Cooperative Regulation of Translational Repression by FMRP and the miRNA Pathway

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Cooperative Regulation of Translational Repression by FMRP and the miRNA Pathway

A Dissertation

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Doctor of Philosophy

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Navneeta Kaul

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Advisor: Dr. Scott A. Barbee
ABSTRACT

Fragile X syndrome (FXS) is the most common inherited monogenic cause of intellectual disability. FXS patients exhibit social and language deficits, hyperactivity, seizures, growth abnormalities, macroorchidism, anxiety, and epilepsy. FXS is caused by the transcriptional silencing of the fragile X mental retardation gene 1 \((Fmr1)\), resulting in the loss of the fragile X mental retardation protein (FMRP). FMRP is a selective mRNA binding protein that plays a role in translation repression. Studies suggest that FMRP utilizes the miRNA pathway to repress translation of its target mRNAs through an unknown mechanism. The aim of my thesis is to investigate the mechanism by which FMRP regulates the translation of specific mRNA targets via the miRNA pathway using \textit{Drosophila melanogaster} as a model system. Here, we demonstrate that FMRP requires the core miRNA-induced silencing complex (miRISC) components, AGO1 and GW182, to mediate translation repression. Moreover, we show that FMRP itself is necessary for miRNAs to repress translation of a reporter mRNA, which suggests an interdependent role of FMRP and miRNA in regulating gene silencing. Finally, our findings elucidate a novel role for GW182 in the maintenance of proper synaptic structure and morphology at the glutamatergic larval neuromuscular junction (NMJ) in an FMRP-dependent manner. We propose that FMRP binds to the 3'UTRs of target mRNAs and controls translation of target mRNAs in a GW182-dependent manner. Our data improve current understanding of the normal FMRP function in neurons. We postulate that this
could assist in the discovery of novel therapeutic targets to treat FXS and related autism spectrum disorders.
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TABLE OF CONTENTS

Chapter One: Introduction
1.1 Overview of Fragile X syndrome.................................................................1
1.2 Structure and function of FMRP .................................................................7
  1.2.1 Role of FMRP in regulating neurodevelopment......................................10
  1.2.2 Function of RNA binding domains of FMRP........................................13
  1.2.3 FMRP binds to distinct mRNA sequence and structural elements.........15
1.3 FMRP acts as a translation repressor through different mechanisms........17
  1.3.1 Evidence that FMRP stalls translation at the elongation stage..............19
  1.3.2 Evidence that FMRP stalls translation at the initiation stage...............22
1.4 miRNA biogenesis and miRISC assembly.................................................25
  1.4.1 miRNA biogenesis, loading, and RNA induced silencing complex formation. 31
  1.4.2 Mechanisms of miRNA mediated target gene silencing.........................32
    1.4.2.1 Translation repression by miRNAs at the initiation stage.................35
    1.4.2.2 miRNA-mediated translation repression at the elongation stage.........39
    1.4.2.3 Role of GW182 in mediating translation repression.......................44
    1.4.2.4 Compartmentalization of miRNA-mediated repression in P bodies......46
1.5 Role of FMRP in miRNA-mediated repression.........................................47
1.6 Summary..................................................................................................50

Chapter Two: Methods
2.1 Key resources table ..............................................................................53
  2.1.1 Reagents and Chemicals ......................................................................53
  2.1.2 Experimental models: cell lines .........................................................55
  2.1.3 Experimental models: Fly strains .......................................................55
  2.1.4 DNA constructs ..................................................................................56
  2.1.5 Oligonucleotides ..............................................................................58
  2.1.6 Antibodies .........................................................................................62
  2.1.7 Media and buffer composition ............................................................64
  2.1.8 Software and algorithms ..................................................................67
2.2 DNA constructs ......................................................................................68
2.3 S2 Cell culture and Transfection ............................................................70
  2.3.1 Tethering assays ..............................................................................71
  2.3.2 Assays with untethered FMRP ............................................................72
2.4 Luciferase Assays ...................................................................................72
2.5 RNAi against miRISC Components in S2 cells ......................................73
2.6 Western blotting .....................................................................................73
2.7 Co-Immunoprecipitation .......................................................................74
2.8 Real Time PCR ......................................................................................75
  2.8.1 RNA Isolation and Purification ............................................................75
  2.8.2 qRT-PCR ..........................................................................................75
2.9 miRNA Target-site prediction .................................................................76
2.10 RNA secondary structure prediction .....................................................76
2.11 S2 cell Immunofluorescence and Microscopy .................................................. 76
  2.11.1 FMRP granules in S2 cells ........................................................................... 76
  2.11.2 Immunofluorescence in S2 cells ..................................................................... 76
  2.11.3 S2 cell colocalization analysis ....................................................................... 77
2.12 Generation and characterization of Drosophila stocks ...................................... 77
  2.13 NMJ dissections and processing ........................................................................ 78
    2.13.1 Image Acquisition and Quantification of NMJ ............................................. 79
2.14 Larval CNS Immunostaining ................................................................................ 79
    2.14.1 Larval CNS colocalization analysis .............................................................. 80
2.15 Larval Crawling Assay ....................................................................................... 80
2.16 Statistical Analysis ............................................................................................. 81

Chapter Three: Results
3.1 Tethered FMRP represses translation of a FLuc reporter in a
dosage-dependent manner ......................................................................................... 82
3.2 The KH domains of tethered FMRP are required for translation repression of
the FLuc-BoxB reporter .............................................................................................. 86
3.3 Tethered FMRP requires miRISC components to mediate repression of
the FLuc-BoxB reporter .............................................................................................. 90
3.4 FMRP interacts biochemically with AGO1 and GW182 in S2 cells ...................... 92
3.5 Untethered FMRP can repress the translation of the FLuc-BoxB reporter .............. 94
3.6 miRNA-958 requires FMRP to mediate translation repression
of the FLuc-BoxB reporter ........................................................................................ 96
3.7 FMRP can repress the translation of FLuc reporters for known target mRNAs
that contain BoxB-like sequences in their 3’ UTR .................................................... 101
3.8 FMRP colocalizes to granules containing AGO1 and GW182 in S2 cells .............. 105
3.9 FMRP is expressed in the same population of neurons as AGO1 and GW182
within the cell bodies in the Drosophila larval CNS. .................................................. 107
3.10 dFmr1 interacts with GW182 to regulate synaptic growth .................................. 111
3.11 dFmr1 and miRNA pathway components do not interact to
control rates of larval locomotion ............................................................................ 117
3.12 The role of dFmr1 in regulating synaptic growth is independent of the dekaylase
components (CCR4 and NOT1), PABP or the decapping complex (Dcp1) ............ 121
3.13 FMRP is expressed with CCR4, CAF1 and NOT1 within the same cells
in the larval CNS ....................................................................................................... 121
3.14 FMRP shows no genetic interaction with CCR4, NOT1, PABP or Dcp1
at the larval NMJ ...................................................................................................... 125
3.15 FMRP does not interact with CCR4, NOT1, PABP or Dcp1 to
to control rates of larval locomotion ..................................................................... 131

Chapter Four: Discussion
4.1 FMRP requires AGO1 and GW182 to mediate translation repression .................. 134
4.2 FMRP requires AGO1 and GW182 to control NMJ development ....................... 135
4.3 Cooperative interaction between FMRP and miRNAs regulate translation .......... 136
4.4 Role of the KH domains in FMRP mediated translation repression ................. 137
4.5 Role of the KH domains in FMRP granule formation……………………………139
4.6 RBPs can promote miRISC targeting at the 3’UTR of target mRNA……………..140
4.7 Future directions……………………………………………………………………..142

References………………………………………………………………………………………..145

Appendix: List of Abbreviations…………………………………………………………………..173
LIST OF TABLES

Chapter Two
1 Reagents and Chemicals.................................................................53
2 Experimental models: cell lines .......................................................55
3 Experimental models: Fly strains.......................................................55
4 DNA constructs ........................................................................56
5 Oligonucleotides.........................................................................58
6 Antibodies.................................................................................62
7 Media and buffer composition.........................................................64
8 Software and algorithms.................................................................67

Chapter Three
9 List of miRNAs predicted by PITA algorithm for binding to SV40 3’UTR in the FLuc reporter.................................................................99
# LIST OF FIGURES

## Chapter One
1. A schematic representation of the FMR1 gene and its various allelic forms ........................................ 3
2. Domain organization of human FMRP ........................................................................................................... 6
3. Domain organization of dFMRP .................................................................................................................. 9
4. FMRP regulates mRNA translation at the elongation stage ........................................................................ 21
5. FMRP regulates mRNA translation at the initiation stage ........................................................................... 24
6. FMRP regulates mRNA translation through the interaction with the miRISC components .................................................. 28
7. A schematic of miRNA biogenesis and their assembly into miRISC complex ............................................. 29
8. Mechanisms of miRNA mediated target gene silencing ............................................................................. 34
9. miRNA mediated translation repression at the initiation stage ................................................................. 38
10. miRNA mediated translation repression at the elongation stage ............................................................. 42
11. miRNA mediated co-translational protein degradation ............................................................................ 43

## Chapter Three
12. Tethered FMRP represses the translation of FLuc reporter in a dosage-dependent manner ................................................................. 84
13. The KH domains of tethered FMRP are required for translation repression of the FLuc-BoxB reporter and FMRP-containing granule formation ............................................................. 88
14. Tethered FMRP requires miRISC components to mediate repression of the FLuc-BoxB reporter ................................................................. 91
15. FMRP co-immunoprecipitates with AGO1 and GW182 in S2 cells ........................................................... 93
16. Untethered FMRP can repress the translation of FLuc-BoxB reporter ....................................................... 95
17. miRNA-958 requires FMRP to mediate translation repression of the FLuc-Box reporter ................................................................. 99
18. Untethered FMRP can repress the translation of FLuc reporters for known target mRNAs that contain BoxB-like sequences in their 3’ UTRs ............................................................................. 103
19. FMRP colocalizes to granules containing AGO1 and GW182 in S2 cells ................................................ 106
20. FMRP is expressed with AGO1 and GW182 in the same population of motor neurons within the *Drosophila* larval CNS ........................................................................................................ 109
21. *dFmr1* interacts genetically with *gawky* to regulate synaptic growth ...................................................... 114
22. Trans-heterozygotes of *gawky* display an increased number of 1b boutons ........................................... 116
23. *dFmr1* and miRNA pathway components do not interact to control rates of larval locomotion ................................................................. 119
24. FMRP is expressed with CCR4, CAF1 and NOT1 within the same cells in the larval CNS ................................................................. 123
25. *dFmr1* shows no genetic interactions with *Twin* or Not1 at the larval NMJ ........................................... 127
26. *dFmr1* shows no genetic interactions with *pAbp* or *Dcp1* at the larval NMJ ......................................... 129
27. *dFmr1* does not interact with *Twin, Not1, pAbp* or *Dcp1* to control rates of larval locomotion ............. 133
Chapter Four
28. Proposed model of how FMRP interacts with the miRNA pathway to regulate translation..............................144
CHAPTER ONE: INTRODUCTION

1.1 Overview of Fragile X Syndrome (FXS).

Fragile X Syndrome (FXS) is one of the most frequent forms of inherited mental
disability in humans and a widely studied monogenic cause of autism (Ascano et al. 2012;
Khayachi et al. 2018). FXS is an X-linked disorder that affects about 1:4000 males and
1:8000 females worldwide (Crawford et al., 2001; Kashima et al., 2017; Khayachi et al.,
2018; Penagarikano et al., 2007). Patients with FXS exhibit severe intellectual disability,
delayed development, hyperactivity, abnormal facial traits, macrocephaly, attention deficit,
social behavioral problems and male macroorchidism (Boyle and Kaufmann, 2010; Drozd
et al., 2018).

Genetic mapping of disease-causing mutations revealed that FXS results from an
abnormal expansion of trinucleotide ‘CGG’ repeats within the 5’ untranslated region (UTR)
of the FMR1 gene (Figure 1; Coffee et al., 1999, 2002; Sutcliffe et al., 1992). These CGG
expansions fall into two classes: premutation and full mutation (Bassell and Warren, 2008;
Hagerman and Hagerman, 2002; Schaeffer et al., 2001). Individuals with relatively modest
expansions (55-200) are considered to have a premutation (Jin et al., 2004a; Todd et al.,
2013). Premutation alleles can cause the increased transcription of FMR1 gene and confer
the risk to develop fragile X-associated tremor/ataxia syndrome (FXTAS) in males and
fragile X-related primary ovarian insufficiency (FXPOI) in females (Chen and Joseph,
2015). In contrast, full mutations are characterized by large expansions (ranging between
200- >1000 repeats) (Fu et al., 1991; Li et al., 2008; Schaeffer et al., 2003). This expansion results in hypermethylation of the promoter region of the \textit{FMR1} gene, causing transcriptional silencing and a loss of the encoded Fragile-X mental retardation protein, FMRP (Eberhart et al., 1996; Loomis et al., 2013; Pieretti et al., 1991). Subsequent studies have shown that loss of FMRP alters synaptic structure and function, which contributes to the profound effects observed on learning, memory and cognitive function (Banerjee et al., 2018; Bassell and Warren, 2008; Gibson et al., 2008; Grossman et al., 2006; Richter et al., 2015). Therefore, FMRP deficiency has been well-characterized to directly affect neurodevelopment in FXS patients.
Figure 1: A schematic representation of the FMR1 gene and its various allelic forms. The 5'UTR of the FMR1 gene has a promoter region, polymorphic CGG repeats (pink box), and the coding region. Based on the number of CGG repeats, the FMR1 gene has three main classes of alleles. A) Normal alleles: characterized by less than 55 repeats, proper transcription of FMR1 and translation of FMRP. B) Premutation alleles: Characterized by 55-200 repeats cause increased transcription of FMR1 gene that leads to FXTAS and FXPOI in individuals. Interesting, this results in increased transcription of a “toxic” mRNA that accumulates in the nucleus. Thus, overall levels of FMRP are lower than in healthy individuals. C) Full mutation alleles: Characterized by more than 200 repeats cause an absence of FMRP transcription and translation and leading to FXS.
FMRP is a selective mRNA binding protein that is highly expressed in the central nervous system and testis in mammals (Ashley et al., 1993a; Devys et al., 1993; Hinds et al., 1993). Neurons in FXS patients are characterized by long, thin immature dendritic spines (Bagni and Greenough, 2005; Irwin et al., 2001). These structural defects result from an abnormal synaptic maturation or a failure in the synaptic elimination process (Khayachi et al., 2018). FMRP plays an important role in the translational regulation of specific neuronal transcripts, many of which encode proteins essential for synaptic structure and function (Bassell and Warren, 2008; Bhakar et al., 2012; Edbauer et al., 2010). Previous studies on the regulation of synaptic translation have shown that mutations in translational regulators including Smaug, Nanos, and Pumilio also lead to a similar dendritic phenotype as observed in FXS patients (Gabriela et al., 2013). Taken together, this suggests that the tight regulation of translation is key to normal synaptic function. Further, this suggests that loss of translation regulation by FMRP deficiency could lead to these phenotypes in FXS patients (Bagni and Greenough, 2005).

Some FXS patients have a normal number of CGG repeats but have missense mutations in the coding region of FMRI (Kenny and Ceman, 2016). Two identified missense mutations are isoleucine to asparagine (I304N) in the RNA binding ribonucleoprotein K homology domain (KH2), and arginine to glutamine mutation (R138Q) in a KH-like domain (KH0) (Figure 2; Collins et al., 2010; Feng et al., 1997a; Kenny and Ceman, 2016; Myrick et al., 2015a). These mutations are linked to defects in the association of FMRP with polysomes, target mRNAs and interacting proteins (Feng et al., 1997; Hu et al., 2015; Myrick et al., 2015b; Siomi et al., 1994). Together, these findings
support an important relationship between FMRP-mediated translational control and synaptic dysfunction in FXS (Bassell and Warren, 2008; Ronesi and Huber, 2008).

