015) to determine which miRNA recognition elements (MRE) were the closest to the CLIP sites of MOV10 and FMRP. We then tested several different MREs that according to TargetScan, were

highly conserved as potential miRNA binding sites of human *DICER1* mRNA. miRNA mimics for miR-103-3p, miR-195-3p and miR-206 were transfected into WT and *Mov10* KO Neuro2A cells followed by the *DICER1* long 3'UTR luciferase reporter. We found that for the miRNAs tested, MOV10 had the strongest effect on the miR-103-3p site ($p < .001$, Fig 6E, Supplemental Fig 5A). Addition of miR-103-3p further suppressed the *DICER1* long luciferase construct in the absence of MOV10, suggesting that MOV10 blocks AGO2+miR-103-3p (Fig 6E, middle columns, green). Moreover, when we introduced a luciferase construct with the miR-103-3p sites deleted, suppression was lifted and expression of the construct in N2A cells was restored (Fig 6E, right columns, red).

Overexpression of MYC-*Dicer1* rescues impaired neuronal phenotype

To definitively show that it is the loss of DICER expression that is the primary cause of the shortened neurites, we expressed MYC-tagged Human *DICER1* in the *Mov10* KO Neuro2A, *Mov10* HET and Fmr1 KO neurons and observed restored neurite length to WT levels ($p < .001$, Fig 7A) and improved dendritic arborization within 100 μM of the soma ($p < .05$, Fig 7B), respectively. Although trending, we did not observe restoration of the dendritic arbor in Fmr1 KO neurons when Dicer1 was OE (Fig 7C). Given that Fmr1 KO neurons had a more severe neuronal phenotype, and that MOV10 and FMRP both regulate many different mRNAs, this result was unsurprising. The data together suggest a mechanism for regulating local DICER expression when MOV10 and FMRP are present.

2.4 Discussion

Our work reveals a new understanding of how FMRP and MOV10 regulate cobound mRNAs and neuronal development. *Fmr1* KO and *Mov10* Het neurons share the features of reduced dendritic arborization, including reduced dendritic length, number of nodes, number of primary branches and reduced soma size. Reducing expression of either protein in Neuro2A cells leads to reduced neurite length and simultaneously reducing both proteins does not lead to a shorter phenotype (Skariah et al., 2017) suggesting that FMRP and MOV10 operate in the same neurite outgrowth pathway and dendritic arborization. Although FMRP and MOV10 bind some of the same mRNAs, which includes *Dicer1*, there is also a large number of RNAs that are unique to FMRP and MOV10 and it is likely that misregulation of these mRNAs cause the unique spine features.

Both proteins participate in the miRNA pathway, which plays an important role in normal dendritic arborization. DICER is the primary producer of miRNAs and its mRNA is directly bound by FMRP, MOV10 and AGO2 (Darnell et al., 2011b; Skariah et al., 2017; Kenny et al., 2020). We observed significantly reduced levels of AGO2-associated miRNAs in the *Fmr1* knockout brain compared to wild type, initially suggesting a global defect in miRNA production in the absence of FMRP; however, that was not the case based on miRNA-seq of both WT and *Fmr1* ko brains, showing that the global miRNA levels were unchanged. To explain our AGO2-eCLIP results, we propose that FMRP participates in loading AGO2 with miRNAs (Fig 7A-B). In fact, it was recently shown that FMRP is able to bind miRNAs in regions outside of the seed

sequence (DeMarco et al., 2019) and miRNAs are present in the FMRP CLIP lists (Ascano et al., 2012).

We observed reduced DICER expression in the absence of MOV10 and FMRP in cell lines and brain, respectively, although since DICER is an enzyme, there must be enough present to produce normal levels of miRNAs in brain. Thus, we propose that the role of FMRP and MOV10 on *Dicer* translation affects local expression of DICER in dendrites. In fact, local activation of DICER activity in neurons has been demonstrated before (Sambandan et al., 2017). Dynamically altering local production of miRNAs by changing expression levels of DICER is one way in which neurons could respond to a wide range of temporal and environmental signals (Pilotte et al., 2011; Daugaard and Hansen, 2017; O'Brien et al., 2018). The DICER protein itself, along with FMRP and MOV10, is found in neuronal granules and thus can be rapidly dendritically and synaptically localized (Kiebler and Bassell, 2006; Mikl et al., 2010; Bicker et al., 2013; Fritzsche et al., 2013). In fact, neuronal granules typically consist of one mRNA and a large composition of RNA binding proteins (Kiebler and Bassell, 2006), suggesting a mechanism in place for rapid control of miRNA synthesis upon signal stimulation. Since RNA binding proteins have different functions in different parts of the cells and FMRP phosphorylation changes its function (Kenny et al., 2020), some mRNAs like *Dicer1* can be prevented from being bound by AGO2 yet FMRP can facilitate loading of AGO2 with miRNAs. For example, phosphorylation of FMRP increases its association with AGO2 (Kenny et al., 2020). Perhaps FMRP protects the *Dicer1* mRNA from AGO association during transport or localization and this protection may be related to the number of mRNAs with GQs as these tend to be protected by FMRP/MOV10 (Kenny et

al. 2020). We propose an additional mechanism for DICER regulation here where its local expression is regulated by FMRP and MOV10 (Fig 7A-B).

There may be many roles for MOV10 in the regulation of miRNA production. A recent study showed that shRNA-mediated knockdown of MOV10 in spermatogonial progenitor cells resulted in a significant decrease in most miRNAs. The authors suggest that MOV10 regulates miRNA biogenesis through nuclear RNA metabolism and splicing control, as levels of the miRNA processing proteins were unchanged (Fu et al., 2019).

In summary, we describe a novel mechanism that has many potential implications. FMRP and MOV10--by regulating the *Dicer1* mRNA, an indispensable element of the canonical miRNA processing machinery--could alter downstream expression of many genes. It has been long known that there is a global increase in protein translation in the absence of FMRP (Darnell and Klann, 2013). Much research has focused on finding a single target of FMRP to develop novel therapeutics for patients with FXS (Bagni et al., 2012). The work presented here might explain why this granular approach has so far not yielded a single gene whose deficiencies explain all of the features of FXS. Our work elucidates how it is possible that so many different genes can be affected by the loss of *Fmr1* and increases our understanding of the role of miRNA biogenesis elements in maintaining proper neuronal cell homeostasis.

2.5 Conclusion

Our work provides a new understanding of how the microRNA processing pathway is regulated and a novel mechanism for how FMRP loss leads to a global

increase in translation. DICER is the primary producer of miRNAs and its mRNA is directly bound by FMRP, MOV10 and AGO2. We observed significantly reduced AGO2-associated microRNAs in the *Fmr1* knockout brain compared to WT. DICER protein is also significantly reduced in both the *Fmr1* knockout brain and in the *Mov10* knockout Neuro2A cells, suggesting that FMRP and MOV10 act to block AGO2-mediated suppression of *Dicer1*. This work describes how the FMRP/MOV10/AGO2 complex regulates *Dicer1* expression and suggests that FMRP facilitates loading AGO2 with miRNAs.

2.6 Methods

Animals

Experiments were performed on C57BL6/J WT, *Mov10* Het and *Fmr1* KO mice from both sexes (The Jackson Laboratory, Bar Harbor, ME). Animals were kept on a 12/12 hour light/dark cycle with food and water *ad libitum*. All experiments were performed during the light phase (7 AM-7 PM). Animals were treated in accordance and with compliance with Institutional Animal Care and Use Committee (IACUC) guidelines, IACUC protocol 19112.

Hippocampal neuron culture

Mov10 heterozygotes were genotyped at postnatal day 0 (P0) using tail samples and DNA was extracted with the KAPA Fast Extract Kit (#KK7103, KAPA Biosystems, Wilmington, MA). After genotyping, mouse hippocampi were dissected and cultured as described (Beaudoin et al., 2012). Coverslips were coated overnight with 10 µg/mL poly-L-lysine (#P4704, Sigma, St. Louis, MO) and 10^5 cells/well were plated for

immunofluorescence (IF) in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). After 24 h, the medium was switched to Neurobasal (NB) medium (#21103049, Gibco, Dublin, Ireland) supplemented with B-27 (#17504-044, Gibco). Half of the media was removed and replaced with fresh NB medium every three days.

Immunofluorescence and microscopy of cultured neurons

Neurons grown on coverslips were fixed in 4% paraformaldehyde for 10 minutes at room temperature. Samples were blocked in 10% normal donkey serum (#017-000-121, Jackson ImmunoResearch, West Grove, PA) for 30 min at room temperature. MAP2 antibody (1:1000 dilution, # AB5622, RRID: AB_91939, Millipore, Burlington, MA) was incubated overnight at 4 °C. Secondary antibody (Alexa 594 goat anti-rabbit [1:4000, RRID:AB_2307325, Jackson ImmunoResearch, 111-585-144,]) was added for 2 h at room temperature. Coverslips were inverted unto glass slides containing mounting media with 1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI). Fluorescence images of DIV14 neurons were obtained with a Zeiss LSM 700 inverted confocal microscope (Zeiss, Oberkochen, Germany) using a 40× and 63× EC Plan-Neufluar 1.30 oil objective respectively. Images were captured with a cooled charge-coupled device (CCD) camera running Zen 2012 software (Zeiss). A total of 10–15 0.2-μM-thick sections were acquired as z-stacks for each neuron imaged.

Neuronal transfection

P0 brains from *Mov10* heterozygotes mice were harvested for hippocampal neuron culture as above and on DIV2 transfected with pDESTmycDICER, (Addgene plasmid # 19873; RRID:Addgene_19873) and empty MYC-vector plasmid DNA (Stratagene)

using Lipofectamine 2000 (Thermofisher #11668019) for 15 minutes. Half of the transfection media was removed and replaced with Neurobasal (NB) medium (#21103049, Gibco, Dublin, Ireland) supplemented with B-27 (#17504-044, Gibco). After four hours, media was fully replaced, and half of the media was removed and replaced with fresh NB medium every three days. At DIV 7, neurons were prepared for immunofluorescence. Transfection efficiency was measured as percentage of MYC-expressing cells per number of cells plated on coverslip (~50,000) cells and for both conditions, was ~ 0.05%.

Sholl analysis

Anonymized Sholl analysis of all orders of branches (Total Sholl) was performed using confocal z-stacks of WT, *Mov10* Het, and *Fmr1* KO DIV14 neurons immunostained for MAP2 and imported into ImageJ (Fiji, RRID:SCR_002285). A dendritic complexity analysis, including Sholl analysis, was performed according to the protocol described (Ferreira et al., 2010). Neurite lengths from the soma and soma size area were traced and measured using Image J software and SNT plugin and the data were compiled and analyzed using the Excel program (RRID:SCR_016137, Microsoft, Redmond, WA) and GraphPad Prism (RRID:SCR_002798, San Diego, CA).

Neurite Outgrowth

WT and *Mov10* KO N2A cells were plated in triplicate (density of 1.5 x 10^4cells/well) and incubated at 37°C in Dulbecco's modified Eagle's medium (DMEM, 10% fetal calf serum). Cells were allowed to differentiate for 48 hours using 0.1% retinoic acid (RA) and imaged under transmitted light using an EVOS cell-imaging microscope (Thermofisher). The images were anonymized and analyzed by an experimenter

blinded to the conditions using the Axiovision Image analysis software (Zeiss). 800-1000 differentiated neurons were counted from 10 images per condition.

Golgi staining

Brain tissue was processed separately in three sets of experiments performed at different times from P14 WT (*n* = 5), *Fmr1* KO (*n* = 5) and *Mov10* Het (*n* = 4). Animals were deeply anesthetized with sodium pentobarbital injection (60 mg/kg, i.p.) and transcardially perfused with 0.9% saline, pH 7.4. The brains were immediately processed for Golgi–Cox analysis using a standard protocol (Glaser and Van der Loos, 1981), embedded in celloidin (Irwin et al., 2002) and sectioned in a coronal plane at 175 µm. Spine analysis was conducted as outlined by (Irwin et al., 2002) from somatosensory cortex.

Western blot

Samples from at least three biological replicates were prepared for immunoblotting after quantification by Bradford assay and suspension in 1× sample buffer, resolved by SDS-PAGE and analyzed by western/immunoblotting. Briefly, membranes were blocked with 10% non-fat dry milk in phosphate-buffered saline (PBS) containing 1% TWEEN-20 for 1 h at room temperature. Primary antibody was applied for 1 h at room temperature or overnight at 4 °C followed by a brief wash in 1% non-fat milk PBS containing 1% TWEEN-20 wash buffer. Horseradish peroxidase (HRP)-conjugated secondary antibody was applied at 1:5000 dilution for 1 h at room temperature and washed 4 × 15 min using wash buffer. The HRP signal was detected using an enhanced chemiluminescent (ECL) substrate and exposed using iBright digital imaging platform. The antibodies used were

anti-Dicer (1:100, #sc-393328, RRID:AB_2802128, Santa Cruz Biotechnology, Santa Cruz, CA). anti-eIF5 (RRID:AB_631427, Santa Cruz) at 1:5,000, anti-KIF1A (1ug/ml), (#ab91029, RRID:AB_10862338, Abcam, Cambridge, United Kingdom), 1:1000 anti-MAP1b (# 21633-1-AP, RRID:AB_10793666, Proteintech Group, Rosemont, IL), and HRP-conjugated anti-rabbit and anti-mouse antibodies (RRID:AB_772191, GE Healthcare, Chicago, IL) and Jackson Immunoresearch, (RRID:AB_2338512) respectively. The level of significance and tests performed are described in the Fig legends for each experiment.

Luciferase reporter assays

Luciferase assay constructs were obtained from Addgene (RRID:SCR_002037, Cambridge, MA). N2A or HEK293T cells were seeded at $5x10^4$ cells into a 24 well plate for 24 hours and transfected with irrelevant or *Fmr1*-specific siRNAs (M-019631-00-0020; D-001810-0X, Dharmacon, Lafayette, CO) using Lipofectamine 2000 (Thermofisher #11668019) for N2A and PEI (Thermofisher #BMS1003-A) for HEK293T for 4 hours. Addition of siRNAs was repeated daily for 72 hours, followed by transfection on Day 4 with luciferase constructs. The procedure was identical for transfection of miRNA mimics (C-310389-05-0002; C-310532-05-0002; C-310427-07-0002, Dharmacon) and pIS1 DICER1 long-mut-miR103/107, (Addgene plasmid # 21652 ; RRID:Addgene_21652) into N2A cells. For N2A cells, seeding was identical, and transfection of control and luciferase reporter constructs was 24 hours after initial seeding. 1 µg of luciferase (renilla only) containing reporter was transfected along with 10-50 ng of pluc vector (firefly) post knockdown and 24 hours after initial seeding in N2A cells. Luciferase activity was measured in quadruplicate using a dual luciferase

reporter assay kit (#E1910, Promega, Madison, WI) on a SynergyTM HT Multi-detection plate reader (Biotek, Winooski, VT) 24 hours post-secondary transfection.

eCLIP of P0 WT and *Fmr1* KO brain

P0 Brains from Jax WT C57BL/6 and Fmr1ko mice were sent to Eclipse BioInnovations (San Diego, CA). eCLIP was performed per (Van Nostrand et al., 2016), using anti-AGO2 antibody (EAG009, Eclipse BioInnovations). Briefly, single-end (75nt) sequencing was performed on the HiSeq 4000 platform (Illumina, San Diego, CA). The first 10 nt of each read contains a unique molecular identifier (UMI) which was extracted from each read with UMI tools (version 5.2) and appended to the end of the read name. Next, sequencing adapters were trimmed from the 3' end of each read. Reads were then mapped to a database of mouse repeats using STAR (version 2.6.0c) Reads that mapped to the repeats were removed. The remaining reads were mapped to the mouse genome (mm10) using STAR (version 2.6.0c). PCR duplication removal was performed using UMI tools (version 5.2). CLIP per (version 1.4) was then used to identify clusters within the IP samples and read density within clusters was compared against the size matched input sample using a custom perl script to identify peaks enriched in the CLIP sample versus the input sample.

miRNAseq of P0 WT and Fmr1 KO brain

University of Illinois Urbana Champaign sequencing center prepared the libraries from three P0 brains from each genotype and sequenced using NovaSeq 6000 and performed FASTQC (version 0.11.8) on individual samples (N=3 of each genotype). Average per-base read quality scores are over 30 until ~90 bp and no adapter

sequences were found indicating those reads are high in quality. The Sequence Length Distribution plot shows a large spike at 22 bp that are the mature miRNA and a smaller spike at 66 which are tRNAs and hairpin/precursor miRNAs. Weighted counts to mature miRNA, hairpin miRNA and tRNAs were generated. Percentages of total reads that mapped to any of these three ranged from 58.6 to 64.8% (Supplemental Fig 4). The mapping percentage did not differ between WT and *Fmr1* KO samples. The unmapped reads were discarded while the number of remaining reads (range: 31.5 - 37.9 million per sample) were kept for further analysis which were then mapped onto each small RNA type.

A total of 3,429 smRNAs (1,978 mature, 1,234 hairpin and 217 tRNA) were detected. The detection threshold was set at 0.25 cpm (counts per million) in at least 3 samples, which resulted in 2,413 genes being filtered out, leaving 1,016 smRNAs (795 mature, 99 hairpin and 122 tRNA) to be analyzed for differential expression that contain 99.99% of the reads. After filtering, Trimmed Mean of M values (TMM) normalization was performed again (Supplemental Fig 4) and normalized log2-based count per million values (logCPM) were calculated using edgeR's cpm() function with prior.count = 2 to help stabilize fold-changes of extremely low expression genes. Differential gene expression (DE) analysis was performed using the edgeR-quasi method (edgeR version 3.30.3). Multiple testing correction was done using the False Discovery Rate method.