Currently, there are no effective FDA-approved treatment options in the United States for FXS patients (Banerjee et al., 2018; Berry-Kravis et al., 2017; Penagarikano et al., 2007). This is partly due to a significant gap in our understanding of which specific aspect of neurophysiology is most affected by FMRP deficiency. This suggests that a deeper understanding of the underlying mechanisms by which FMRP can regulate mRNA translation is necessary to better understand the pathogenesis in FXS patients and in order to identify novel therapeutic targets (Banerjee et al., 2018; Belmonte and Bourgeron, 2006; Darnell et al., 2011; Gross et al., 2012; Hagerman et al., 2012; Krueger and Bear, 2011).
Figure 2: Domain organization of human FMRP.
The human FMRP protein has three putative mRNA binding domains: KH0 (yellow box), KH1 (pink) KH2 (green), and the RGG box (orange). KH0 contains a nuclear localization signal (NLS) required for nuclear localization of FMRP. There is also a C-terminal nuclear export signal (NES) required for its role as a nucleocytoplasmic shuttling protein. Also shown are the R138Q, G266E, and 1304N point mutations linked to the severe form of FXS. Indicated above are the amino acid residues.
1.2 Structure and function of FMRP.

Multiple isoforms of FMRP exist in humans due to alternative splicing but much remains to be learned about the precise role and functional significance for each of these variants (Ashley et al., 1993b; Sittler et al., 1996; Verkerk et al., 1993). In mammals, FMRP belongs to a family of RNA binding proteins that include its autosomal paralogs, the Fragile X-Related proteins FXR1 and FXR2 (Ceman et al., 1999; Khandjian, 1999; Kirkpatrick et al., 2001; Siomi et al., 1995; Zhang et al., 1995). FMRP contains three well-defined RNA binding domains: two KH domains (KH1 and KH2) and an arginine-glycine-glycine (RGG) box motif (Ashley et al., 1993a; Siomi et al., 1993). FMRP is enriched in the cytoplasm and binds selectively to about 4% of target mRNAs in the mammalian brain (Ashley et al., 1993a; Brown et al., 2001; Santos et al., 2014).

*Drosophila melanogaster* has been widely used as a genetic model system to study FXS pathology, in part due to the ease of working with fruit flies and conducting genetic studies in drosophila neurons (Drozd et al., 2018; Weisz et al., 2015). *Drosophila* expresses only a single ortholog of FMRP which is called dFMRP (Wan et al., 2000). Sequence comparison shows that dFMRP has 56% overall amino acid similarity to mammalian FMRP (Figure 3; Bagni and Oostra, 2013). However, the RNA binding domains are about 75% identical between dFMRP and human FMRP (Chen et al., 2014; Wan et al., 2000). Moreover, dFMRP has similar biochemical and neuronal functions compared to mammalian FMRP (Dockendorff et al., 2002; Inoue et al., 2002; Ishizuka et al., 2002; Morales et al., 2002; Siomi and Dreyfuss, 1997; Wan et al., 2000; Zhang et al., 2001). These characteristics make *Drosophila* a viable system to study FMRP function.
In both mammalian and fly neurons, FMRP with its multiple conserved domains interacts with specific mRNAs to form membrane-less RNA-protein granules (Aschrafi et al., 2005; Barbee et al., 2006; Bassell, 2011; Mazroui, 2002). In neurons, FMRP is involved in the transport, targeting and translational regulation of synaptically localized mRNAs in response to synaptic stimulation (Antar et al., 2004, 2005, 2006; Bassell and Warren, 2008; Dictenberg et al., 2008; Kiebler and Bassell, 2006; Otero et al., 2002). Additional details of the functions of FMRP are discussed in detail below.
Figure 3: Domain organization of dFMRP.
The dFMRP protein is 56% similar overall to human FMRP with conserved mRNA binding domains: Shown are the conserved KH0 (yellow), KH1 (pink), KH2 (green), and RGG box (orange). Also depicted are NES domains (blue) with amino acids on the top. As with mammalian FMRP, there is an NLS located within the KH0 domain.
1.2.1 Role of FMRP in regulating neurodevelopment.

Proteins encoded by synaptically localized mRNAs modulate the structure and strength of synapses, memory formation, and cognitive functions (Bramham and Wells, 2007; Costa-Mattioli et al., 2009; Lin and Holt, 2008; Martin and Zukin, 2006; Steward and Schuman, 2003; Zukin, 2009). Dysregulation of local protein synthesis and mRNA transport is closely linked with improper neurodevelopment and synapse maturation (Bassell, 2011; Gabriela et al., 2013; Xing and Bassell, 2013). FMRP is thought to be involved in maintaining proper synaptic plasticity by binding to several key neuronal transcripts and maintaining them in a translationally inactive state in mRNA-containing ribonucleoprotein (mRNP) granules (Bagni and Greenough, 2005; Ferrari et al., 2007; Miyashiro et al., 2003; Muddashetty et al., 2007; Todd et al., 2003; Zalfa et al., 2003; Zukin, 2009).

FMRP is involved in the transport, stability and translation of several neuronal transcripts (Bagni and Oostra, 2013; Fernández et al., 2013). FMRP transports several mRNAs, including the Fmr1 mRNA itself, from the cell body towards synapses in an activity-dependent manner through an association with motor proteins including kinesin (Fernández et al., 2013; Ferrari et al., 2007). For example, the loss of FMRP impairs the localization of several important mRNA targets such as MAP1B and the PSD-95-associated protein 4 (Sapap4) mRNA to synapses (Dictenberg et al., 2008; Kao et al., 2010). Furthermore, FMRP can modulate the stability of some mRNAs by preventing or promoting mRNA decay (Fernández et al., 2013; Zalfa et al., 2010; Zhang et al., 2007). For example, previous studies have shown that the association of hippocampal FMRP can
protect PSD-95 transcript from decay (Zalfa et al., 2010). In contrast, it can promote the decay of nuclear RNA export factor 1 (NXF1) transcript in mouse neuroblastoma (N2a) cells (Zhang et al., 2007). Together, this suggests that FMRP’s interaction with specific transcripts regulates their stability and localization in a transcript-dependent manner.

FMRP plays an important role in repressing the translation of a subset of mRNAs. Studies in cells derived from FXS patients as well as mice and flies lacking FMRP show an increased rate of translation for mRNAs targeted by FMRP (Bolduc et al., 2008; Brown et al., 2001; Dolen et al., 2007). These data support a role for FMRP as a translation repressor. Based in part on these data, it is hypothesized that translation dysregulation of FMRP associated mRNAs is the major contributor to FXS pathology (Bagni and Oostra, 2013; Fernández et al., 2013; Richter et al., 2015). In the absence of FMRP, key target mRNAs are excessively translated in dendritic spines leading to defects in synaptic morphology (Bagni and Greenough, 2005; Cruz-Martin et al., 2012; Darnell and Klann, 2013; Grossman et al., 2006; Mientjes et al., 2006; Nimchinsky et al., 2001; The Fragile X Consortium, 1994; Zhang et al., 2001).

In mammalian neurons, FMRP activity is regulated by metabotropic glutamate receptor (mGluR) stimulation (Antar et al., 2004; Bear et al., 2004; Dictenberg et al., 2008; Weiler et al., 1997). Activation of mGluR induces the rapid dephosphorylation of FMRP followed by ubiquitination and subsequent proteasome-mediated degradation (Hou et al., 2006; Nalavadi et al., 2012). As a result, specific FMRP-bound mRNAs are released from RNP granules and translated to make proteins (Aschrafi et al., 2005; Bear et al., 2004; Muddashetty et al., 2011). Conversely, phosphorylation of FMRP inhibits the translation
of its associated mRNA (Narayanan et al., 2007; Niere et al., 2012). Interestingly, recent studies suggest that the activation of mGLuR can also lead to the reversible sumoylation of FMRP, suggesting that mGLuR activation can regulate FMRP’s function through different mechanisms (Khayachi et al., 2018).

Many studies have focused on identifying the mRNAs whose translation is regulated by FMRP in order to understand the neuropathology of FXS (Banerjee et al., 2018; Darnell and Klann, 2013; Richter et al., 2015). Several approaches have been used to identify FMRP associated mRNAs in neuronal mRNA granules (Brown et al., 2001; Darnell et al., 2011; El Fatimy et al., 2016). Isolation and purification of FMRP-containing neuronal granules from mouse brain homogenates has led to the identification of several associated transcripts (El Fatimy et al., 2016). Additionally, crosslinking-immunoprecipitation combined with high-throughput sequencing (HITS-CLIP) has been used to identify FMRP-associated target mRNAs (Darnell et al., 2011). Finally, photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) approach was utilized to isolate FMRP bound target mRNAs and to identify binding sites (Ascano et al., 2012). Collectively, these studies have demonstrated that FMRP-associated target mRNAs that encode for key proteins involved in the control of synaptic structure and function. Some notable examples of these mRNAs include the microtubule-associated protein 1b (MAP1B), Down syndrome cell adhesion molecule (DSCAM), Postsynaptic density protein 95 (PSD-95), FMRP, Ataxin, Human antigen R (HuR), Calcium/Calmodulin-dependent protein kinase II (CaMKII), Amyloid precursor protein (APP), the Activity-regulated cytoskeleton-associated protein (ARC), Zinc-finger
CysCysCysHis [CCCH]-type 14 (ZC3H14), Ras-related C3 botulinum toxin substrate 1 (Rac1) and Staufen2 (Bienkowski et al., 2017; Brown et al., 2001; Darnell et al., 2011; Davis and Broadie, 2017; Elvira et al., 2006; El Fatimy et al., 2016; Kanai et al., 2004; Muddashetty et al., 2007; Sudhakaran et al., 2014; Suhl et al., 2015; Zalfa et al., 2003; Zhang et al., 2015). FMRP granules are also enriched in mRNAs which encode for molecular motor proteins (Dync1h1, Myo18a, Myh10, Myo5a) and several members of the spectrin and ankyrin families, which are essential for synaptic stability and maintenance (Darnell et al., 2011; El Fatimy et al., 2016; Lindsay and Mccaffrey, 2014). Targets of dFMRP have also been identified in Drosophila neurons, including Futsch (the fly orthologue of MAP1B), Dscam (the fly orthologue of DSCAM), chickadee (the fly orthologue of Profilin), Rac1 (the fly ortholog of Rho GTPase RAC1) and pickpocket1 (the fly ortholog of PPK1) (Lee, 2003; Reeve et al., 2005; Sterne et al., 2015; Xu et al., 2004; Zhang et al., 2001). These studies provide an extensive list of mRNAs whose activity is regulated by FMRP, and mis-regulation of which may contribute to aberrant dendritic spine development in Fragile X patients (El Fatimy et al., 2016).

1.2.2 Function of RNA binding domains of FMRP.

FMRP has multiple conserved RNA binding domains that allow it to bind to and regulate the translation of several mRNAs (Ashley et al., 1993a; Gareau et al., 2013a; Siomi et al., 1996). Previous studies have demonstrated that essential functions of FMRP rely on functional KH domains, as missense mutations within these domains can cause a severe form of FXS. These mutations (G266E within KH1), I304N in KH2 and R138Q in KH0) disrupt the association of FMRP with target mRNA and polyribosomes (Figure 2;
De Boulle et al., 1993; Feng et al., 1997a; Hu et al., 2015; Myrick et al., 2014, 2015b, 2015a). However, the precise role of these KH domains in mediating the interaction of FMRP with target mRNAs is not clear. Darnell et al demonstrated that KH2 domain binds to a double stem-loop structure in RNAs known as “kissing complex” or “loop-loop pseudo-knot” (Darnell et al., 2005; Kenny and Ceman, 2016). The authors showed that RNAs with this structure could compete FMRP off polyribosomes, suggesting that FMRP strongly binds to RNAs containing this secondary structure (Darnell et al., 2005). However, no structure of this type has been reported in any known endogenous target mRNAs (Santoro et al., 2012). The identity of specific structures in mRNA targets bound by KH1 and KH0 domains is not known (Kenny and Ceman, 2016). Thus, the precise role and function of KH domains in binding target mRNAs needs to be studied in detail.

FMRP also possesses a well-characterized RGG box motif, which selectively binds to G-rich secondary structures known as G-quadruplexes (GQs) (Blackwell et al., 2010; Darnell et al., 2001, 2004; Dolzhanskaya, 2006; Menon and Mihailescu, 2007; Phan et al., 2011; RAMOS, 2003; Schaeffer et al., 2001). G-quadruplexes are formed by guanine tetrads held together by Hoogsteen base-pairing and stabilized by monocations (Blice-Baum and Mihailescu, 2014; Joachimi et al., 2009; Santoro et al.; Schaeffer et al., 2001; Williamson et al., 1989). Studies show FMRP binds to several target mRNAs including MAP1B which are capable of folding into distinct G-quadruplex structures (Darnell et al., 2001; Schaeffer et al., 2001). This suggests that FMRP can regulate the activity of specific mRNAs that can fold into G-quadruplex substructures.
Recent studies also have indicated that low complexity domains (LCDs) within the conserved RGG motif can modulate the flexible interactions of FMRP with several mRNA ligands and proteins (Kenny and Ceman, 2016; Ozdilek et al., 2017). These domains are unstructured and disordered in solution, become structured when bound to a target mRNA, and are proposed to drive RNP granule formation (Coletta et al., 2010; Kenny and Ceman, 2016). However, it is not clear whether these domains co-operate with other domains of FMRP to mediate its activity (Davis and Broadie, 2017).

1.2.3 FMRP binds to distinct mRNA sequence and structural elements.

The RNA binding domains of FMRP recognize a variety of specific sequence and structural motifs in target mRNAs (Ashley et al., 1993a; O’Donnell and Warren, 2002; Siomi et al., 1993). Recent studies using PAR-CLIP indicate that the KH2 and KH1 domains of FMRP preferentially bind to ACUK, WGGA and GAC sequences in target mRNAs (in which K=G or U and W= A or U) (Ascano et al., 2012; Ray et al., 2013; Suhl et al., 2014b, 2014a). However, the frequency of these sequences to be present in all human mRNAs is very high (1 in 128 nucleotides) (Chen et al., 2014). Therefore, it is unknown how these sequences can provide FMRP the specificity required to bind to its target mRNAs (Chen et al., 2014). G-quadruplex structures are found in several (but not all) FMRP-associated targets including the *Fmr1, MAP1b*, and *Sema3F* mRNAs (Didiot et al., 2008; Menon and Mihaiiescu, 2007; Menon et al., 2008; Santoro et al., 2012; Schaeffer et al., 2001). However, an analysis of FMRP associated neuronal mRNAs in mice by HITS-CLIP did not show an enrichment for G-rich quadruplexes or pseudoknot forming
sequences (Darnell et al., 2011; Kenny and Ceman, 2016). Therefore, specific RNA elements that recruit FMRP to target mRNAs are not well understood.

FMRP has been shown to bind to other structural motifs in specific target mRNAs. For example, FMRP binds to a unique structural motif composed of three independent stem-loops in the superoxide Dismutase 1 (Sod1) mRNA through the RGG box domain (Anderson et al., 2016). This motif termed as ‘Sod1 mRNA Stem Loops Interacting with FMRP’ (SoSLIP) competes with the GQs for binding to the RGG box (Bechara et al., 2009; Santoro et al., 2012). Surprisingly, these motifs are structurally modified upon interaction with FMRP and promote the translation of Sod1, in contrast to FMRP’s canonical role as a translation repressor (Bechara et al., 2009). Other RNA secondary motifs have also been identified in FMRP targets, including U-rich sequences with 5-23 bases of U-rich pentamer repeats in some FMRP target mRNAs (Chen et al., 2003; Dolzhanskaya et al., 2003; Fähling et al., 2009). The U-rich motifs need further characterization as not much is known about the role of these motifs or the specific domains in FMRP that mediate this interaction (Santoro et al., 2012).
1.3 FMRP acts as a translation repressor through different mechanisms.

FMRP is characterized as a protein that regulates the translation of several mRNAs in mammalian cells. An early study identified FMRP to be associated with the 60S ribosomal subunit (Siomi et al., 1996). However, the functional consequences of that association were not known. Laggerbauer et al. provided the first evidence for a function of FMRP in translation regulation (Laggerbauer et al., 2001). The authors incubated recombinant FMRP with in vitro transcribed mRNAs encoding for the survival of motor neuron (SMN) protein, FMRP or the luciferase protein in rabbit reticulocyte lysate assays. In this system, FMRP strongly inhibited the translation of all these mRNAs in a sequence-independent manner. Moreover, Xenopus oocytes injected with the FMRP-mRNA complexes showed identical results (Laggerbauer et al., 2001). Immortal cells from Fmr1 KO mice provided the first in vivo evidence for FMRP function as a translation repressor (Mazroui, 2002). Fmr1 KO cells were co-transfected with human FMRP and reporter genes encoding for FXR1P and the sine oculis homeobox homolog transcription factor (SIX3) (Mazroui, 2002). The transfection of human FMRP led to a reduction in the translation of reporter genes, suggesting FMRP to be a negative regulator of translation.

Consistent with the role of FMRP as a translation repressor, in vivo assays in Fmr1 KO mice exhibited an increase in the expression of FMRP-target proteins including MAP1B, Arc and CamKII (Santoro et al.; Zalfa et al., 2003). dFmr1 null flies also showed an increase in the expression of the MAP1B ortholog, futsch (Zhang et al., 2001). Moreover, subcellular fractionation of synaptoneurosomes from Fmr1 KO mice show elevated expression levels for other FMRP target proteins (Zalfa et al., 2003). Together,
these results highlight the ability of FMRP to repress the translation of several mRNAs in both mammalian cells and in *Drosophila* neurons.

Several *in vitro* studies suggested that direct interaction of FMRP with its target mRNA is critical for translation repression (Li et al., 2001). The authors removed the 3'UTR from the myelin basic protein mRNA (*MBP*), a known target of FMRP regulation in mammalian oligodendrocytes, and observed an inability of FMRP to repress its translation (Li et al., 2001; Wang et al., 2004; Zalfa et al., 2010). In contrast, FMRP repressed the translation of the full length MBP transcript (Li et al., 2001). This suggests that the association of FMRP with its target regulates its ability to repress translation.

In addition to interacting with polyribosomes, FMRP also localizes to RNP granules known as P-bodies and stress granules, which are high-order complexes that contain repressed mRNAs (Barbee et al., 2006; Mazroui, 2002; Santoro et al., 2012). FMRP is hypothesized to control the translation of mRNAs in part by promoting their movement between these “storage” granules and polysomes (Aschrafi et al., 2005; Bassell and Warren, 2008). However, the mechanism behind this process remains unknown.

FMRP is suggested to be an effector of translational repression through several different mechanisms (Ascano et al., 2012; Brown et al., 2001; Chen et al., 2014; Darnell et al., 2001, 2005; Jin et al., 2004b; Kenny and Ceman, 2016; Stefani, 2004; Zalfa et al., 2003). However, these mechanisms by which FMRP regulates translation of neuron targets remain poorly understood (Bagni, 2008; Chen et al., 2014; Costa-Mattioli et al., 2009; Iacoangeli et al., 2008a; Kenny and Ceman, 2016). FMRP can repress the translation of target mRNAs at both initiation and elongation stages (Bhakar et al., 2012; Ceman et al.,
2003; Santoro et al., 2012). FMRP also regulates mRNA translation via the miRNA pathway in P-bodies (Barbee et al., 2006; Eulalio et al., 2007a; Parker and Sheth, 2007). It is important to note that these mechanisms are not mutually exclusive. It is likely that the repression of FMRP targets by FMRP binding is mRNA specific.

1.3.1 Evidence that FMRP stalls translation at the elongation stage.

Previous studies suggest that FMRP causes ribosomes to stall during the elongation stage of translation (Figure 4). In-vivo CLIP studies of FMRP demonstrate that it can directly bind to the coding regions of some target mRNAs (Stefani, 2004). Subsequent studies by Darnell and colleagues demonstrated that FMRP co-sediments with polyribosomes and confers resistance to puromycin, which releases elongating ribosomes (Darnell et al., 2011; Harigaya and Parker, 2014). These results indicate that by binding to coding regions of mRNA, FMRP could either block puromycin action or could impede ribosome movement to slow translation elongation. Interestingly, after puromycin treatment, target mRNAs of FMRP were deeper in the polysome gradient compared to non-targets (Darnell et al., 2011; Harigaya and Parker, 2014). These results suggest FMRP causes increased retention of ribosomes on target mRNA. The authors further characterized FMRP-stalled complex with electron microscopy and demonstrate target mRNAs to be complexed with multiple ribosomes (Darnell et al., 2011). Ribosomal run-off assays on these transcripts also demonstrate that FMRP is associated with stalled ribosomes on these transcripts (Ceman et al., 2003). Phosphorylation of FMRP at serine residues correlates with its association with stalled polyribosomes, and dephosphorylated FMRP associates with actively translating polyribosomes (Ceman et al., 2003). These results suggest that the
molecular mechanism controlling FMRP phosphorylation may thereby regulate translation elongation of FMRP targeted mRNAs.

Recent cryo-electron microscopic studies in Drosophila suggest that FMRP stalls ribosome movement on mRNAs to repress their translation (Chen et al., 2014; Harigaya and Parker, 2014). The authors showed that FMRP directly binds to the L5 protein of 80S subunit of the ribosome, blocking the access of tRNAs and essential translation elongation factors to inhibit translation (Chen et al., 2014). According to Chen et al., FMRP binds to specific structural elements in target mRNA, which leads to the KH2 domain of FMRP interacting with the 80S subunit of the ribosome (Chen et al., 2014; Harigaya and Parker, 2014). This interaction causes a steric conflicts with the tRNA at the P-site, leading to ribosomal stalling (Chen et al., 2014). These results also explain why FMRP-bound mRNAs are resistant to puromycin treatment, since FMRP’s interaction with the 80S subunit will block access of puromycin to the ribosome.

Recent studies directly compared the movement of ribosomes along mRNAs in brain lysates from wild-type and Fmr1 KO mice (Richter et al., 2015; Udagawa et al., 2013). These lysates were supplemented with hippuristanol, a drug that blocks new translation initiation, allowing ribosomes already associated with transcripts to runoff the transcript after completing translation (Bordeleau et al., 2006; Richter et al., 2015; Udagawa et al., 2013). The authors found that the rate of radioactive amino acid incorporation was higher in Fmr1 KO lysates compared to wild-type lysates. Given that new translation initiation was blocked in these experiments, these results strongly suggest that FMRP stalls ribosomes on target mRNAs and blocks translation elongation.
Figure 4: FMRP regulates mRNA translation at the elongation stage.
FMRP can stall the progression of ribosomes along the target mRNA. Electron microscopy shows FMRP to be in a complex with polyribosomes. Recent cryo-EM studies demonstrate that dFMRP can directly bind to the 80S ribosome through KH2 domains and interfere with the binding of essential elongation factors to the ribosome. Post-transcriptional modification of FMRP such as phosphorylation at serine residues and methylation on arginine residues can modulate the association of FMRP with ribosomes.
1.3.2 Evidence that FMRP stalls translation at the initiation stage.

While a significant amount of research points to a role of FMRP in regulating translation at elongation stage, some data suggests that FMRP blocks the translation of specific mRNAs at the initiation stage. Specifically, it was shown that FMRP can block translation initiation by modulating the interaction between the cytoplasmic FMRP-interaction protein (CYFIP1) and eukaryotic initiation factor eIF4E which binds to the m7G cap and initiates translation (Figure 5; Napoli et al., 2008; Richter et al., 2015; Schenck et al., 2001). Bulk cap-dependent translation requires the formation of the eIF4A-eIF4G-eIF4E (eIF4F) complex in association with the 5’ m7G cap structure (Richter and Sonenberg, 2005). eIF4E-binding proteins (4E-BPs) regulate the formation of eIF4E complex by interfering with eIF4E-eIF4G interaction (Banko et al., 2007; Klann and Richter, 2007; Marcotrigiano et al., 1999; Richter and Sonenberg, 2005), and phosphorylation of 4E-BPs results in their dissociation from eIF4E and initiation of translation (Gingras et al., 2001). FMRP interacts biochemically with CYFIP1, a protein which has a non-canonical eIF4E-binding protein (4E-BP) domain (Marcotrigiano et al., 1999). Another binding partner of FMRP, the noncoding brain cytoplasmic RNA 1 (BC1), helps to facilitate the interaction between FMRP and the CYFIP-eIF4E complex in the mammalian brain (Napoli et al., 2008; Zalfa et al., 2003, 2005).

FMRP-CYFIP1-eIF4E is transported as a translationally inactive complex in dendrites stabilized by target mRNAs and responds to synaptic stimulation induced by either brain-derived neurotrophic factor (BDNF) or group 1 mGluR signaling (DeRubeis et al., 2013; Napoli et al., 2008). Evidence suggests that local synaptic stimulation results
in the release of FMRP-CYFIP1 from eIF4E to rapidly alleviate translation repression (DeRubeis et al., 2013; Napoli et al., 2008; Richter et al., 2015). Consistent with these results, genetic loss of CYFIP1 in the brain results in an increase in the expression of known FMRP targets including MAP1B, APP, CaMKII (Bagni and Greenough, 2005; Hou et al., 2006; Napoli et al., 2008; Westmark and Malter, 2007).

The in vivo significance of the FMRP-BC1-CYFIP interactions, however, has been challenged (Iacoangeli et al., 2008a, 2008b; Stefani, 2004; Wang et al., 2005). In one specific study, the authors were unable to detect specific interactions between FMRP and BC1 mRNA in vitro (Iacoangeli et al., 2008b). Moreover, competitor tRNA abolished the association between FMRP and BC1 mRNA in mobility shift assays, suggesting that the binding of FMRP to BC1 may be non-specific (Iacoangeli et al., 2008b; Wang et al., 2005). Therefore, the model of FMRP-mediated interaction between CYFIP1 and eIF4E and its role in blocking translation initiation is contested.
**Figure 5: FMRP regulates mRNA translation at the initiation stage.**
FMRP has been shown by some to recruit CYFIP1 (orange) and sequester the general translation initiation factor eIF4E (green) from binding to eIF4G (light green). The inability to form the pre-initiation complex leads to translation repression. BC1 mRNA increases the affinity of FMRP-CYFIP1 interaction *in vitro*. Studies propose that in response to mGLuR activation, the CYFIP1-FMRP complex gets released from eIF4E for the local translation at the synapses. This model of action is highly contested.
1.4 miRNA biogenesis and miRISC assembly.

FMRP can also regulate the translation of target mRNAs through its interaction with specific miRNAs and conserved components of the RNA-induced silencing complex (RISC) (Figure 6; Fabian and Sonenberg, 2012). Recent studies suggest that FMRP cooperates with miRNAs to modulate synaptic structure and function (Loffreda et al., 2015). Before addressing the details of how FMRP interacts with miRNAs and miRISC, an overview of the regulation of miRNA biogenesis and function is summarized below.

miRNAs are small ∼22 nucleotide non-coding mRNAs with an important role in regulating gene expression by binding to complementary mRNAs and regulating translation (Baek et al., 2008; Bartel, 2009; Eulalio et al., 2008a; Fabian et al., 2010; Ha and Kim, 2014; Krol et al., 2010; Lim et al., 2005; Selbach et al., 2008). Published evidence indicates a role for miRNAs in nearly every biological process ranging from development to the regulation of the cell cycle and synaptic plasticity (Ambros, 2011; Bushati and Cohen, 2007; Eulalio et al., 2008a; Kim et al., 2009; McNeill and Van Vactor, 2012; Nesler et al., 2013; Shenoy and Blelloch, 2014). miRNA biogenesis and function are tightly regulated, and dysregulation of their expression or function is linked to human diseases ranging from cancer to neurodevelopment disorders (Chang and Mendell, 2007; Garzon et al., 2009; McNeill and Van Vactor, 2012; Saugstad, 2010).

miRNA genes are transcribed by RNA polymerase II (Pol II) to form the long primary miRNA (pri-miRNA) (Figure 7; Filipowicz et al., 2008a; Ha and Kim, 2014; Kim, 2005; Lee et al., 2004a). Following transcription, the pri-miRNA undergoes several steps of processing (Denli et al., 2004; Ha and Kim, 2014). The pri-miRNA folds into a dsRNA
hairpin structure with the embedded miRNA sequences and undergoes cleavage by the microprocessor complex to form precursor miRNAs (pre-miRNA) (Kim et al., 2009; Lee et al., 2002). The microprocessor complex is composed of dsRNA binding proteins with endonucleolytic activity: DROSHA and DGCR8 (Pasha in flies) (Du, 2005; Gregory et al., 2004; Han et al., 2004, 2006; Kim et al., 2009; Landthaler et al., 2004). The pre-miRNA is exported from into the cytoplasm by Exportin 5 (XPO5) in a RAN-GTP dependent manner (Bohsack et al., 2004; Kim, 2004; Lund et al., 2004). Upon export, the pre-miRNA is further cleaved by Dicer to produce a miRNA duplex of approximately 22 nucleotides (Bernstein et al., 2001; Grishok et al., 2001; Hutvágner et al., 2001; Ketting et al., 2001; Knight and Bass, 2001). Like DROSHA, Dicer also belongs to an RNAase endonuclease family and cleaves dsRNAs (Ma et al., 2004; Song et al., 2003; Zhang et al., 2004). Dicer acts as a molecular ruler and cuts miRNAs at specific cleavage sites with a fixed distance of about 22-25 nucleotides from either 3' or 5' end (Coffee et al., 2002; Macrae et al., 2006; MacRae et al., 2007; Vermeulen et al., 2005; Zhang et al., 2002, 2004). Although mammals have a single Dicer gene, *Drosophila* expresses two Dicer paralogs (Ha and Kim, 2014; Kim et al., 2009). Dicer 1 is required for miRNA biogenesis whereas Dicer 2 is involved in short interfering (siRNA) processing (Lee et al., 2004b). Several RNA binding proteins (RBPs) modulate the activity of Dicer for efficient processing of most miRNAs (Chendrimada et al., 2005; Förstemann et al., 2005; Fukunaga et al., 2012; Lee and Doudna, 2012; Lee et al., 2013; Saito et al., 2005; Trabucchi et al., 2009). In *Drosophila*, Dicer1 associates with Loquacious (Loqs) for efficient pre-miRNA processing (Kim et al., 2009), whereas in humans, Dicer interacts with TAR RNA binding protein (TRBP) and
Protein activator of the interferon-induced protein kinase (PACT) protein for formation of the miRISC complex (Ha and Kim, 2014; Kim et al., 2009).
Figure 6: FMRP regulates mRNA translation through the interaction with the miRISC components.
Genetic and biochemical studies show that FMRP associates directly with miRNAs, Dicer, and AGO1. AGO1 is a core component of the miRNA-containing RISC complex (miRISC). FMRP can recruit the miRISC complex to facilitate the recognition of target mRNAs and regulate the translation of a target mRNA using miRNAs. The FMRP-miRISC complex can repress translation at either the initiation or at the elongation stage. The mechanisms regulating the interaction between FMRP and the RISC remain unclear.
Figure 7: A schematic of miRNA biogenesis and their assembly into miRISC complex.

miRNA genes are transcribed by RNA polymerase II to generate the pri-miRNA. The nuclear endonuclease DROSHA and DGCR8 process the pri-miRNA to form the pre-miRNA hairpin structure. Then, XPO5 recognizes the pre-miRNA and exports it to the cytoplasm for further processing by Dicer. Dicer catalyzes the formation of the pre-miRNA to generate miRNA duplex. One strand of the duplex, known as the guide strand, is preferentially loaded onto the AGO proteins to form the miRISC complex. The miRISC complex binds to miRNA binding sites, primarily in the 3'UTR of the target mRNA to mediate translation repression or decay. The nucleotides at the 5'end of the miRNA known as the miRNA seed are crucial for the recognition of the target mRNA.
1.4.1 miRNA loading and RNA induced silencing complex formation.