2.7 Figures

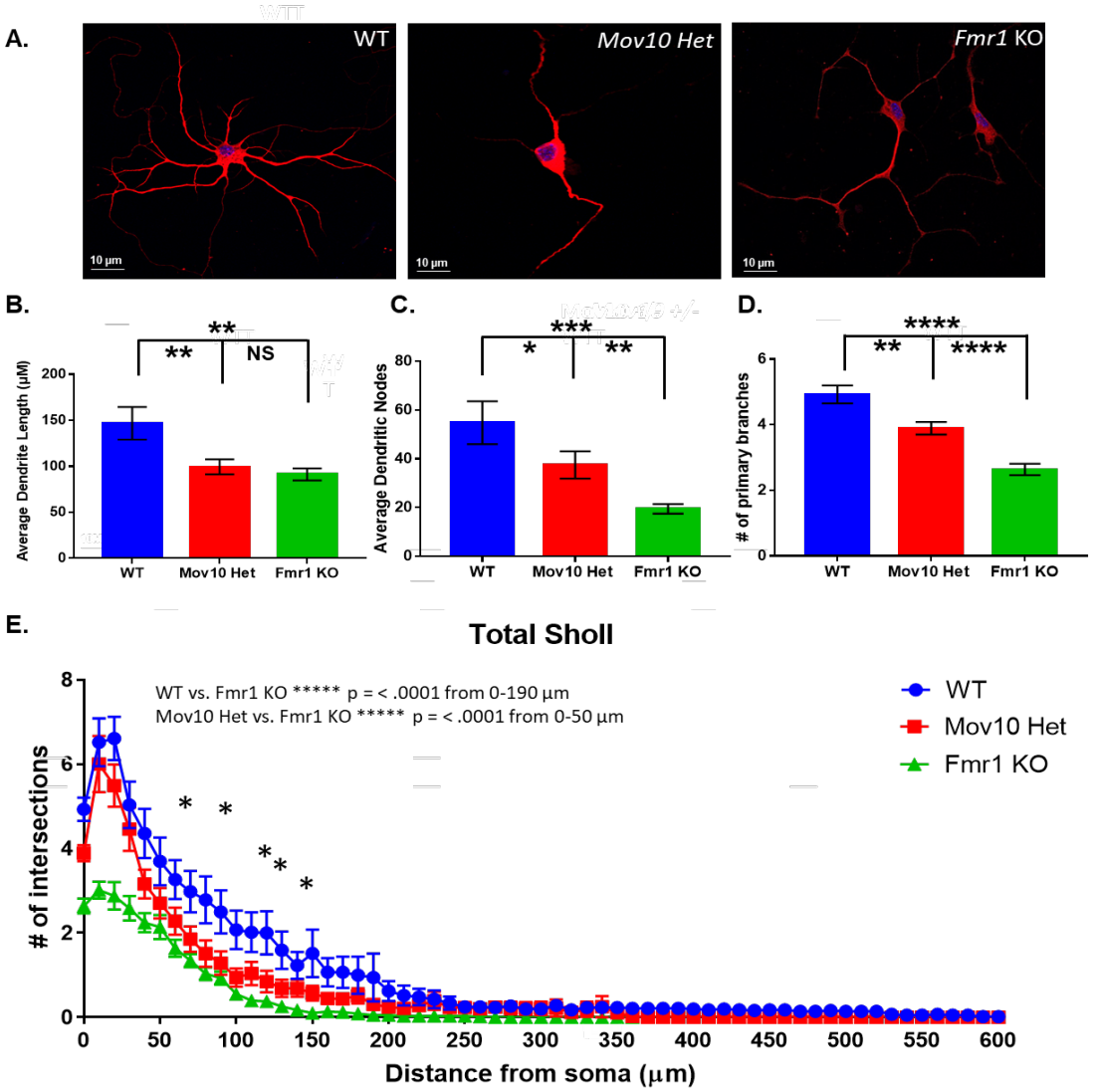


Fig 2.1. **Mov10 Het hippocampal neurons show an abnormal morphology similar to Fmr1 KO neurons. (A)** MAP2/DAPI immunostaining of hippocampal neurons from DIV14 WT, Mov10 het, and Fmr1 KO neurons. **(B-D)** Dendritic morphology analysis. Confocal z-stacks of MAP2-stained WT, Mov10 het and Fmr1 KO DIV14 neurons were analyzed. **(E)** Dendritic morphology analysis. Confocal z-stacks of MAP2-stained WT, Mov10 het and Fmr1 KO DIV14 neurons were analyzed using Sholl. Statistics were calculated using two-way ANOVA followed by Bonferroni multiple comparisons test. Error bars indicate SEM and *$p < 0.05$; ****$p < 0.0001$ ($n = 56$ neurons for WT, $n = 94$ neurons for Mov10 Het, n = 58 for Fmr1 KO). Neurons were cultured in 24-well plates and imaged from three independent experiments, and N compiled from all three experiments. Scale bar = 10 µm.

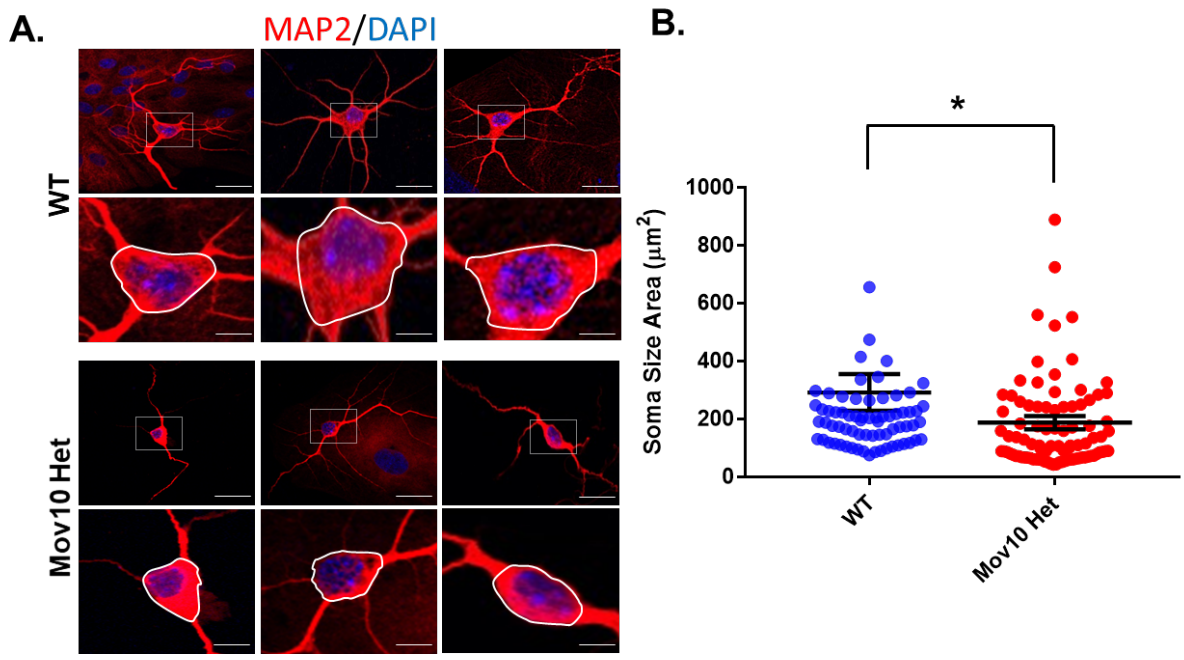


Fig 2.2. ***Mov10* Het neurons have a reduction in total soma area *in vitro*.**
(A) Immunofluorescence microscopy of control (WT) and *Mov10* Het primary hippocampal neural cultures at 14 days *in vitro* (DIV14) showing MAP2 (red) and DAPI (Jentarra et al., 2010). The dashed box indicates the region shown at higher magnification. The area encircled by the white line indicates the region of soma size analysis. **(B)** Measurements of soma size area in DIV14 primary hippocampal neurons revealed a significant reduction in *Mov10* Het (n = 94) cell body size compared to WT (n = 56). Data are presented as mean ±SEM; *p* values in relation to control (*$p < 0.05$), (Student's *t*-test). Scale bar: 25 µm.