Once the mature miRNA duplex is generated following several processing steps, the duplexed RNA is loaded onto the Argonaute (AGO) protein (Figure 7; Elkayam et al., 2012; Gregory et al., 2005; MacRae et al., 2008; Maniataki and Mourelatos, 2005; Nakanishi et al., 2012; Schirle and MacRae, 2012). Loading of the miRNA duplex is followed by a subsequent unwinding of the strands. The non-associated strand, called the “passenger strand”, is released and degraded, whereas the active strand, called the “guide strand” is retained to complete the formation of the miRNA-containing RISC (Diederichs and Haber, 2007; Ha and Kim, 2014; Kawamata and Tomari, 2010). The guide strand is selected on the basis of the relative thermodynamic stability of the 5' and 3' end of the small RNA duplex (Khvorova et al., 2003; Schwarz et al., 2003).

Mammals express four AGO paralogs (AGO1-4) which are capable of inducing translation repression or decay of target mRNAs to varying degrees utilizing either the miRNA or the siRNA pathways (Ha and Kim, 2014; Huntzinger and Izaurralde, 2011; Ipsaro and Joshua-Tor, 2015; Yoda et al., 2010). In contrast, *Drosophila* expresses two paralogs of the AGO proteins (dAGO1 and dAGO2) (Förstemann et al., 2007; Okamura et al., 2004; Tomari et al., 2007). dAGO1 is utilized by the miRNA pathway and dAGO2 is utilized by the siRNA pathway (Czech et al., 2009; Kawamata et al., 2009).

Thermodynamic stability of base-pairing between the miRNA and complementary sequences generally located in the 3'UTR of target mRNA determines the ability of miRNA to repress translation of target mRNA (Clarke et al., 2012). The nucleotides at positions 2-8 at the 5' end of the miRNA, known as seed region, are crucial for miRNA target
recognition (Ameres and Zamore, 2013; Bartel, 2009; Brennecke et al., 2005; Doench and Sharp, 2004; Grimson et al., 2007; Pasquinelli, 2012). Following miRNA binding, the regulation of target mRNAs is facilitated by another core protein component of the miRISC, the trinucleotide repeat containing protein (TNRC; also known as GW182; Eulalio et al., 2008b, 2009; Fabian and Sonenberg, 2012; Huntzinger and Izaurralde, 2011; Pfaff and Meister, 2013). The primary function of GW182 is to acts as a scaffold for other accessory proteins that mediate either translation repression or mRNA degradation (Braun et al., 2011, 2013; Chekulaeva et al., 2011; Fabian and Sonenberg, 2012; Fabian et al., 2012; Huntzinger et al., 2010, 2013; Zekri et al., 2009). How the bound miRNA regulates translation of the target mRNA is described in detail below.

1.4.2 Mechanisms of miRNA-mediated gene silencing.

miRNAs have been shown to target mRNAs for translational repression via four distinct mechanisms: translation initiation, translation elongation, premature termination (ribosome drop off) and co-translational protein degradation (Chendrimada et al., 2007; Eulalio et al., 2008a; Hu et al., 2010; Humphreys et al., 2005; Kiriakidou et al., 2007; Maroney et al., 2006; Nottrott et al., 2006; Petersen et al., 2006; Pillai et al., 2005; Seggerson et al., 2002; Tat et al., 2016). miRNAs can also directly target mRNAs for degradation (Figure 8; Bagga et al., 2005; Braun et al., 2011; Chekulaeva et al., 2011; Eulalio et al., 2007b; Fabian et al., 2012; Rehwinkel et al., 2005; Wu et al., 2006). mRNA degradation is initiated by a gradual shortening of the poly (A) tail by components of the deadenylase complexes, CCR4-NOT1 and PAN2-PAN3 (Antic et al., 2015; Decker and Parker, 2012; Houseley and Tollervey, 2009; Parker and Song, 2004). In eukaryotes, the
poly (A) tail is initially shortened by the PAN2-PAN2 complex, followed by rapid deadenylation by the CCR4-NOT complex (Wahle and Winkler, 2013; Yamashita et al., 2005). Following deadenylation, mRNAs can either be degraded by the exosome in a 3'-5' manner or undergo decapping by the DCP1-DCP2 complex followed by 5'-3' decay by the XRN1 exonuclease (Decker and Parker, 2012; Meyer et al., 2004).

There is a lack of clear understanding of what factors determine whether a specific mRNA-regulated mRNA will undergo translation repression or degradation (Eulalio et al., 2008a). Thus, whether or not miRNAs elicit mRNA degradation is likely to be strongly dependent upon specific features of the miRNA-binding site, miRISC factors, specific accessory proteins associated with the miRISC, target mRNA and various additional interacting RBPs (Eulalio et al., 2007b, 2008a; Grimson et al., 2007). The evidence supporting known mechanisms of miRNA-mediated translation repression at both the initiation and elongation stages are outlined below.
Figure 8: Mechanisms of miRNA mediated target gene silencing.
miRNAs (red) can either inhibit translation of target mRNAs or facilitate their deadenylation followed by subsequent decapping, and decay. These mechanisms require core components of the miRISC complex, AGO1 (orange), and GW182 proteins (green). miRNAs can repress the translation of target mRNAs by blocking translation initiation, elongation or by premature termination of ribosomes. Alternatively, GW182 can recruit the components of the deadenylase complex (CAF1, CCR4 and the NOT proteins) to facilitate deaadenylation of the poly (A) tail. Following deadenylation, the Dcp1-Dcp2 complex removes the m7G cap to facilitate 5’ to 3’ exonucleolytic decay. Repressed mRNAs can move to P-bodies for either storage or degradation. Deadenylation as well as the displacement of poly(A)-binding protein (PABP) through GW182 and CCR4–NOT also contribute to the overall miRNA-mediated translational repression.
1.4.2.1 Translation repression by miRNAs at the initiation stage.

Several studies suggest that miRNAs inhibit translation of target mRNAs at the initiation stage (Figure 9; Eulalio et al., 2008a). Pillai et al demonstrated that miRNAs and target mRNAs do not co-sediment with the polysomal fractions in sucrose gradients, but rather with the free mRNP pool in mammalian cells (Humphreys et al., 2005; Pillai et al., 2005). These results are indicative of a defect in translational initiation due to an impaired ribosome recruitment (Ding and Großhans, 2009; Fabian and Sonenberg, 2012). This work, coupled with several other studies, showed that mRNAs lacking the m7G cap can bypass miRNA mediated repression. This indicates that miRNAs can target the m7G cap-dependent translation and inhibit translation at the initiation stage (Filipowicz et al., 2008; Pillai et al., 2005; Wu and Belasco, 2008). Moreover, studies in cell free assays provide support for a role of miRNAs in inhibiting translation initiation (Mathonnet et al., 2007; Wakiyama et al., 2007). In cell lysates, miRNAs repressed translation of m7G-capped mRNAs but not mRNAs with an artificial unmethylated cap analog (ApppN) (Huntzinger and Izaurralde, 2011). Further, miRNAs also did not repress the translation of mRNAs containing an internal ribosome entry site (IRES) (Filipowicz et al., 2008; Mathonnet et al., 2007; Wakiyama et al., 2007). The importance of m7G cap was further supported by experiments with bi-cistronic reporters. The translational activity of the first cap-dependent cistron was repressed by the endogenous let-7 miRNA. On the other hand, the second cistron was unaffected by let-7 miRNA (Filipowicz et al., 2008; Pillai et al., 2005). Taken together, these results suggest that cap-dependent translational initiation is repressed via the miRNA-mRNA interaction.
In agreement with a role for miRNAs inhibiting translation initiation, several studies suggest that translation initiation factors play a critical role in miRNA-mediated repression (Iwakawa and Tomari, 2015; Kiriakidou et al., 2007; Mathonnet et al., 2007). Supplementation or depletion of translation initiation factors in HEK293 cells, S2 cells and *in vitro* cell-free translation system results in an attenuation of miRNA-mediated translation repression (Fukao et al., 2014; Fukaya et al., 2014; Meijer et al., 2013). The eIF4F complex is composed of three proteins: the cytoplasmic cap-binding protein eIF4E, the scaffolding protein eIF4G and the RNA helicase eIF4A (Huntzinger and Izaurralde, 2011). Interestingly, Kiriakidou et al., (2007) demonstrated that central domains of AGO proteins exhibit sequence similarity to the cap binding region of eIF4E (Kiriakidou et al., 2007). The authors found that mutations of two crucial phenylalanines in this region severely compromised the ability of tethered AGO proteins to repress reporter mRNAs. These data suggest that AGO proteins might compete with eIF4E for m7G binding, block the eIF4E-eIF4G interaction, abolish mRNA circularization and thus prevent the translation of capped mRNAs (Hurschler et al., 2010; Kiriakidou et al., 2007).

More recently, studies using *in vitro* UV crosslinking indicate that miRNAs dissociate the eIF4A protein from target mRNAs (Fukaya et al., 2014). eIF4A is an RNA helicase normally involved in unwinding the secondary structures within target mRNA to allow the 43S pre-initiation complex to scan the 5'UTR for translation (Jackson et al., 2010). The authors showed that crosslinking of eIF4A to reporter mRNA was abolished in the presence of miRNAs, whereas crosslinking of eIF4E showed no change. These results indicate that miRISC targets eIF4A and not eIF4E for repression. The interaction between
eIF4A proteins and miRISC components is proposed to block 43S scanning, resulting in translation repression (Fukaya et al., 2014; Meijer et al., 2013; Ricci et al., 2013). Fukao et al. used multiple approaches in HEK293 cells to show the significance of the dissociation of eIF4A for miRNA-mediated translational repression. The authors used in vitro translation assays to assess the effect of eIF4A proteins on target mRNA repression. In this system, miRNAs failed to repress the translation of a Hepatitis C virus (HCV) IRES-containing reporter mRNA, which is known to be translated independently of eIF4A (Fukao et al., 2014). Furthermore, HuD and silvestrol, which facilitate the retention of eIF4As on mRNAs attenuated miRNA-mediated dissociation of eIF4As and translational repression in vitro (Fukao et al., 2014). Future studies are needed to determine the mechanism by which miRNAs dissociate eIF4A from target mRNAs. Taken together, these results suggest that miRNAs can repress the translation of target mRNAs by inhibiting formation of the pre-initiation complex. mRNAs that can undergo cap-independent translation are immune to miRNA-mediated repression.
Figure 9: miRNA mediated translation repression at the initiation stage.
The miRISC can inhibit translation initiation by interfering with the m7G cap recognition. The miRISC proteins can either compete with the eIF4E for binding to the m7G cap or impede the association of the 40S and the 60S subunit to form the 80S ribosomal subunit. The interaction between the GW182 protein and the PABP protein can interfere with the closed-loop mRNA configuration thus contributing to the translation repression at the initiation stage. Alternatively, miRNAs can inhibit the association or activity of eIF4A (not shown). These effects are likely to be miRNA/mRNA specific.
1.4.2.2 miRNA-mediated translation repression at the elongation stage.

While extensive studies have suggested that miRNA can block translation at the initiation stage, other data suggests that some mRNAs can also repress translation at elongation stage. For example, some miRNA-bound mRNAs remain associated with polysomes (Maroney et al., 2006; Nottrott et al., 2006; Olsen and Ambros, 1999; Petersen et al., 2006; Seggerson et al., 2002; Wu and Belasco, 2008). Additional studies using IRES-containing reporters support miRNA repression after translation initiation. Lytle et al. and Peterson et al. demonstrated that specific miRNAs can mediate repression of IRES-containing reporters (Lytle et al., 2007; Petersen et al., 2006). Because IRES elements initiate translation independently of the m7G cap, these results indicate that miRNAs repress translation at a step after cap recognition (Filipowicz et al., 2008). The interpretation of polysome profiles of miRNAs and their associated target mRNAs have led to several possible mechanisms by which miRNAs might regulate mRNA repression. Some specific miRNAs are proposed to repress translation at the elongation step through mechanisms including co-translational protein degradation, premature translational termination, or ribosome stalling (Eulalio et al., 2008a; Filipowicz et al., 2008).

In support of the co-translational protein degradation model, sucrose sedimentation assays demonstrated that some miRNAs co-sediment with target mRNAs and polysomes but have no detectable protein product (Olsen and Ambros, 1999; Seggerson et al., 2002). Based on these results, Nottrott et al. proposed that the nascent polypeptide chain might be degraded co-translationally when miRNAs are bound to mRNAs (Figure 11; Nottrott et al., 2006). However, the identity of the protease remains unknown and treatment with
proteosome inhibitors did not restore protein expression of these reporters (Eulalio et al., 2008a; Nottrott et al., 2006). Therefore, additional experiments are needed to validate this mechanism. Petersen et al. proposed that some miRNAs cause premature termination of ribosomes (known as “ribosome drop off”) (Petersen et al., 2006). To test this hypothesis, the authors used a reporter with target sites for miRNAs. They performed a sedimentation assay on the repressed reporter and an unrepressed mRNA followed by treatment with the translation inhibitor hippuristanol. They found that ribosomes on repressed miRNA reporter were released rapidly compared to the ribosomes on non-repressed mRNAs. These correlative results suggest that miRNAs can lead ribosomes to fall off during translation (Petersen et al., 2006).

An alternative mechanism for miRNA mediated repression at the post-initiation stage was proposed by Chendrimeda et al. (Chendrimada et al., 2007). The authors identified that eIF6 is involved in miRNA-mediated translation repression. Previous studies have demonstrated that eIF6 plays a crucial role in the assembly of the 60S ribosomal subunit. eIF6 inhibits the premature assembly of the 60S ribosomal subunits with the 40S ribosomal subunit (Russell and Spremulli, 1978). The authors showed that AGO proteins can repress translation by recruiting eIF6, which prevents the association of the 60S and 40S ribosomal subunits (Chendrimada et al., 2007). However, the mechanism by which AGO proteins recruit eIF6 to target mRNAs is not exactly clear. Moreover, the involvement of eIF6 in this process has been challenged (Ding et al., 2008; Hurschler et al., 2010). Knocking down eIF6 in Drosophila S2 cells did not affect miRNA mediated repression (Fabian et al., 2010). eIF6 knockdown in C. elegans enhanced miRNA mediated...
translation repression (Ding et al., 2008). Other studies have suggested that eIF6 may instead affect either miRNA maturation or miRNA loading onto the miRISC complex (Hurschler et al., 2010).