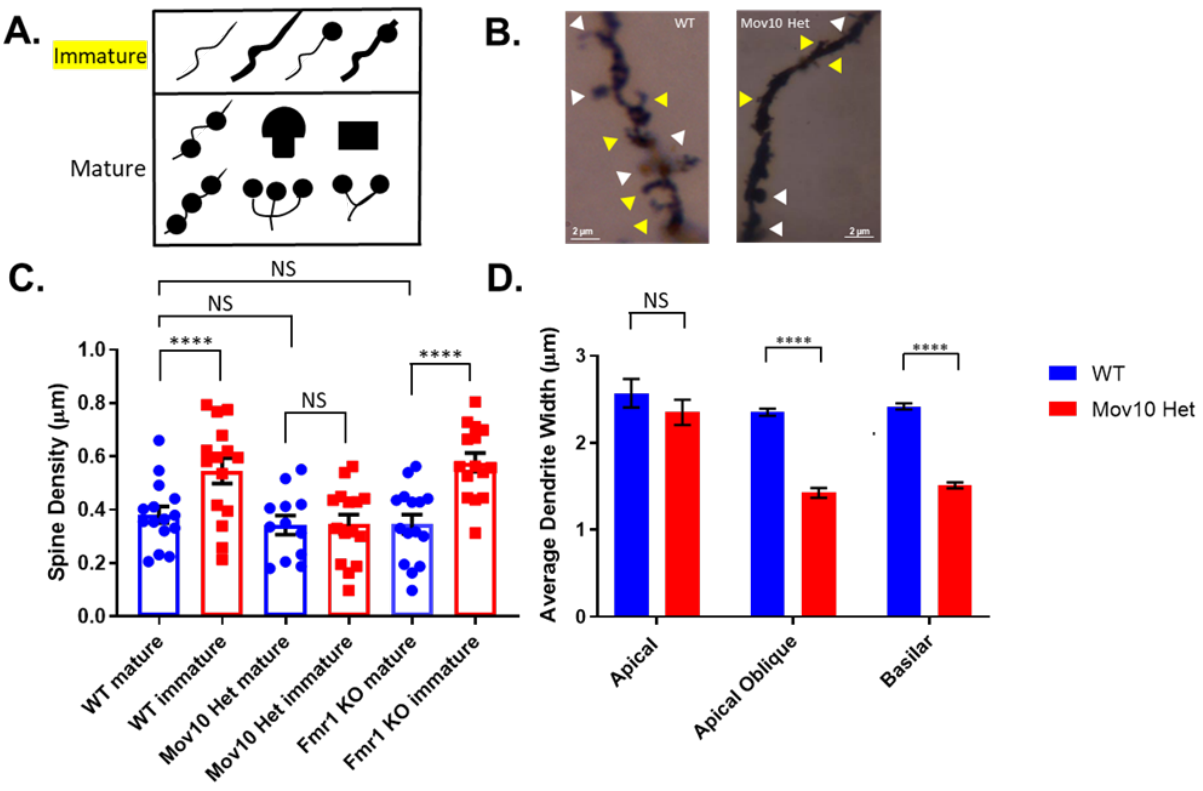


Fig 2.3. *Mov10* Het mice have fewer immature spines and wider apical oblique and baslar (secondary and tertiary) branches. (A) Spine morphology analysis was classified into one of ten different groups, which were further subdivided into immature or mature spines based on [28]. **(B)**. Representative images of cortical neurons from WT and *Mov10* Het mice using Golgi staining of somatosensory cortex, scale bar = 2 µm. **(C-D)** Spine density and diameter of spines were measured per 10 µm dendritic segments of n = 5 (WT animals (8 neurons total), n = 4 *Mov10* Het animals (8 neurons total), n = 5 (Fmr1 KO animals (8 neurons total). All measured data are expressed as means ±SEM. ****p < 0.0001; NS = not significant; p > 0.05 (Student's t-test).

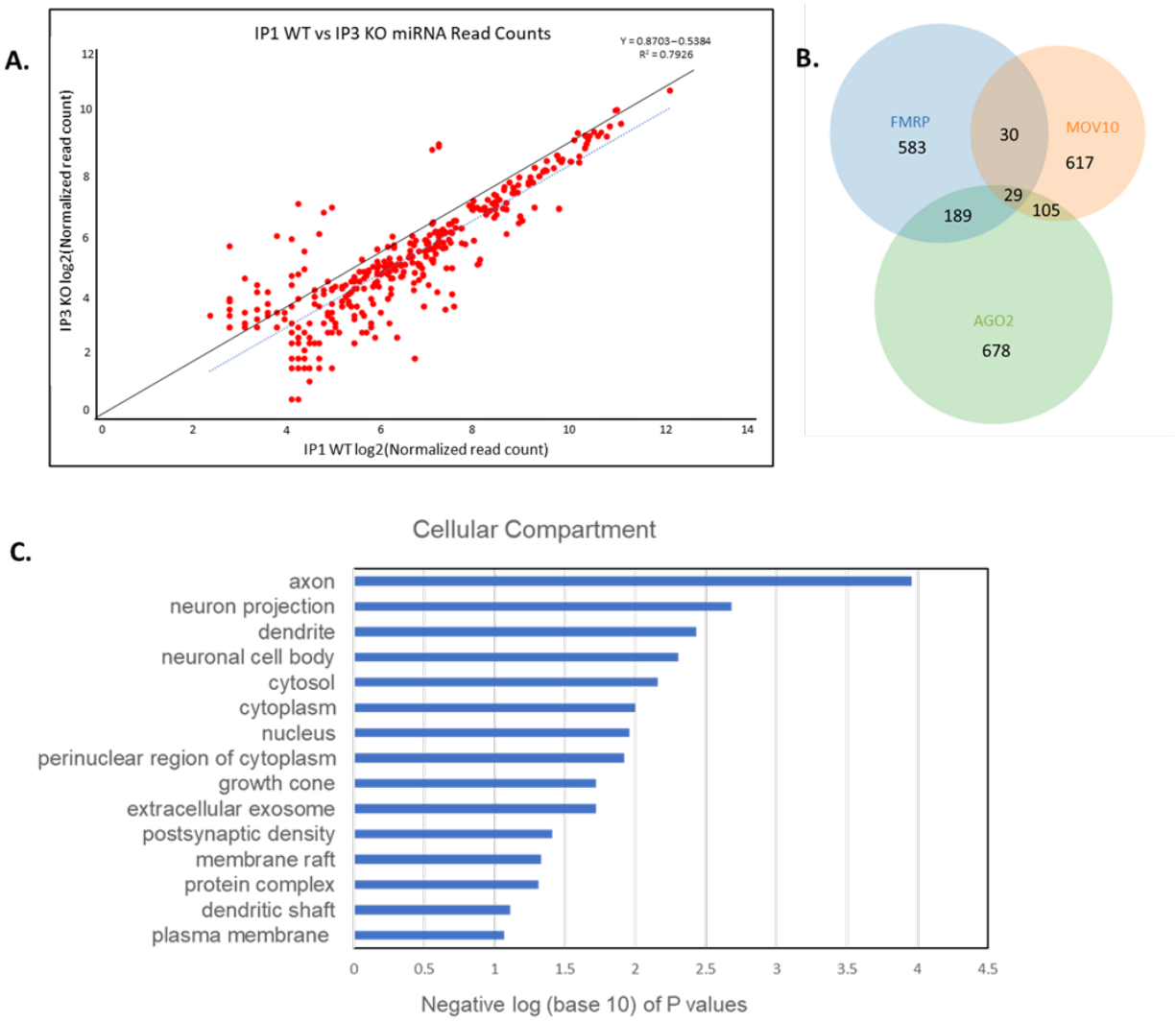


Fig 2.4. Global miRNA reduction in brain in the absence of Fmrp. (A) Two-fold less miRNAs associate with Ago2 in the absence of Fmrp. Reads per million of WT (X-axis) and Fmr1 KO (Y-axis) Ago2-IPs at each cluster that maps to miRNAs. The IPs had a log2 fold change ≥3 over input and p-value ≤ 0.001. Solid black line = best fit of data. Dashed blue line = actual fit of data. **(B)** GO analysis of iCLIP mRNAs from postnatal brain. *Y axis*: GO terms for Cellular Compartment; *X axis*: negative log (base 10) of the 15 lowest *p* values showing FMRP binds mRNAs encoding proteins involved in neuron projection. **(C)** Venn diagram showing the overlap between brain-derived iCLIP targets of Fmrp (Darnell, 2011), Mov10 (Skariah et al., 2017), and Ago2. All three proteins in the brain commonly bound 29 mRNAs (Dicer1 included).

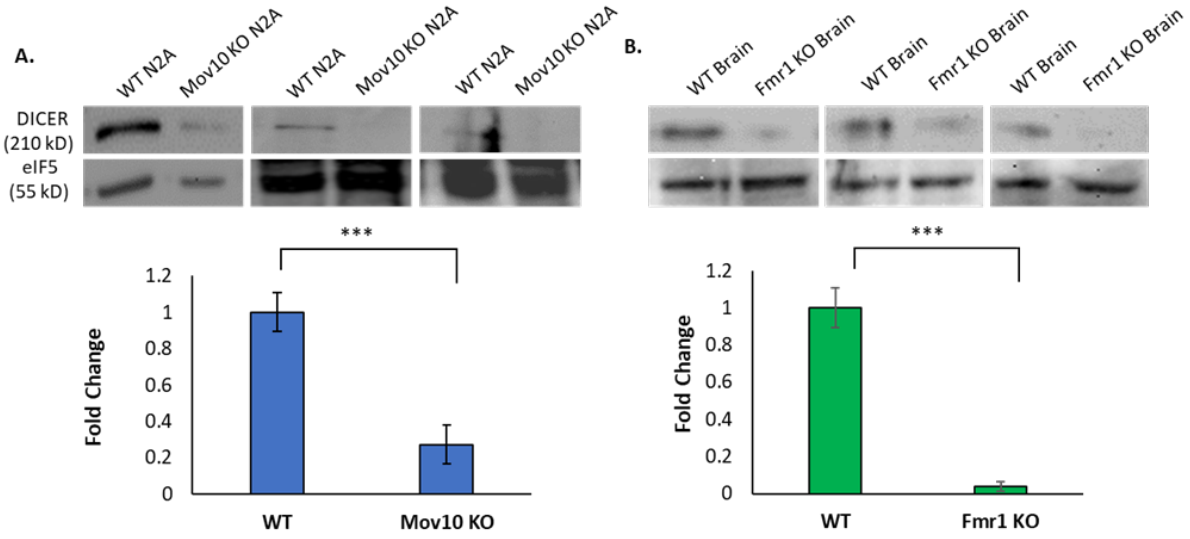


Fig 2.5. DICER protein expression is significantly decreased in the absence of MOV10 and FMRP. (A-B) Three representative images of WT and *Mov10* KO N2A cells (50 µg) and brain extract (50 µg) from P2 C57BL/6 WT and *Fmr1* KO mice immunoblotted for DICER and eIF5 as a loading control. Bar graphs of four and six biological replicates, respectively, are shown below. All measured data are expressed as means ±SEM. ***$p < 0.001$ (Student's *t*-test).