Taken together, these data provide evidence that for some mRNAs, miRNAs can repress translation by releasing elongating ribosomes, as well as through co-translational protein degradation. However, this data is contradictory to previous studies that suggested that miRNAs primarily affected translation initiation. Different experimental conditions, cell lines and reporters can explain some of these contradictions. However, it is also possible that specific miRNAs can utilize both pre-initiation and post-initiation translation inhibition mechanisms to regulate translation of a wider variety of mRNAs. This versatility could especially be useful in physiological conditions where cap-dependent translation is limited or when certain mRNAs utilize both cap-dependent and IRES-dependent translation. Further investigation into the processes is required.
Figure 10: miRNA mediated translation repression at the elongation stage.
The miRISC proteins have been suggested to either slow the movement of ribosomes along
the target mRNA or to induce ribosomal drop-off. The premature dissociation of ribosomes
is proposed to cause the ribosomes to drop off the target mRNA. The precise mechanisms
underlying these models are currently not clear.
Figure 11: miRNA mediated co-translational protein degradation.
Nascent polypeptides are proposed to be degraded co-translationally via the association of specific miRNAs with target mRNAs. However, the identity of the putative protease involved in this process remains unknown.
1.4.2.3 Role of GW182 in mediating translation repression.

The GW182 family of proteins is important for miRNA-mediated gene silencing (Braun et al., 2013; Eulalio et al., 2008b). GW182 designates target mRNAs for translation repression or degradation through multiple protein-protein interactions (Braun et al., 2013; Fabian and Sonenberg, 2012). Besides interacting with the AGO proteins, GW182 interacts with Poly-A binding protein (PABP) and the CCR4-NOT deadenylase complexes to contribute to miRNA-induced translation repression (Eulalio et al., 2008a; Fabian and Sonenberg, 2012; Fabian et al., 2009, 2010).

Several studies suggest that the PABP–GW182 interaction is important for miRNA mediated gene silencing (Fabian et al., 2009; Huntzinger et al., 2010; Jinek et al., 2010; Zekri et al., 2009). One model posits that GW182 competes with eIF4G for PABP binding, thereby interfering with the PABP-eIF4G association, mRNA circularization, and cap-dependent translational initiation (Fabian et al., 2009; Zekri et al., 2009). A second model proposes that GW182–PABP interaction may reduce the association between PABP and the poly-A tail, leading to translation repression (Huntzinger et al., 2010). In contrast to these observations, several other studies have demonstrated that GW182-PABP interaction does not affect miRNA-mediated repression. Notably, in S2 cell lysates and zebrafish embryos, depletion of PABP or addition of PABP-binding protein 2 (PAIP2), which inhibit both the eIF4G–PABP and the PABP–poly (A) interactions, did not affect miRNA-mediated translational repression (Fukaya and Tomari, 2011; Iwakawa and Tomari, 2015; Mishima et al., 2012). These results suggest that although PABP’s interaction with GW182 is important for miRNA-mediated repression, inhibition of GW182-PABP interaction is
likely not the only mechanism for miRNAs to mediate translation repression (Iwakawa and Tomari, 2015).

Previous studies in S2 cells and human cells demonstrate that the GW182 proteins act as docking platforms to recruit CCR4-NOT1 and PAN2-PAN3 deadenylase components to regulate deadenylation of polyadenylated mRNAs (Braun et al., 2011; Chekulaeva et al., 2011; Fabian et al., 2012). Interestingly, knockdown of the deadenylase components severely impairs GW182-mediated repression of non-adenylated mRNAs (Braun et al., 2011; Chekulaeva et al., 2011). These results led to the model that deadenylase complexes facilitate a deadenylation-independent mode of translation repression (Braun et al., 2013; Fabian and Sonenberg, 2012). However, mechanistic details on how GW182 might either recruit deadenylase components to mediate deadenylation or how GW182 mediates translation repression independent of deadenylation remain elusive (Fabian et al., 2010). Recent structural and biochemical studies demonstrated that the deadenylase NOT1 interacted directly with the DEAD-box RNA helicase DDX6. DDX6 is believed to function primarily as a translation repressor in mammalian cells (Chen et al., 2014b; Mathys et al., 2014; Rouya et al., 2014). Therefore, the GW182 proteins might recruit DDX6 to target mRNAs through an interaction with the CCR4-NOT1 complex (Iwakawa and Tomari, 2015). Recent studies also indicate that CCR4-NOT1 recruits the eIF4E-binding protein 4E-T through interaction with DDX6 (Nishimura et al., 2015; Waghray et al., 2015), and 4E-T represses translation by binding to eIF4E proteins and inhibit cap-dependent translation initiation. However, further studies are needed to identify
the specific step of translation blocked by the interaction between GW182 and DDX6 (Wilczynska and Bushell, 2014).

1.4.2.4 Compartamentalization of miRNA-mediated repression in P bodies.

mRNAs subject to translational repression are localized to cytoplasmic RNP granules known as P bodies along with miRNAs and the miRNA machinery in eukaryotic cells (Eulalio et al., 2007a; Liu et al., 2005; Parker and Sheth, 2007). Cellular components that are involved in miRNA function, including AGO1, GW182, the CCR4-NOT1 deadenylase complex, the decapping enzyme DCP2, decapping enhancers (DCP1, EDC3) and the RNA helicase DDX6/RCK/ME31B, also localize to P bodies (Anderson and Kedersha, 2006; Eulalio et al., 2007a; Parker and Sheth, 2007). P bodies are devoid of ribosomal subunits and translation initiation factors (Filipowicz et al., 2008; Liu et al., 2005; Mitchell and Parker, 2014; Teixeira et al., 2005). Previous studies have indicated a correlation between miRNA-mediated translational repression and the accumulation of target mRNAs in P bodies (Bhattacharyya et al., 2006; Filipowicz et al., 2008; Liu et al., 2005; Pillai, 2012). Specifically, miRNAs, AGO1 and GW182 interact with other P body components, and the knockdown of ME31B or other decapping enhancers weakens miRNA-mediated translation repression (Chu and Rana, 2006; Fabian et al., 2010). Importantly, a functional miRNA pathway is important for P body formation (Eulalio et al., 2007c; Filipowicz et al., 2008), and inhibition of miRNA biogenesis or depletion of GW182 or AGO1 protein results in a dispersal of P bodies in mammalian and S2 cells (Filipowicz et al., 2008). However, knockdown of some P-body components (for example LSM1, LSM3) result in the dispersal of P-bodies but have no effect on miRNA function.
(Filipowicz et al., 2008). Therefore, P bodies are likely not required for miRNA function, and their formation is a consequence rather than the cause of translational silencing (Chu and Rana, 2006; Eulalio et al., 2007c).

1.5 Role of FMRP in miRNA-mediated repression.

Previous studies have demonstrated that FMRP interacts with some miRNAs and core miRISC components to regulate translation. This association provides selective, bidirectional, spatial and temporal control for regulating mRNA translation (Liu-Yesucevitz et al., 2011; Muddashetty et al., 2011). FMRP is genetically and biochemically linked to the miRNA pathway (Edbauer et al., 2010; Li and Jin, 2009). dFMRP and mammalian FMRP interact directly with Dicer1, AGO1, and the miRISC-associated RNA helicase MOV10 (Cheever and Ceman, 2009; Jin et al., 2004b; Kenny et al., 2014; Li and Jin, 2009; Loffreda et al., 2015; Plante et al., 2006). Moreover, translationally repressed mRNAs along with FMRP, miRNAs, core miRISC components (AGO1, GW182) and other RNA binding proteins are localized in motile neuronal mRNA granules in axons and dendrites (Barbee et al., 2006; Muddashetty et al., 2011).

*In vitro* studies have indicated that FMRP can also act as an acceptor of miRNAs derived from Dicer processing of pre-miRNAs through its KH2 domain (Plante et al., 2006). Moreover, phosphorylation of FMRP inhibits its association with Dicer (Cheever and Ceman, 2009). These results suggest that cooperation between Dicer and FMRP is important for miRNA function and mRNA regulation. KH domains of FMRP have been demonstrated to be crucial for the association of FMRP and miRISC components. A specific mutation in the KH2 domain of FMRP (I304N) causes a severe form of FXS. This
mutation causes defects in association with ribosomes and target mRNA (Caudy et al., 2002). The analogous mutation (I307N) in *Drosophila* has been shown to disrupt the association between FMRP and AGO proteins (Caudy et al., 2002; Loffreda et al., 2015). These results suggest the importance of a direct interaction between FMRP and miRISC components in the function of FMRP- and miRNA-mediated repression in cells.

Recent studies have proposed that modification of the secondary structure of mRNA can facilitate the interaction between FMRP and miRISC components. The 3’UTRs of mRNAs contain secondary structures and have embedded seed regions for miRNAs. FMRP was shown to interact with an RNA helicase, MOV10, which is a component of the miRISC (Kenny and Ceman, 2016; Kenny et al., 2014). FMRP recruits MOV10 to target mRNA for unwinding the secondary structure for subsequent access of miRISC complex to repress target mRNA (Kenny and Ceman, 2016; Kenny et al., 2014). Taken together, these results support a model in which the binding of FMRP to target mRNAs contributes to the ability of miRISC to repress target mRNAs (Banerjee et al., 2018; Edbauer et al., 2010; Kenny et al., 2014; Li et al., 2014; Muddashetty et al., 2011).

Other studies have demonstrated that a functional relationship between FMRP and specific miRNAs is essential to repress target mRNAs (Li and Jin, 2009). It is unclear if this interaction involves the RISC but it is important for proper synaptic structure and function (Edbauer et al., 2010; Kenny and Ceman, 2016; Loffreda et al., 2015). Edbauer et al. demonstrated that FMRP associates with miR-125b and miR-132 to control dendritic spine morphology in mice (Kenny and Ceman, 2016). The authors showed that overexpression of miR-125b and miR-132 resulted in dendritic spine defects in hippocampal neurons.
Importantly, a knockdown of FMRP rescued these phenotypes in mice, suggesting that FMRP interacts with specific miRNAs to control dendritic spine development (Banerjee et al., 2018; Edbauer et al., 2010; Richter et al., 2015).

Additional studies have demonstrated the existence of a cooperative mechanism between FMRP and miRISC components to reversibly and selectively regulate synaptic mRNAs in response to mGLuR signaling (Loffreda et al., 2015; Muddashetty et al., 2011). FMRP recruits the miR-125a-AGO2 complex to the 3'UTR of the PSD-95 mRNA to repress translation of the postsynaptic density protein 95 (PSD-95) at the synapse in response to mGLuR stimulation. Phosphorylation of FMRP at a specific serine residue (Ser499) results in the formation of the miR-125a-AGO2 inhibitory complex on PSD-95. Dephosphorylation of FMRP, on the other hand, relieves PSD-95 from repression (Kenny and Ceman, 2016; Muddashetty et al., 2011). Similarly, dephosphorylation of FMRP by the specific phosphatase 2A (PP2A) plays an important role in the bidirectional regulation of mRNA translation in neurons (Loffreda et al., 2015).

Although previous studies demonstrated that interactions between FMRP and miRNA pathway are critical to regulating translation of target mRNAs, the mechanism of FMRP’s interaction with various miRNA components is not well understood. Studying this interaction will shed light on an important function of FMRP in cells. Because FMRP deficiency and loss of function is implicated in FXS, understanding of the function of FMRP in miRNA-mediated translation repression (or vice versa) has the potential to identify underlying mechanisms that contribute to the pathology of FXS in patients.
1.6 Summary

Previous studies indicate that FMRP interacts biochemically and genetically with Dicer1 and miRISC proteins such as AGO1 and MOV10 (Caudy et al., 2002; Jin et al., 2004b; Kenny et al., 2014). FMRP is also known to associate with specific miRNAs to regulate translation repression (Edbauer et al., 2010; Muddashetty et al., 2011). These interactions are required to control normal synaptic development in mammals and flies. A growing amount of evidence points towards a cooperative mechanism of FMRP-mediated translation repression of target mRNAs via miRNAs. However, the details of this mechanism remain elusive. The underlying objective of this thesis is to explore the mechanism of FMRP-mediated translation repression via the miRNA pathway. To address this, we used Drosophila as our model as it offers multiple advantages compared to the other model organisms. Drosophila are easier to maintain, less expensive, have a shorter lifespan and generation time with a completely sequenced genome (Drozd et al., 2018). Moreover, dFMRP, the only Drosophila ortholog of human FMRP, is highly conserved with a similar amino acid sequence, RNA-binding properties and biochemical function in vivo (Bhogal and Jongens, 2010). dFmr1-null flies recapitulate many of the FXS phenotypes, such as defects in memory, social behavior, circadian rhythmicity and sleep (Weisz et al., 2015). Further, dFmr1 nulls display defects in synaptic growth mimicking dendritic spine overgrowth in FXS patients (Weisz et al., 2015).

In this study, we have used an in vitro system to study FMRP mediated translation control by using a translational reporter system in Drosophila S2 cells. Our results suggest that FMRP requires AGO1 and GW182 to mediate translation repression when FMRP is
tethered to a luciferase reporter mRNA. We have also examined the role of the RNA binding domains (RBDs) of tethered FMRP in translation repression and found that KH1 and 2 domains contribute to its ability to repress translation. Further, we show that untethered FMRP can repress translation of the reporter by recognizing and binding to a small stem-loop structure within its 3’UTR. These results point towards the importance of structural elements in target mRNAs for FMRP mediated translation regulation. Remarkably, our analysis suggest role for FMRP in the recruitment of miRNAs to a target mRNA for translation repression, suggesting a role for FMRP in miRNA-mediated repression.