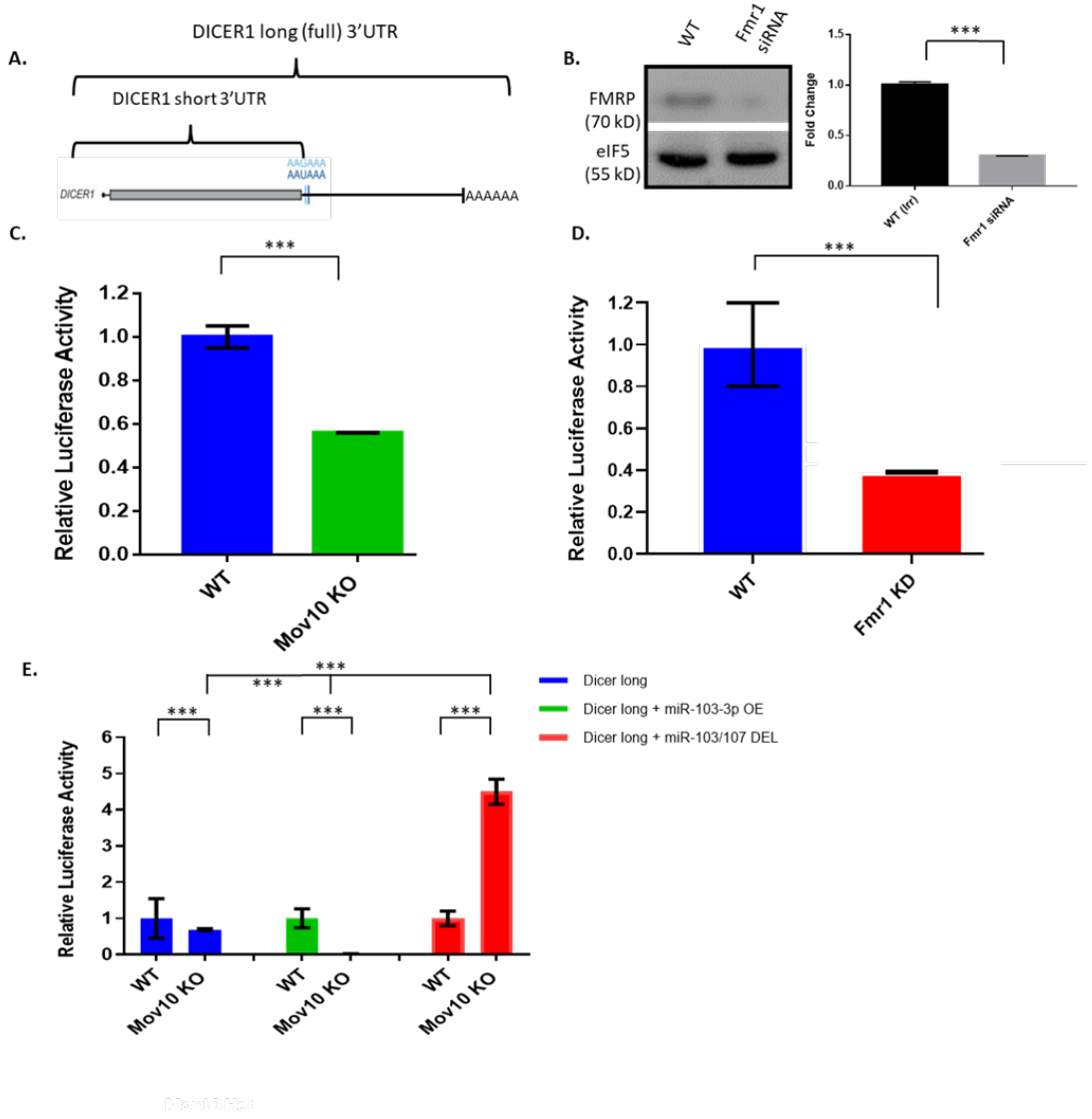


Fig 2.6. MOV10 and FMRP modulate expression of *Dicer1* mRNA via the 3'UTR. **(A)** Schematic illustration of *Dicer1* mRNA with alternative isoforms. Grey boxes show protein coding region; black line represents untranslated regions and AAAAAA represents the poly(A) tail. **(B)** Blot (25ug) and graph showing Hek293T cells were treated with Irr small interfering RNA (*siRNA*) or Fmr1 siRNA (n = 3). **(C)** Effect of Mov10 loss (in N2A cells) and **(D)** FMRP KD (in Hek293T) on luciferase expression of full-length 3 'UTR of *Dicer1* and the shortest *Dicer1* isoform. Mov10 KO and Fmr1 KD samples were normalized to WT Dicer long 3'UTR and Dicer short 3'UTR values were subtracted for final graph to account for differences in plasmid transfection. **(E)** Effect of miR-103-3p overexpression and deletion in the absence of Mov10. Assays were performed in quadruplicate from three independent experiments. All measured data are expressed as means ±SEM. ***$p < 0.001$ (Student's *t*-test).