FMRP associates with mRNAs as part of a large mRNP complex in distinct cytoplasmic foci (Kedersha and Anderson, 2007; Kenny and Ceman, 2016). Therefore, we also examined the intracellular localization of FMRP with miRISC components within S2 cells and the larval CNS. Our results demonstrate that FMRP is present in same cells as core miRISC components. We performed co-immunoprecipitation to test the direct association of FMRP with core miRISC components. Our results indicate a strong association of FMRP with AGO1 and a weak association of FMRP with GW182 under low salt conditions. Under high salt conditions, we found a dissociation of FMRP with GW182 with not much effect on the association of FMRP-AGO1. Therefore, the association between FMRP and AGO1 appears to be stronger than GW182. Next, we used the larval neuromuscular junction (NMJ) to study genetic interactions between FMRP and AGO1 or GW182. We demonstrate that genetic interactions of FMRP with AGO1 and GW182 are crucial to regulate synaptic structure at the larval NMJ.
FMRP-associated mRNAs that are targeted for translation repression are found to localize in cytoplasmic foci such as P-bodies and stress granules. Previous studies also indicate that miRISC regulates repression by targeting mRNAs for repression via deadenylation followed by translation repression in P bodies (Braun et al., 2013; Eulalio et al., 2007c; Fabian and Sonenberg, 2012). Therefore, we screened for a genetic interaction between the key proteins involved in deadenylation and repression: CCR4, NOT1, and PABP, in order to identify the downstream mechanism for FMRP-miRISC mode of repression. We demonstrate that FMRP is present in the same cells as the components of deadenylase enzyme (CAF1, CCR4, and NOT1) within larval CNS. However, FMRP does not interact genetically with CCR4, NOT1 or PABP to control synaptic morphology. These results suggest that FMRP does not interact with CCR4, NOT1 or PABP within the same pathway. Therefore, FMRP-GW182 utilizes an alternative mechanism to control synaptic structure and morphology in Drosophila.
CHAPTER TWO: METHODS

2.1 Key resources table

2.1.1 Reagents and Chemicals

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2.1.2 Experimental models: Cell lines

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2.1.3 Experimental models: Fly strains

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<td>pAc5.1B-lambdaN-HA-ΔRGG FMRP</td>
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<td>pAc5.1B-EGFP-ΔKH0 FMRP</td>
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<td>pAc5.1B-EGFP-ΔKH1/2 FMRP</td>
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### 2.1.5 Oligonucleotides

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<td>CACCTGCCGCCGCCAGTGGAA</td>
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<td>Reverse primer to amplify the Tral3’UTR</td>
<td>AGTAAATAATTTCAGTTATAAAATTATAT</td>
</tr>
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<td>CGGAACACGATTATTTGCCT</td>
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<td>CGCAACACGGTGACAAATATC</td>
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<td>----------------------------------------</td>
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<td>Reverse primer for amplifying miR-33a</td>
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<td>Forward primer for amplifying miR-13b</td>
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60
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<td>Forward primer for amplifying miR-2a</td>
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### 2.1.6 Antibodies

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<tr>
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</thead>
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<tr>
<td>Mouse anti-FMRP (6A15)</td>
<td>Abcam</td>
<td>AB-10299</td>
</tr>
<tr>
<td>Rabbit anti-Dicer1</td>
<td>Abcam</td>
<td>AB-4735</td>
</tr>
<tr>
<td>Rabbit anti-AGO1</td>
<td>Abcam</td>
<td>AB-5070</td>
</tr>
<tr>
<td>Rabbit anti-AGO2</td>
<td>Abcam</td>
<td>AB-5072</td>
</tr>
<tr>
<td>Guinea-pig anti-GW182</td>
<td>(Schneider et al., 2006)</td>
<td>N/A</td>
</tr>
<tr>
<td>Mouse anti-Armitage</td>
<td>(Saito et al., 2010)</td>
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<td>Rabbit anti-CCR4</td>
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<td>Torey Pines</td>
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<td>Mouse anti-FLAG (M2)</td>
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<td>Alexa Fluor 594 goat anti-guinea pig IgG</td>
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<td>Alexa Fluor 647 goat anti-HRP</td>
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### 2.1.7 Media and buffer composition

<table>
<thead>
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<th>Media</th>
<th>Composition</th>
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</thead>
<tbody>
<tr>
<td><strong>M3 media (10 L)</strong></td>
<td>393.6 g Shields and Sang powdered medium, 5g KHCO₃, 10g yeast extract, 25g bactopeptone (pH 6.6)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Buffers for western blot</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S2 lysis buffer</strong></td>
<td>50mM Tris-HCl (pH 7.4), 150mM NaCl, 1mM EDTA, 0.1% Triton, 0.5% NP40, supplemented with EDTA free protease inhibitor tablets</td>
</tr>
<tr>
<td><strong>Sample buffer</strong></td>
<td>2X Lamelli buffer and β-mercaptoethanol</td>
</tr>
<tr>
<td><strong>10 X TGS (Tris Glycine SDS) Running buffer</strong></td>
<td>250 mM Tris, 1.92M Glycine and 1% w/v SDS</td>
</tr>
<tr>
<td><strong>1X Transfer buffer (2 L)</strong></td>
<td>6.0 g Tris, 28.8 g Glycine, 200ml Methanol, 1.6ml water</td>
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<tr>
<td><strong>1X Tris-buffered saline (1X TBS)</strong></td>
<td>150mM NaCl, 50mM Tris-Cl (pH 7.5)</td>
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<tr>
<td><strong>TBST (Tris-buffered saline, 0.1% Tween 20) - (1L)</strong></td>
<td>100 mL of TBS 10X – 900 mL of distilled water – 1 mL Tween 20</td>
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<tr>
<td>Blocking buffer</td>
<td>1X TBST with 5% w/v dry milk</td>
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<tr>
<td>-----------------------------------------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>Gentle stripping buffer (1L)</td>
<td>15 g glycine, 1 g SDS, 10 ml Tween20 (pH 2.2)</td>
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<td>Harsh stripping buffer (0.5 L)</td>
<td>20 ml 10% SDS, 12.5 ml Tris HCl (pH 6.8), 67.5 ml water, 0.8 ml of β-mercaptoethanol</td>
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<tr>
<th>Buffers for co-immunoprecipitation</th>
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<td>1X PBS (Phosphate buffered saline) (pH 7.4)</td>
<td>0.137M NaCl, 0.0027M KCl, 0.01M Na₂HPO₄, 0.0018M KH₂PO₄</td>
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<tr>
<td>S2 lysis buffer</td>
<td>150mM NaCl, 0.5% NP40, 20mM HEPES (pH 7), 2mM MgCl₂, 1mM DTT supplemented with EDTA free protease inhibitor tablets and RNAase inhibitor</td>
</tr>
<tr>
<td>Low-salt wash buffer</td>
<td>150 NaCl, 0.5% NP40, 20mM HEPES (pH 7), 2mM MgCl₂, 1mM DTT</td>
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<tr>
<td>High-salt wash buffer</td>
<td>300mM NaCl, 0.5% NP40, 20mM HEPES(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 2mM MgCl₂, 1mM DTT (pH 7),</td>
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<tr>
<td>Buffers for CNS dissections</td>
<td>Composition</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>1X PBS (Phosphate buffered saline) (pH 7.4)</td>
<td>0.137M NaCl, 0.0027M KCl, 0.01M Na₂HPO₄, 0.0018M KH₂PO₄</td>
</tr>
<tr>
<td>Permeabilization buffer (PBS-T)</td>
<td>1X PBS with 0.3% Triton X-100</td>
</tr>
<tr>
<td>Blocking buffer</td>
<td>1X PBS-T with 5% Normal Goat Serum and 2% Bovine Serum Albumin (BSA)</td>
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<table>
<thead>
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<th>Buffers for NMJ dissections</th>
<th>Composition</th>
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<td>Calcium-free HL-3 buffer (1L) (pH 7.2)</td>
<td>70mM NaCl, 5mM KCl, 20mM MgCl₂.6H₂O, 10mM NaHCO₃, 115mM Sucrose, 5mM Trehalose, 5mM HEPES</td>
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<tr>
<td>Permeabilization buffer (PBS-T)</td>
<td>1X PBS with 0.1% Triton X-100</td>
</tr>
<tr>
<td>Blocking buffer</td>
<td>1X PBS-T with 5% NGS and 2% BSA.</td>
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2.1.8 Software and Algorithms

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<tr>
<td>LarvaTrack</td>
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<tr>
<td>algorithm</td>
<td><a href="https://github.com/plredmond/larva-tracker">https://github.com/plredmond/larva-tracker</a></td>
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METHOD DETAILS

2.2 DNA Constructs

For tethering assays, we obtained pAc5.1C- FLuc-Stop-5BoxB, pAc5.1C- RLuc-Stop-V5His6 and pAc5.1B-lambdaN-HA vectors from Prof. Elisa Izzauralde via Addgene as listed in Table 2.1 (Behm-Ansmant et al., 2006; Rehwinkel et al., 2005). To generate the pAc5.1B-λN-HA FMRP, the full sequence of FMRP was amplified by PCR and cloned into the pAc5.1 backbone vector in frame with the λN-HA tag. The pAc5.1C-FLuc-Stop-3XBoxB, pAc5.1C-FLuc-Stop-1XBoxB and pAc5.1C-FLuc-Stop were generated by removing the 5BoxB sequence from the pAc 5.1C-FLuc-Stop-5XBoxB and cloning oligos containing the indicated number of BoxB repeat sequences.

Snapdragon was used to design optimal RNAi primers with no off-target effects against Dicer1, AGO1, AGO2, Armitage and GW182 in S2 cells. Templates for dsRNA synthesis were either cDNA isolated from Drosophila S2 cells or cDNA clones obtained from DGRC. cDNAs were PCR amplified using primers listed in Table 2.1.5. The amplified products were extended to have convergent T7 promoters. Following cDNA clones were obtained from DGRC: AGO1 (CG6671) and GW182 (CG31992). Dicer1, AGO2, and Armitage were amplified using primers listed in Table from cDNA of Drosophila S2 cells. The EGFP sequence was amplified via PCR from the pAC5.1-EGFP plasmid. Predicted dsRNAs were 300-566 nucleotide long and corresponded to the coding sequences for control EGFP dsRNA, GW182, AGO1, AGO2, Armitage, and Dicer1. PCR products were isolated, purified and transcribed into dsRNA using MEGAscript T7TM Transcription Kit. Transcription reactions were treated with DNAase and purified using
phenol-chloroform extraction. dsRNAs were ethanol precipitated and resuspended in nuclease-free water. Agarose gel and UV absorbance values were used to assess the yield and the quality of dsRNAs.

The 3'UTR sequences of known FMRP target mRNAs including Trailer-hitch, pickpocket, Rac, Chicadee, Arc1 were amplified by PCR and cloned into pENTR and subsequently into pAc5.1-FLuc2 [dPolyA] destination vector using the Gateway system. These constructs were used for transfection in S2 cells for analysis of repression by FMRP. For primer sequences, refer to Table 2.1.4.

For miRNA expression in S2 cells, PCR primers were designed to amplify a sequence 200 nucleotide sequence upstream and downstream of each miRNA hairpin from genomic DNA to allow proper processing. The PCR products of miR-958, Bantam, miR-13b, miR-33, miR-2b, miR2a were cloned into pENTR and then into the pAC5.1 (dV5-HisA) expression vector for transfections in S2 cells as we have previously described (Nesler et al., 2013). The primers are described in Table 2.1.4.

For the pAFW-GW182 construct, cDNA clone obtained from DGRC was used to amplify GW182 using primers described in table 2.1.4. The product was cloned into the pENTR vector as an entry vector for LR recombination into the pAFW expression vector (Drosophila Gateway Vector Collection, Carnegie Institution). Using primers listed in table 2.1.4, Dicer1 was amplified from the cDNA of S2 cells and inserted into the sites between Not1 and SacII sites in the pAc5.1-EGFP plasmid to generate pAc5.1-EGFP Dicer1. These plasmids were used for S2 cell transfection.
The pAc5.1B plasmids containing EGFP-FMRP and deletions for EGFP FMRP (ΔRGG), FMRP(ΔKH1/2), FMRP(ΔKH0) were cloned as described (Gareau et al., 2013b). The first PCR product was amplified from pAc5.1 λN-HAFMRP using oligos with BamHI and HindIII sites, and the second PCR fragment was amplified using oligos with BamHI and EcoR1 sites. The amplified products were digested and ligated into pAc5.1 EGFP FMRP construct, previously digested with HindIII and EcoRI, to generate pAc5.1 EGFP FMRP(ΔKH1/2) and FMRP(ΔKH0) constructs respectively. To generate the pAc5.1 EGFP FMRP(ΔRGG), the first PCR product was amplified from pAc5.1 λN-HAFMRP using oligos with HindIII and EcoR1 sites at the end and the second product was amplified using primers with BamHI and HindIII sites. These PCR products were digested with restriction enzymes for ligation into the pAc5.1 EGFP FMRP previously digested with EcoRI and HindIII for directional cloning.

2.3 S2 Cell Culture and Transfection

*Drosophila* S2 cells were maintained in vented 75cm² cell culture-treated flasks and cultured in M3 media, supplemented with 10% FBS, 1% Penicillin-Streptomycin and Fungizone. Cells were incubated at room temperature in an incubator and passaged every 72 hours at a ratio of 4 ml cells to 12 ml cell culture media. The day before transfection, cells were split into 250 ml suspension flasks in M3 media without Fungizone at 1:1 dilution.

On the day of transfection, cells were vigorously suspended and detached from tissue culture flasks with a pipette. Hemocytometer was used to determine the number of viable cells in the original cell suspension. The volume of cell suspension corresponding
to a density of $1 \times 10^6$ cells/ml was determined. Cells were centrifuged at 2,500 rpm for 3 minutes and resuspended in 1X PBS for 15 minutes followed by resuspension in an appropriate volume of cell culture media (0.8ml/well). All transfections were performed in three biological replicates in 12 well plates using Effectene transfection reagent. Manufacturer's instructions were followed to prepare transfection mixtures, as described below.

Briefly, the transfection mixture contained reporter plasmid, plasmid encoding gene of interest, control plasmids, DNA condensation buffer (Buffer EC), Enhancer solution and Effectene solution. After incubation for 10 minutes, 1.5 ml of media was added directly to the tubes and mixed with a pipette. The cells were dispensed drop-wise (500ul) into individual wells while gently swirling the plate. Post-transfection, the cells were incubated for 72 hours until harvested, and were processed for gene expression or biochemical analysis as described below.

2.3.1 Tethering Assays

In tethering experiments, cells were co-transfected with 0.05µg of the FLuc-BoxB reporter plasmid, 0.2µg of RLuc plasmid, 0.25µg of plasmid-expressing the λN-HA protein per well of a 12-well plate. The plasmid expressing the λN-HA-tagged FMRP was added in increasing amounts from 0.125µg to 0.5µg per well. The modified FLuc reporter with 3 (FLuc-3xBoxB), 1(FLuc-1xBoxB) or no BoxB sites (FLuc-0xBoxB) was transfected using similar procedure and concentration as described above. At least three independent transfection experiments were performed, each with three technical replicates.
2.3.2 Assays with untethered FMRP

Transfection assays with untethered FMRP (FMRP lacking λN-HA tag) followed a similar transfection procedure as described above. In assays with miR-958, transfection mix contained FLuc-Box B reporters mainly 0.05µg of FLuc-1XBoxB or FLuc-0XBoxB reporter, 0.2µg of RLuc plasmid, 0.25µg of empty vector (no miRNA control) and 0.5µg of untethered FMRP.

The entire SV40-3'UTR sequence of the FLuc-BoxB reporter was replaced by the 3'UTR of ppk, chic, tral, rac, and arc as described previously. These reporters were co-transfected with increased concentration of untethered FMRP (0.125-0.5 µg), 0.2 µg of RLuc, and 0.25 µg of λN-HA control.

2.4 Luciferase Assays

The FLuc and RLuc luciferase activities were measured sequentially with the Dual-Luciferase Reporter Assay System. The ratio of the FLuc/RLuc was measured for each well in three technical replicates on an infinite M1000 plate reader.

For the measurement of FLuc activity, 75μl of cell suspension was dispensed into the wells of a 96-well plate, followed by the addition of an equal volume of Dual-Glo® Reagent. FLuc values were quenched with Stop & Glo® reagent, followed by the measurement of the RLuc activity in the same wells. The FLuc/RLuc ratio for each sample was normalized to the ratio of the well with transfected λN-HA. Cells were frozen at -80°C for detecting protein and mRNA expression levels.
2.5 RNAi Against miRISC Constructs in S2 Cells

1x10^6 S2 cells were seeded in 12 well plates and transfected with 2µg of the appropriate dsRNA per well along with 0.05µg of the FLuc-BoxB reporter plasmid, 0.2µg of RLuc plasmid, and 0.25µg of plasmid-expressing the λN-HA protein. All transfections were performed using the Effectene transfection reagent. Post-transfection, the cells were incubated for 72 hours in an incubator at 25°C to be used for luciferase assays and western blotting. Primers for dsRNA synthesis have been listed in Table 2.1.4.

2.6 Western blotting

S2 cells were scraped in 1 ml of ice-cold 1X PBS buffer and centrifuged at 1000xg for 10 min at 4°C. Cell pellets were resuspended in ice-cold lysis buffer and mixed intermittently on ice for 15 minutes to ensure proper lysis. The samples were mixed in loading buffer, boiled for 3 min at 100°C and loaded in an 8% SDS-polyacrylamide gel. For detailed composition of the buffers and refer to table 2.1.6. The resolved proteins were then transferred onto a PVDF membrane. A stack consisting of filter papers and sponges soaked in transfer buffer was assembled to ensure the transfer of proteins onto PVDF membrane. The PVDF membrane, soaked in methanol and equilibrated in transfer buffer was placed in this stack with the resolved polyacrylamide gel. This stack was placed in the blotting apparatus for overnight transfer at 22V.

Before incubation with antibodies, the membrane was blocked for an hour in 5% non-fat milk in 1X TBST. The lysates were immunoblotted with primary antibodies against FMRP (1:1000) or HA tag (1:500) for 2 hours to examine the expression of FMRP. We
used antibodies against B-actin for loading control. Antibodies have been listed in table 2.1.5.

To confirm the efficiency of RNAi in S2 cells, blots were probed with primary antibodies against Dicer1 (1:1000),AGO1 (1:2000), Armitage (1:1000),AGO2 (1:1000) and GW182 (generously gifted to us by Dr. Andrew Simmonds) (1: 2000) (Schneider et al., 2006). The PVDF membrane was washed with 1xTBST thrice for 5 min followed by incubation HRP-conjugated secondary antibodies. We used super signal solution to detect the proteins on the PVDF membrane and developed the blot on an X-ray film.

2.7 Co-Immunoprecipitation

For FMRP immunoprecipitation, we seeded 4x 10^6 S2 cells in 25 cm² flasks and transfected with plasmids expressing 1 µg of EGFP-FMRP, FLAG-AGO 1, FLAG-AGO2, and FLAG-GW182 respectively. Three days after transfection, S2 cells were harvested, washed with ice-cold 1X PBS, centrifuged at 1000 x g for 10 min at 4°C followed by washing with ice-cold 1X PBS buffer. Cells were re-suspended in 200µl lysis buffer, incubated on ice for 30 minutes and centrifuged at 16000xg for 20 minutes at 4°C. We added 300 µl of the lysis buffer to the lysate and discarded the cell pellet. 50µl of the ‘Input fraction’ was saved from the lysate for western blotting and 250 µl of the lysate was used for FMRP-immunoprecipitation (‘IP fraction’).

The GFP-trap bead slurry (25 µl) was equilibrated as per manufacturer's instructions and incubated with the IP fraction for about 2 hours at 4°C. The beads were washed five times with 500 µl of low and high salt wash buffer (150 mM or 300 mM NaCl) and magnetically separated from the supernatant. The proteins bound to beads were eluted.
directly off beads with 100 µl of the 2X SDS buffer, resolved by 8% polyacrylamide gel electrophoresis (PAGE) and probed with anti-FLAG (1:1000) and anti-EGFP antibody (1:1000). The buffers for co-immunoprecipitation experiments have been listed in table 2.1.6.

2.8 Real-Time PCR

2.8.1 RNA Isolation and Purification

Total RNA was isolated from S2 cells transfected with 0.05µg of FLuc, 0.2µg of RLuc, 0.25µg of λN-HA and 0.125-0.50µg of λN-HAFMRP or untethered FMRP using 500µl of TRIzol. The RNAeasy mini kit was used to isolate and purify mRNA from cell lysates. Briefly, the total isolated mRNA was incubated with RNase-free DNase I using the DNA-free kit and dissolved in 50 µl of nuclease-free water. 1µg of DNA-free RNA was used as a template for cDNA synthesis with RNA to cDNA EcoDry Premix as per manufacturer’s instructions.

2.8.2 qRT-PCR

We used iQ SYBR Green Supermix and designed gene-specific primers for FLuc and RLuc as described in Table 2.1.4. Melt curve analysis indicated that the primers generated a single PCR product. Quantitative RT–PCR was performed on an iCycler thermocycler as per the manufacturer’s instructions. For relative quantification of the transcripts, we averaged the results from three technical replicates to generate Ct values for each biological replicate. The Ct results were analyzed for differential fold change as described by Livak (ΔΔCt) method (Livak and Schmittgen, 2001).
2.9 miRNA Target-Site Prediction

Potential miRNAs targeting the SV40 3’UTR of the FLuc-BoxB reporter were identified using the PITA algorithm. PITA scans miRNA-mRNA interactions based on the seed sequence of the miRNA, secondary structure of the target mRNA and free energy (Kertesz et al., 2007). For URL to the website refer to Table 2.17.

2.10 RNA Secondary Structure Prediction

RNAfold web server was used to predict secondary structures of 3'UTR sequences of known FMRP targets. The 3’UTR sequences of known FMRP target mRNAs including Trailer-hitch, pickpocket, dRac, Chicadee, Arc1 were shown to have stem-loops very similar in structure to the BoxB stem-loop. Predicted secondary structures are represented in Figure 18A-B.

2.11 S2 Cell Immunofluorescence and Microscopy

2.11.1 FMRP Granules in S2 Cells:

S2 cells transfected with plasmids expressing pAc5.1B EGFP-FMRP, and deletions for EGFP FMRP (ΔRGG), FMRP(ΔKH1/2), FMRP(ΔKH0) were used to characterize the phenotype of FMRP granules. Cells were transfected with 0.25µg of plasmid DNA as described above and incubated for about 72 hours. Images were acquired using a 100x objective (N.A.= 1.40) on an FV1000 Laser Scanning Confocal Microscope.

2.11.2 Immunofluorescence in S2 cells

S2 cells were examined for endogenous expression of FMRP and with AGO1, GW182, or DCR1 respectively. S2 cells were allowed to adhere to a poly-d-lysine coated dish and fixed using 4% paraformaldehyde. Cells were treated with 1 ml of
permeabilization buffer and after incubation with blocking buffer cells were immunostained with primary antibodies against FMRP, AGO1 and GW182 as described previously. Images were acquired using a 100x objective (N.A. = 1.40) on a FV1000 Laser Scanning Confocal microscope. Buffers for S2 cell immunofluorescence have been described in Table 2.1.6.

2.11.3 S2 cell colocalization analysis

The raw images were resized using Adobe Photoshop and resolution was adjusted to 300 pixels/inch. The images were split using the split channels feature on Fiji into green, red and blue channels. The green channel was for FMRP, the red channel was for the proteins of interest, and the blue channel was for DAPI. For colocalization analysis, six independent S2 cells were measured for colocalization of FMRP with the miRISC proteins. The images were thresholded manually before Mander's coefficient calculation using the ‘Just another colocalization plug-in’ (JaCOP) from ImageJ/Fiji (Bolte and Cordelieres, 2006). Mander's coefficient varies from 0 to 1 corresponding to overlapping images, and 1 represents 100% colocalization. The Mander's coefficient of green channel (FMRP) colocalizing with red channel (the miRISC proteins), M2 value, was imported into Prism for statistical analysis.

2.12 Generation, and characterization of Drosophila stocks

All stocks used in the study were raised at 25⁰C on standard Bloomington media. The fly stocks are listed in table 2.1.2. The Drosophila isogenized strain w¹¹¹⁸ (or Iso31) was used as a wild-type control. dFmr1 heterozygotes were generated by crossing flies
from \textit{dFmr1}^{\Delta113M} with flies from the \textit{w^{1118}} genetic background. The \textit{Not1}^{M10761}, \textit{pAbp}^{k10109} lines were balanced over \textit{CyoGFP} balancer to allow for larval NMJ analysis.

Trans-heterozygotes were generated by crossing \textit{dFmr1}^{\Delta113M} to all the indicated lines and selecting against markers on the balancer chromosome. Transgenic lines with the \textit{gawky (gw182)} mutation were a kind gift from Prof. A. Simmonds (Schneider et al., 2006). The \textit{gawky} gene is located on the fourth chromosome which significantly complicates genetic analysis. To obtain trans-heterozygous \textit{gawky} mutant lines for larval NMJ analysis, flies from \textit{w^{1118}}, \textit{pan}^{2}/P\{\textit{ActGFP}\}unc-13^{Gf} were crossed with flies from \textit{Tm3Sb/Ly;} \textit{gw}^{1}/ciD. F1 progeny from the cross were selected against \textit{Lyra} and for GFP, \textit{Tm3Sb} to generate \textit{gw}^{1}/\textit{ActGFP}.

Transgenic short-hairpin (\textit{UAS} -\textit{RNAi}) targeting \textit{Dcr1}, \textit{Ago1}, and \textit{gawky} were expressed in larvae using a specific pan-neuronal driver \textit{elav-Gal4} for larval CNS staining.

\textbf{2.13 NMJ Dissections and Immunostaining}

All indicated genotypes were crossed with \textit{w^{1118}} or \textit{dFmr1}^{\Delta113M} to generate heterozygotes or trans-heterozygotes for larval NMJ analysis respectively. Wandering third instar larvae were collected and pinned in Sylgard dishes. Larval fillets were dissected in a calcium-free HL-3 buffer and fixed in 4% Paraformaldehyde for 15 minutes.

Fixed larvae were then rinsed in 1X PBS, permeabilized in 1X PBS-T and blocked in the blocking solution for 1 hour. Larval files were incubated with primary antibody against discs large (1: 100) at 4°C overnight. Post-incubation, larvae were washed with 1X PBS thrice for 5 minutes followed by incubation with anti-mouse Alexa Fluor 488 (1:500) and goat Anti-HRP-Dylight-647 (1:500) for 2 hours at room temperature. Antibody against
HRP was used to label presynaptic neuronal membranes. Post-incubation, larvae were washed with 1X PBS thrice for 5 minutes followed by incubation with anti-mouse Alexa Fluor 488 (1:500) and goat Anti-HRP-DyLight-647 (1:500) for 2 hours at room temperature. Following the antibody incubation, the samples were washed extensively with 1XPBS, mounted on a charged slide in DAPI Fluoromount G (Southern Biotech) and stored at -20°C for analysis as described below. The buffer compositions for NMJ dissections and immunostaining have been described in Table 2.1.6.

2.13.1 Image Acquisition and Quantification of NMJ

The images of NMJs were acquired at muscle 6/7 in larval abdominal segment 3(A3) using an FV1000 Laser Scanning Confocal microscope using 20X (N. A=0.85) and 60X (N. A=1.35) objectives. Max-intensity projections were obtained using the FV1000 software. All images were randomized and scored blindly to avoid potential bias in quantification. Using cell counting plugin for ImageJ/Fiji, NMJs were manually counted for a total number of boutons, 1b boutons, 1s boutons, branch points and tips. Based on the size and intensity of Dlg staining, type 1b and type 1s boutons were classified. Synaptic boutons were normalized to muscle surface area (MSA) calculated using the 20X objective.

2.14 Larval CNS Immunostaining

For immunohistochemistry, larval brains from w1118 were dissected in ice-cold 1X PBS under 20 minutes and fixed in 4% Paraformaldehyde for 20 minutes at room temperature. Fixed CNSes were permeabilized with 1XPBST for 20 minutes at room temperature followed by blocking for an hour at room temperature. After blocking, the samples were incubated with primary antibodies against FMRP (1:1000), Dicer1(1:1000),
AGO1 (1:1000), and GW182 (1:2000) overnight at 4\(^{\circ}\)C. Post-incubation, larval CNSs were washed with PBST thrice for 20 minutes and incubated in PBT with secondary antibodies conjugated to anti mouse Alexa Fluor 488 (1:500) and anti-rabbit Alexa Fluor 568(1:500) for two hours. Larval CNSes were then washed with PBST thrice for 10 min and mounted on a slide using DAPI Fluoromount G (Southern Biotech). Buffer compositions for the larval CNS immunostaining have been listed in Table 2.1.6.

Immunostained brains were imaged using a FV1000 Laser Scanning Confocal microscope using 40X (N. A=0.90) and 100X (N. A=1.40) objectives. Maximum intensity Z projections were assembled from 0.4 um sections using Olympus Fluoview software.

2.14.1 Larval CNS colocalization analysis

We used three independent CNSs for the analysis of protein colocalization. The images were split into green, red and blue channels using Fiji. FMRP was imaged in green channel, the protein of interest was imaged in the red channel and the nuclei were imaged in the blue channel. The images were thresholded manually before the calculation of Mander's coefficient (M1 and M2 values) using the JaCoP plugin. The M2 values for green channel (FMRP) colocalization with red channel (miRISC/deadenylase proteins) were imported into Prism for statistical analysis.

2.15 Larval crawling assay

Larval crawling behavior was analyzed for \(\mu^{118}\), heterozygotes and trans-heterozygotes of the indicated genotypes as described previously (Kashima et al., 2017). Average velocity and relative distance were computed from 3 independent experiments for each genotype. Ten individual wandering third-instar larvae were rinsed briefly in 1XPBS
and transferred from vials onto a 90 mm petri dish containing 2% agarose. The crawling behavior was digitally recorded using iPhone 7 for 1 min in QuickTime movie (MOV) format with time-lapse settings using the constraints described by Kashima et al. The videos were analyzed computationally for relative crawling distance and velocity using the LavaTrack algorithm. The URL of the website is listed in Table 2.1.7. Briefly, the position of each larva was recorded on most frames, and the cumulative distance was computed for four 15-second intervals. The velocity was calculated by determining the average speed from 30 to 45 seconds as larvae were slow in first 10 seconds and some of them reached the edge of the petri dish in last 10 seconds.

2.16 Statistical analyses

All statistical analyses including graphing was performed using ANOVA Turkey’s one-way posthoc test or Kruskal-Wallis; Dunn’s posthoc test for the NMJ boutons. Data are normalized to the controls and errors are presented as ± SEM with p values calculated using GraphPad software. For larval crawling, a two-way ANOVA test was used to analyze the data for distance and one-way ANOVA test was used to analyze average velocity. GraphPad Prism was used to graph all data.
CHAPTER THREE: RESULTS

3.1 Tethered FMRP represses translation of a FLuc reporter in a dosage-dependent manner.

To understand the role of FMRP in translation repression, we developed an in vitro translational reporter-based assay. The experiments were based on an existing tethering assay in Drosophila S2 cells (Pillai et al., 2004; Rehwinkel et al., 2005). We fused FMRP with λN-HA tag that can to bind to five tandem BoxB sites cloned within the 3’UTR of the firefly luciferase (FLuc) reporter. The λN tag is derived from the N protein of the bacteriophage λ to enable tethering to a FLuc reporter via the BoxB structural element in its 3’UTR (Keryer-Bibens et al., 2008) and an HA (hemagglutinin) tag for detection of the expressed protein by Western blot. The UTR in the FLuc reporter is derived from the Simian virus 40 (SV40) small T antigen (Figure 12A).

We transfected S2 cells with a plasmid expressing firefly luciferase (FLuc) and Renilla luciferase (RLuc), co-transfected with and increasing concentration of plasmid expressing λN-HA-FMRP. As a negative control, we co-transfected with a plasmid expressing λN-HA alone. We used RLuc as a transfection control since it did not have BoxB sites within the 3’UTR (Figure 12B). We hypothesized that tethered FMRP might repress the translation of FLuc-BoxB reporter in a dosage-dependent manner.