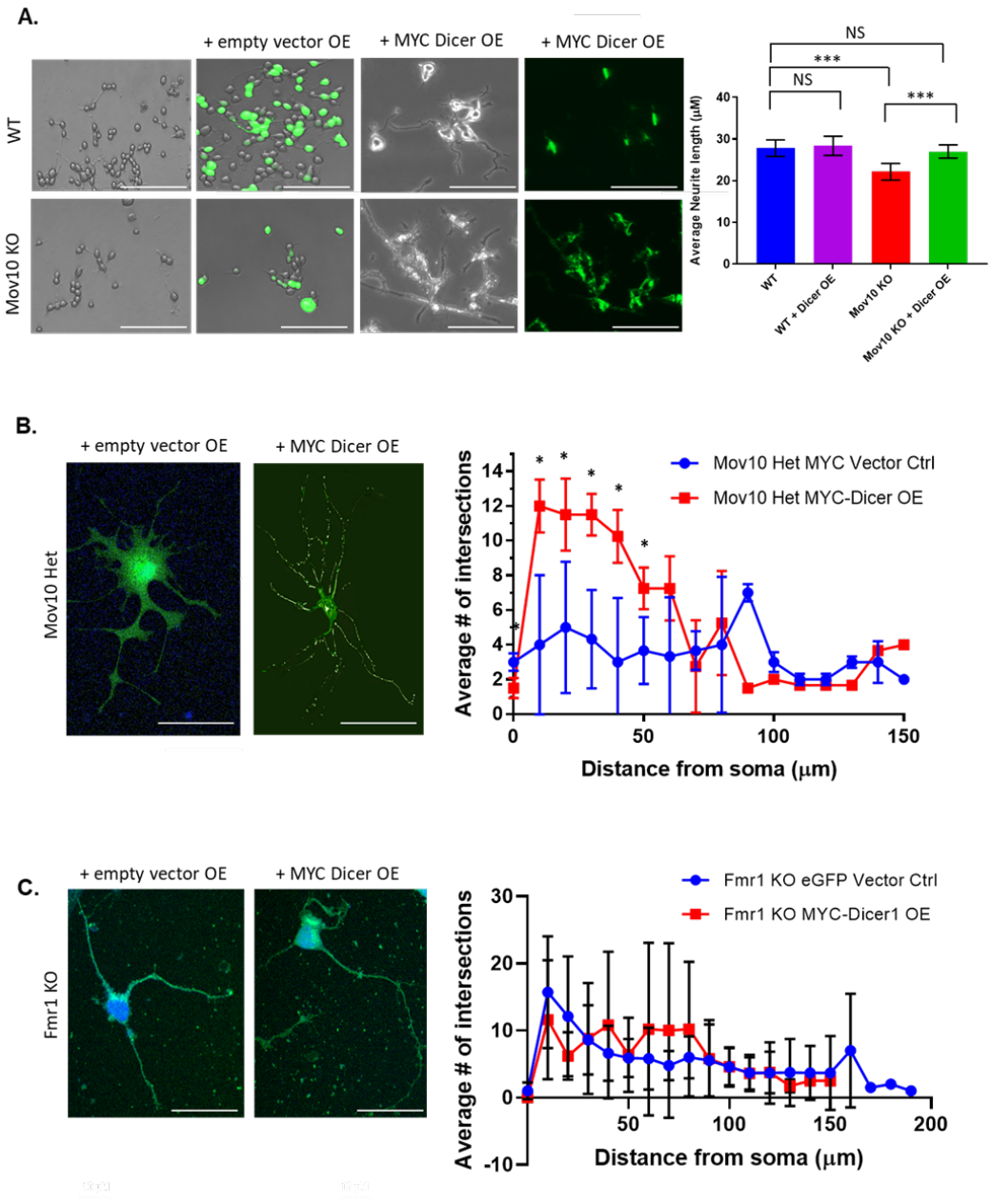


Fig 2.7. Overexpression of MYC-*Dicer1* rescues impaired neuronal phenotype. **(A-C)** Brightfield and immunofluorescence images of WT, Mov10 KO, MYC-tagged Human Dicer1, and empty vector control (MYC and eGFP). MYC-tagged Human Dicer1 was overexpressed (OE) in Mov10 KO N2A cells (A), Mov10 HET hippocampal neurons **(B)** and Fmr1 KO hippocampal neurons **(C)** at DIV 2 followed by immunofluorescence at DIV 7. Sholl statistics were calculated using two-way ANOVA followed by Bonferroni multiple comparisons test. Error bars indicate SEM and $^*p < 0.05$ ($n = 3$ neurons for Mov10 Het empty MYC-vector control, $n = 5$ neurons for Mov10 Het MYC Dicer overexpression, n = 5 for Fmr1 KO MYC Dicer overexpression. Scale bar = 100 μm.

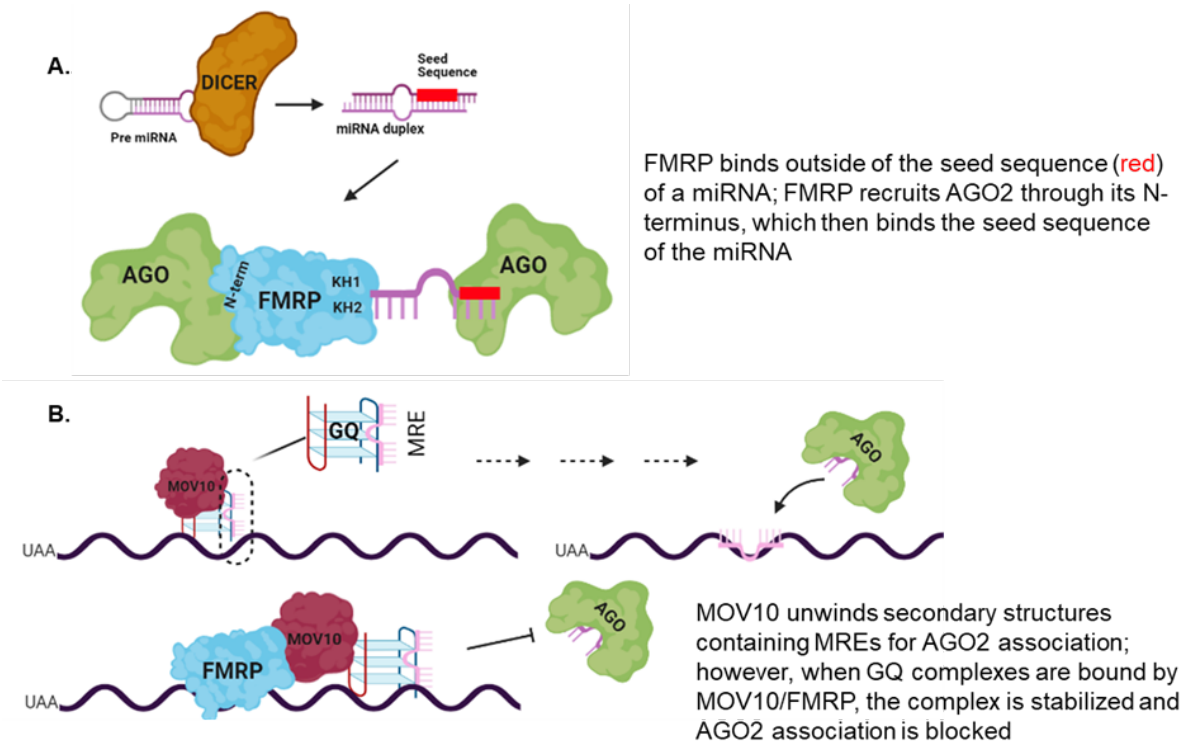
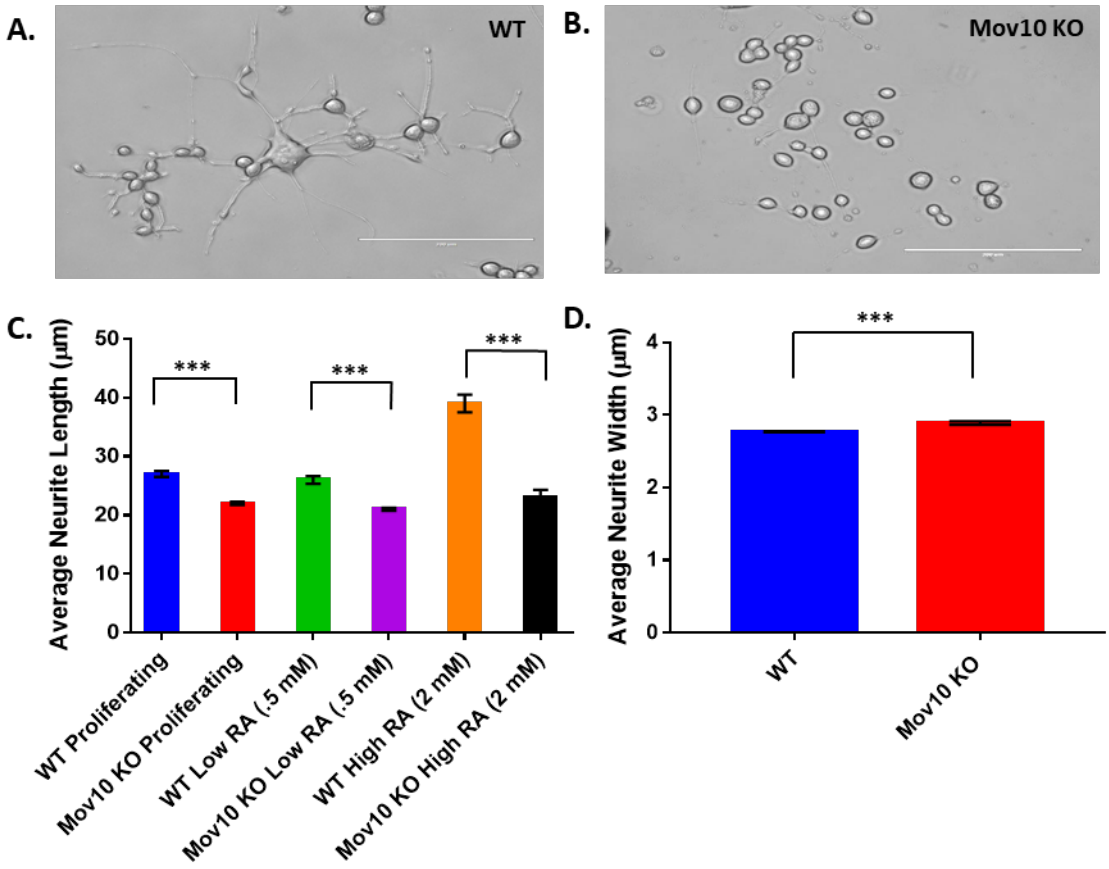
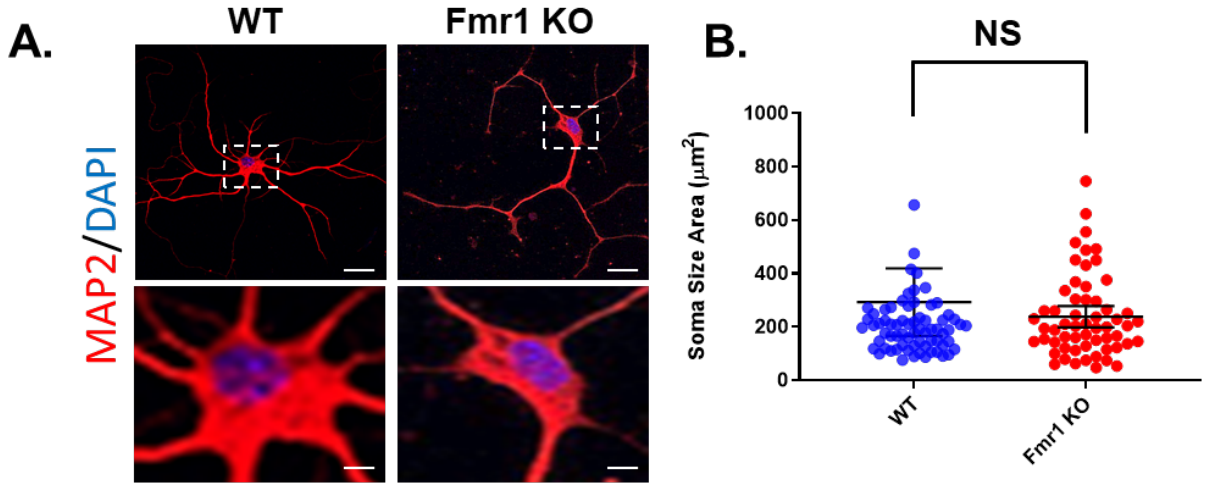


Fig 2.8. MOV10 and FMRP interact with AGO2 to regulate *Dicer1* mRNA expression. (A) Part I of model showing the recruitment of AGO to a miRNA following interaction with FMRP. **(B)** Part II of model showing fate of Dicer1 mRNA bound by MOV10 and FMRP. Binding of both FMRP and MOV10 in proximity of MRE blocks association with AGO2 (Kenny et al., 2014). In the absence of MOV10 and FMRP, AGO2 is free to bind the MRE and translational regulation takes place. Pink line indicates MRE.

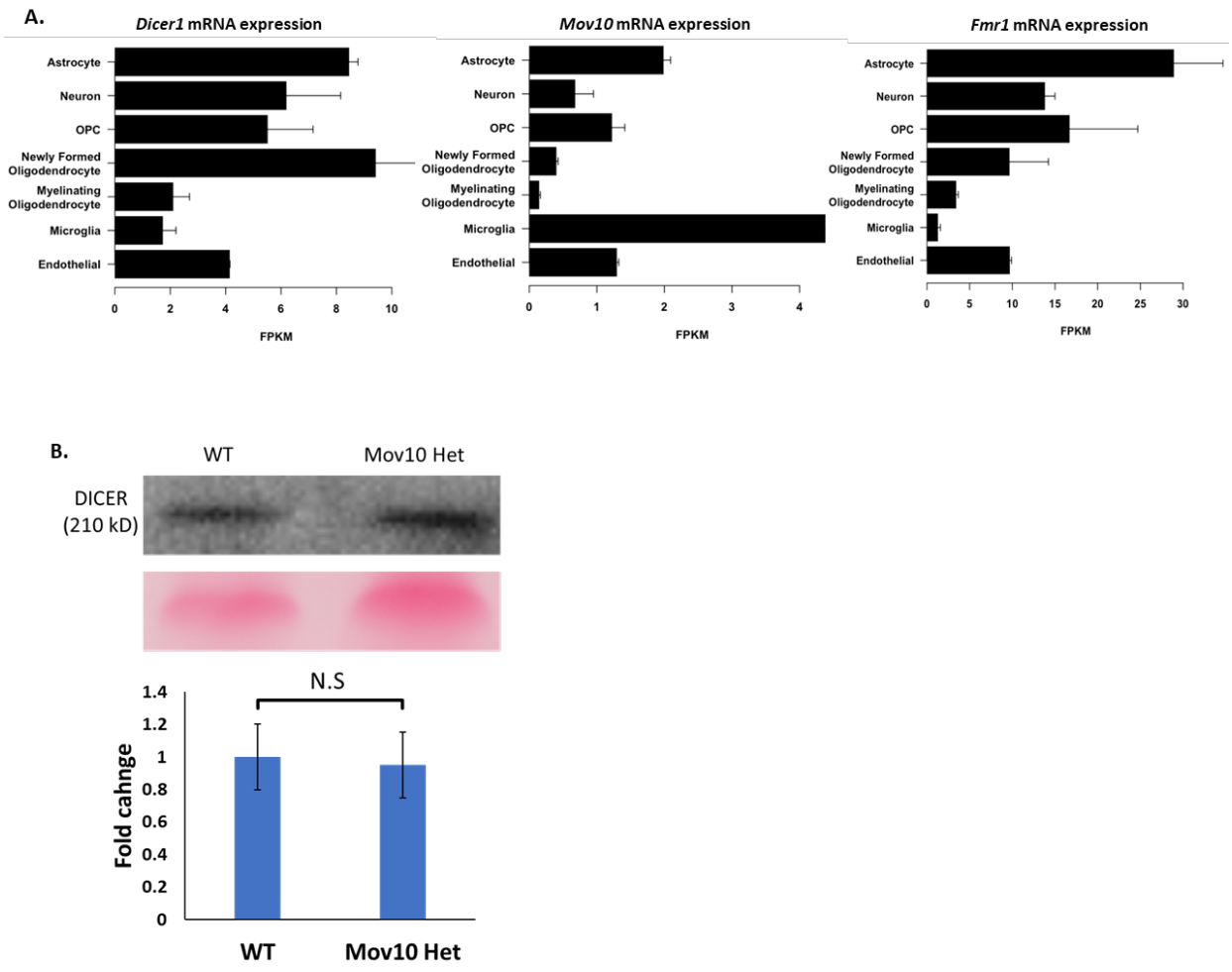
2.8 Supplemental Information



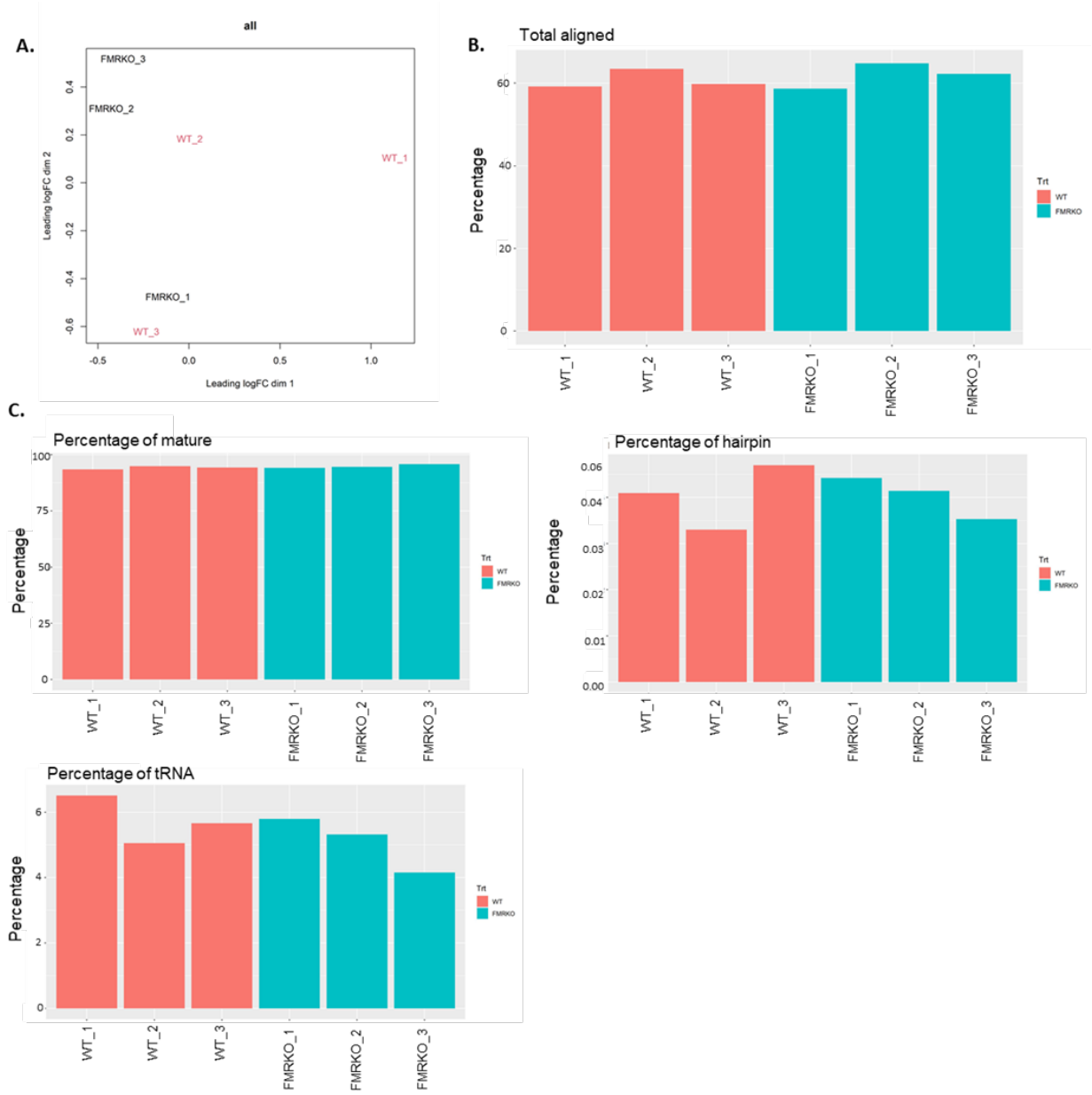
Supplementary Fig 2.1. Mov10 KO Neuro2A cells have shorter and wider neurites compared to WT. (A-B) Brightfield images of N2A WT and Mov10 KO cells. Scale bar = 200 µm. **(C)** Quantification of neurite length of WT and Mov10 KO in the presence of different concentrations of retinoic acid (RA). Between 800-1000 proliferating and differentiated cells were counted from triplicate experiments, and a total of 10 images were counted per condition. **(D)** Average neurite width in differentiated WT and Mov10 KO N2A cells (1 mM RA) were measured n = 100-250 (WT and Mov10 KO). All measured data are expressed as means ±SEM. ***$p < 0.001$ (Student's *t*-test).



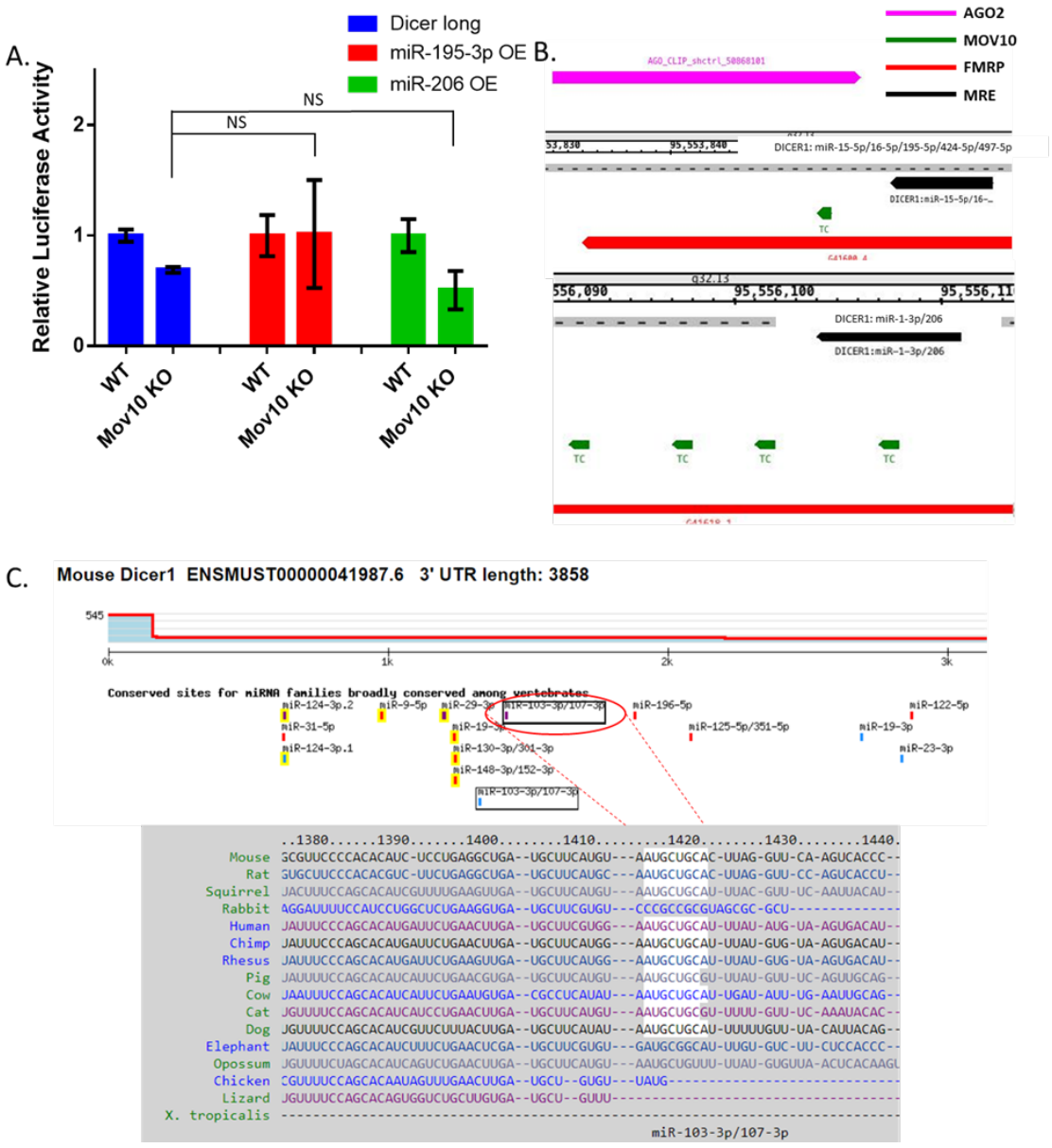
Supplementary Fig 2.2. No change in total soma area *in vitro* between WT and Fmr1 KO neurons. (A) Immunofluorescence microscopy of control (WT) and Fmr1 KO primary hippocampal neural cultures at 14 days *in vitro* (DIV14) showing MAP2 (red) and DAPI (Jentarra et al., 2010). The dashed box indicates the region shown at higher magnification. **(B)** Measurements of soma size area in DIV14 primary hippocampal neurons in Fmr1 KO (n = 58) compared to WT (n = 56). Scale bar: 25 μm. Data are presented as mean ±SEM; *p* values in relation to control (NS = $p > 0.05$), (Student's *t*-test).



Supplementary Fig 2.3. High expression of *Dicer1*, *Mov10* and *Fmr1* mRNA in brain but DICER protein levels do not change in brain when Mov10 is reduced (A) RNA-Seq analysis revealed cell type-specific gene expression profiles in brain for *Dicer1*, *Mov10* and *Fmr1*. RNA-seq data was collected using transcriptome library constructed by (Mathews et al., 1999). Cufflinks algorithm is used to estimate FPKM values (Schuster et al., 1994). **(B)** Whole P2 WT and Mov10 Het (25 µg) were immunoblotted against DICER with Ponceau S as a loading control in three independent experiments. Error bars represent SD, and p values were obtained by Student's t test (NS > 0.05).



Supplementary Fig 2.4. miRNAseq reveals no difference in the level of mature vs. immature microRNAs in the absence of FMRP. (A) Multidimensional scaling on the top 500 most variable genes in brain samples. **(B)** Percentages of total reads in the sequencing report that mapped to any of these three: mature, hairpin and tRNA ranged from 58.6 to 64.8%. The mapping percentage does not differ between WT and FMRKO samples. **(C)** Percentage of mapped reads coming from mature miRNA, hairpin miRNA, and tRNA.



Supplementary Fig 2.5. Overexpression of miR-195-3p and miR-206 does not significantly decrease Dicer 3'UTR expression in the absence of Mov10. (A) Effect of Mov10 KO on luciferase Dicer1 3′ UTR and miR-195-3p and miR-206 site overexpression. **(B)** Screenshot from Integrated Genome Browser (IGB) of Dicer1 3'UTR (running from right to left) with human AGO2 CLIP sites (pink), human MOV10 CLIP sites (green) and human FMRP CLIP sites showing relative locations to the MRES containing sites for miR-195-3p and miR-206 binding. **(C)** TargetScan screenshot of Dicer1 3'UTR (running from right to left) with red circles showing location of MRE sites whose miRNAs were overexpressed in A as well as the degree of conservation among species of the particular MRE. Assays were performed in quadruplicate from three independent experiments. All measured data are expressed as means ±SEM. ***$p < 0.001$ (Student's *t*-test).

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CHAPTER 3

CONCLUSIONS

This thesis is the first systematic study of the local effects of MOV10 and FMRP in neurons on a component of the miRNA pathway, *Dicer1* mRNA. The introductory chapter outlines the known roles of FMRP and MOV10 and their connection to the miRNA pathway. The chapter delves into the importance of the miRNA pathway in proper neuronal development and brain function and addresses gaps in the field as to the mechanism of action of FMRP and MOV10 on their mRNA targets in cells. Chapter 2 elucidates an important role for MOV10 and FMRP on neuronal architecture. When MOV10 is reduced, neurons do not possess normal dendritic arborization which can impact neuronal circuitry. We and others have observed a similar effect in the absence of FMRP. We characterized the morphological defects observed *in vitro* and *in vivo* and then asked what could be causing these defects. We uncovered *Dicer1* as an mRNA that is regulated by both FMRP and MOV10 and whose rescue can improve neuronal morphology even in the absence of both RNA binding proteins. We have proposed a model for how FMRP and MOV10 are able to regulate *Dicer1* locally in neurons. Future work would probe the underlying mechanism of this local regulation by examining local regulation of *Dicer1* expression after stimulation. It would be challenging examine if DICER is translated in response to stimulation at the synapse, but perhaps live cell imaging of a miRNA reporter could be used. It would also be interesting to examine whether FMRP, MOV10 and DICER are present in transport granules in the dendrite, suggesting that the regulation of *Dicer1* translation occurs in the cytoplasm, leading to

formation of the FMRP-MOV10-DICER complex, which functions in dendritogenesis or synaptic activity.

3.1 Future directions

Future work would probe the underlying mechanism of this local regulation by MOV10 and FMRP by examining local regulation of *Dicer1* expression after stimulation. It would be challenging examine if DICER is translated in response to stimulation at the synapse but perhaps live cell imaging of a miRNA reporter could be used. It would also be interesting to examine whether FMRP, MOV10 and DICER are present in transport granules in the dendrite, suggesting that the regulation of *Dicer1* translation occurs in the cytoplasm, leading to formation of the FMRP-MOV10-DICER complex, which functions in dendritogenesis or synaptic activity. Finally, determining if there are any other RNA binding proteins that participate in *Dicer1* regulation in neurons will give us further insight into how the miRNA pathway is controlled in the brain.