We observed that λN-HA-tagged FMRP repressed the translation of FLuc reporter, relative to λN-HA alone (Figure 12C) (p < 0.0001). At 0.125µg, 0.25µg and 0.5µg of λN-
HA tagged FMRP repressed the translation of FLuc-5xBoxB reporter was repressed by 60%, 70%, and 80% compared to controls (Figure 12C) (p < 0.0001). These results suggest that FMRP can repress translation when tethered to the reporter mRNA in a concentration-dependent manner. We confirmed a concentration dependent increase in the levels of λN-HA-tagged FMRP by Western blotting (Figure 12D).

We proposed that it was possible that FLuc protein levels in our reporter assays could be reduced either due to a mechanism involving translational repression or mRNA decay. To investigate whether FLuc mRNA levels decreased upon binding to FMRP, we measured FLuc mRNA levels using qRT-PCR. We observed that FLuc mRNA levels remained constant irrespective of the concentration of FMRP transfected in S2 cells (Figure 12E). This observation suggests that FMRP-dependent decrease in the expression of the FLuc protein is not due to the degradation of the FLuc mRNA.
**Figure 12: Tethered FMRP represses the translation of FLuc reporter in a dosage-dependent manner.**

A) A schematic of λN-HA-tagged FMRP tethered to the FLuc-5xBoxB reporter in S2 cells. λN-HA-tagged FMRP binds with high affinity to 5 tandem BoxB sites inserted into the SV40 3’UTR of the FLuc-5xBoxB reporter. B) A schematic of the RLuc construct (transfection control) with no BoxB sites. C) Results of S2 cells transfected with a mixture of the FLuc-5xBoxB reporter, RLuc control, and λN-HA (empty vector) or increasing concentrations of λN-HA-tagged FMRP (0.125 µg, 0.25 µg and 0.50 µg). FLuc expression was normalized to the RLuc transfection control. D) S2 cell lysates were immunoblotted with antibodies against the HA tag and endogenous dFMRP. An antibody against Actin served as a loading control. Arrow represents endogenous FMRP and star represents an
isoform of approximately same size as λN-HA-FMRP. E) qPCR and luciferase activity to quantify the expression levels of FLuc-5xBoxB reporter show consistent mRNA levels with reduced protein expression in response to higher concentration of tagged FMRP (****; p<0.0001). The error bars are ±SEM.
3.2 The KH domains of tethered FMRP are required for translation repression of the FLuc-BoxB reporter.

We investigated whether domains within FMRP are required for translation repression by tethered FMRP. We designed a series of λN-HA and EGFP tagged FMRP deletion constructs that excluded these conserved domains: ∆RGG (470-507), ∆KH0 (116–212) and ∆KH1/2 (226–335) (Figure 13A). Surprisingly, our results suggest that deleting the KH1 and 2 domains significantly rescued the translation repression (p<0.0001) by tethered FMRP (Figure 13C). We observed a 20% repression of the FLuc reporter with the 0.50µg of ∆KH1/2 FMRP plasmid (Figure 13C). Similar, albeit weaker results were observed when the KH0 domain was deleted (Figure 13C B, 70% p<0.0001) and observed a 50% repression of the FLuc reporter with 0.50µg of ∆KH0 FMRP plasmid (Figure 13C). These results suggest that the KH domains of FMRP are required for mediating repression of its target mRNAs. Since, FMRP is already tethered to the reporter via the high-affinity λN/BoxB interaction, the requirement for these domains in translational repression appears to be independent of their RNA binding activity.

FMRP has previously been shown to localize to cytoplasmic foci in cells that contain mRNA (Barbee et al., 2006; Gareau et al., 2013b). Formation of these structures is facilitated, at least in part, through the activity of FMRP’s functional domains (Gareau et al., 2013b). We, therefore, next investigated whether deletion of the FMRP domains required for translation repression are involved the formation of FMRP granules in the cytoplasm. Our results suggest that deletion of the KH1 and 2 domains results in distinct cytoplasmic puncta (Figure 13B). These structures appear to be very large and roughly
spherical. Deletion of the RGG domains did not result in significant changes on the morphology of FMRP granules (Figure 13B). On the other hand, deletion of KH0 domains result in diffused localization of FMRP (Figure 13B). Since KH1 and 2 domain deletions also rescues translation repression by FMRP, these results suggest there might be a correlation between the size of the granules and de-repression. Taken together, we conclude that FMRP requires the KH domains for translation repression of its target mRNAs. This function may be associated with the morphology and dynamics of FMRP granules.
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B) 

Images showing different constructs: EGFP, dFMRE, dFMRE[ΔRGG], dFMRE[ΔKH1/2], dFMRE[ΔKH0].

C) 

Graph showing FLuc/RLuc levels against dosage of λN-FMRP.
**Figure 13: The KH domains of tethered FMRP are required for translation repression of the FLuc-BoxB reporter and FMRP-containing granule formation.**

A) A schematic representation of dFMRP showing the positions of the mRNA binding domains in dFMRP. Deletion versions of dFMRP were cloned into EGFP and λN-HA backbone vectors. The KH0 domains are boxed in yellow, KH1 in pink, KH2 in green, RGG in orange, and NES in blue. B) Live S2 cells expressing EGFP, EGFP-dFMRP, EGFP-tagged dFMRP deletion variants were imaged by confocal microscopy to detect EGFP. All images were taken using a 100X objective. Scale bar: 20µm C) Luciferase reporter assay for the FLuc-5xBoxB reporter with λN-HA-dFMRP and indicated λN-HA-tagged deletion versions of dFMRP. S2 cells transfected with λN-HA-dFMRP (ΔKH1/2) relieve FMRP mediated repression (****; p<0.0001). The error bars are ± SEM.
3.3 Tethered FMRP requires miRISC components to mediate repression of the FLuc-BoxB reporter.

Previous results suggest that FMRP interact with miRNAs to regulate the repression of target mRNAs (Edbauer et al., 2010; Jin et al., 2004b; Muddashetty et al., 2011). Therefore, we hypothesized that if miRNAs are involved in FMRP mediated repression, then depleting components of the miRNA pathway should attenuate the repression of the FLuc-BoxB reporter. Taking advantage of our translational reporter, we sequentially knocked down Dicer-1, core miRISC components (AGO1 and GW182), by RNAi in S2 cells and examined translation repression by tethered FMRP.

Knocking down Dicer1 (p<0.05), AGO1 (p<0.01) and GW182 (p<0.01) by transient transfection with the FLuc and RLuc plasmids rescued FMRP-mediated translation of the FLuc-BoxB reporter (Figure 14A). As a control, the introduction of dsRNA targeting EGFP had no significant impact on reporter expression (Figure 14A). We confirmed the knockdown efficiency of RNAi constructs targeting Dicer1, AGO1, and GW182 in transfected cells using antibodies and detected expression of these proteins by Western blotting (Figure 14B-D). Taken together, our data suggest that tethered FMRP requires the components of the miRNA pathway to repress translation of the FLuc-BoxB reporter.
Figure 14: Tethered FMRP requires miRISC components to mediate repression of the FLuc-BoxB reporter.
A) S2 cells were transfected with the FLuc-5xBoxB reporter, RLuc, vectors expressing λN-HA or λN-HA-tagged FMRP and co-transfected with dsRNA targeting Dicer1, AGO1, or GW182. We used RNAi against EGFP as our negative control, which did not affect the expression levels of FLuc reporter. RNAi against Dicer1 and candidate miRISC components (AGO1, GW182) significantly attenuated FMRP mediated repression. Data is shown from three independent experiments (*; p<0.05, **; p<0.01). The error bars are ± SEM. B-D) We used Western blotting to visualize the efficiency of Dicer1, AGO1, and GW182 RNAi knockdown using antibodies specific to the indicated proteins. Actin was used as a loading control. All target protein levels were significantly reduced.
3.4 FMRP interacts biochemically with AGO1 and GW182 in S2 cells.

Our results suggest that FMRP requires AGO1, GW182, and Dicer1 to repress translation of the reporter. Next, we investigated the nature of this interaction using co-immunoprecipitation assays in S2 cell lysates. We co-transfected S2 cells with FLAG-tagged AGO1 or GW182 and EGFP-tagged FMRP and isolated binding partners of FMRP using GFP-Trap (Figure 15A). We found that FMRP pulled down both AGO1 and GW182 under low salt conditions (150mM NaCl) (Figure 15B). The pull-down of GW182 was not nearly as efficient as that of AGO1. We propose that this could be due to an indirect interaction between FMRP and GW182, which is directly mediated by AGO1. To test this possibility, we co-transfected with an additional construct expressing HA-tagged AGO1 in order to alter the stoichiometry of the FMRP: AGO1 complex. Surprisingly, this had no impact on the amount of GW182 that was pulled down with FMRP (Figure 15B). It is possible that this is due to the abundance of target mRNAs for the miRISC components or the kinetics of the interaction of FMRP with AGO1.

In contrast, immunoprecipitation under high salt conditions (300mM NaCl) completely abolished the interaction between FMRP and GW182 (Figure 15C). However, interactions between FMRP and AGO1 were unperturbed. In these experiments, AGO2 was used as a positive control, as previous studies have shown a strong biochemical interaction between FMRP and both AGO1 and AGO2 (Caudy et al., 2002) (Figure 15C). Together, our data support the conclusion that FMRP interacts in a complex with the miRISC components, AGO1 and GW182. Moreover, these results also suggest an indirect relationship between FMRP and GW182 mediated either by AGO1 or some other protein.
Figure 15: FMRP co-immunoprecipitates with AGO1 and GW182 in S2 cells.
A) GFP-trap beads were used to pull-down EGFP-tagged FMRP and EGFP protein from S2 cell extracts. The proteins were resolved by SDS-PAGE and probed with antibodies against GFP and FMRP. Endogenous FMRP was detected in both non-transfected cells and cells transfected with EGFP-FMRP. EGFP-FMRP was detected in the IP fraction of transfected cells. B) Lysates from S2 cells transfected with plasmids expressing FLAG-tagged AGO1, GW182 were co-immunoprecipitated using GFP-trap under low salt conditions (150mM NaCl) C) Lysates from S2 cells transfected with plasmids expressing FLAG-tagged AGO1, GW182 were co-immunoprecipitated using GFP-trap under high salt conditions (300mM NaCl). FLAG-AGO2 was used as a positive control.
3.5 Untethered FMRP can repress the translation of the FLuc-BoxB reporter.

Previous studies suggest that FMRP is capable of recognizing and binding to small stable stem loops that are similar in structure to BoxB motifs and regulate the translation of specific target mRNAs (Bechara et al., 2009; Zalfa et al., 2003). Thus, we next asked whether transfected FMRP lacking the λN-HA tag was also able to repress the translation of the FLuc-5xBoxB reporter in a dosage-dependent manner.

To test this hypothesis, we modified the FLuc-BoxB reporter and reduced the number of BoxB motifs or remove them altogether (Figure 16A). We generated a series of FLuc-BoxB reporter with 3x, 1x, and 0x copies (essentially the “wild-type” SV40 3’UTR) of the BoxB sequence. Surprisingly, we found that FMRP was capable of repressing the translation of the FLuc reporter containing only a single BoxB stem-loop just as well as the reporter containing five BoxB stem-loops (Figure 16C; p<0.0001) and as well as tethered FMRP. In contrast, translation of a reporter containing no copies of the BoxB sequence was unaffected by untagged FMRP (Figure 16C). These results suggest that mRNAs with small stem-loops could be targets for FMRP, expanding the toolbox for the study of the mechanism of FMRP-mediated translation repression.
Figure 16: Untethered FMRP can repress the translation of FLuc-BoxB reporter. A) A schematic representation of FLuc-1xBoxB reporter showing a single BoxB site and a binding site for miR-958. B) A schematic representation of FLuc-0xBoxB reporter with no stem loops for FMRP to bind. C) Results for luciferase reporter assay for FLuc-5xBoxB, FLuc-3xBoxB, FLuc-1xBoxB and FLuc-0xBoxB reporter with 0.125μg, 0.25μg, and 0.50μg of FMRP. (**; p<0.01, ****; p<0.0001). The error bars are ±SEM.
3.6 miRNA-958 requires FMRP to mediate translation repression of the FLuc-BoxB reporter.

Our data provide evidence for a requirement of the miRISC complex for FMRP-mediated translation repression. However, it was unclear whether this process required miRNA binding to the target mRNA in order to repress its translation. In order to identify miRNAs that bind to the FLuc reporter, we ran the SV40 3’UTR (with the BoxB sequence) through a miRNA prediction algorithm, PITA, which scans for miRNA-mRNA base pair interactions and predicts a score for the interaction (Kertesz et al., 2007; Peterson et al., 2014). The prediction is based on the seed sequence of the miRNA, secondary structure of the target mRNA, and free energy of the interaction. miR-958 was predicted to have the highest score for FLuc mRNA interaction (Figure 17B). Moreover, the putative binding site for miR-958 is only 80 nucleotides from the BoxB stem-loop sequence, which is just outside the “footprint” of the RISC complex when it interacts with a target mRNA. Therefore, we transfected S2 cells with a plasmid expressing miR-958 to determine if it can repress translation of the minimal FLuc-1xBoxB reporter.

We found that transfected miR-958 represses translation of the FLuc mRNA by 34% compared to control (Figure 17A p<0.01). To elucidate the nature of the interaction between FMRP and miR-958, we used RNAi to knock down endogenous FMRP expression and asked if it disrupted miR-958-mediated repression of the FLuc-1xBoxB reporter. We found that miR-958 was unable to repress the translation of the FLuc reporter in the absence of FMRP in S2 cells (Figure 17A). This suggests that FMRP and the miR-958 are both required for effective translation repression of target FLuc mRNA.
To further validate the requirement of FMRP, we used the FLuc-0xBoxB reporter lacking the BoxB stem-loop sequences (Figure 16B). This reporter is not repressed by untagged FMRP (Figure 16C). We found that miR-958 did not repress the translation of the FLuc-0xBoxB reporter even in the presence of endogenous FMRP (Figure 17A). This suggests that miR-958 mediated translation repression requires FMRP’s interaction with the target mRNA via the BoxB sequence. These results suggest that FMRP binding is required for miR-958 to mediate translation repression. Moreover, these findings suggest that FMRP and miRNAs can function cooperatively in the repression of a shared target mRNA. However, miR-958 is expressed in S2 cells at extremely low abundance (Figure 17B). Therefore, we rule out endogenous miR-958 in repressing the translation of the FLuc-1xBoxB reporter in the tethered FMRP experiments described above.

Next, we sought to determine the identity of actual miRNA that might work in concert with FMRP to repress translation of the FLuc-BoxB reporter. We used PITA to identify other endogenous, abundant miRNAs in S2 cells that predicted to bind to the 3'UTR of the FLuc-BoxB reporter. In our analysis, bantam, miR-33, miR-13, miR-2a, and miR-2b were predicted to bind to the FLuc-BoxB 3'UTR (Figure 17B). We have cloned the indicated miRNAs in the miRNA-expression plasmid for future analysis.

To validate an interaction between FMRP and the identified miRNAs, we pulled down FMRP-containing RNPs from S2 cells expressing EGFP-tagged FMRP using a GFP trap system. The isolated mRNA and miRNA levels from FMRP-IP fraction were analyzed by quantitative real-time PCR (qRT-PCR). Compared to non-transfected control cells, we observed an enrichment of bantam, miR-2b, miR-13b and miR-33 in cells expressing
tagged FMRP (Figure 17C). We also observed non-specific binding using the IgG antibody. Future experiments are needed to optimize the RNA-IP experiment to explore the coordinated nature of FMRP and miRNA in target mRNA repression.
Figure 17: miRNA-958 requires FMRP to mediate translation repression of the FLuc-BoxB reporter.

A) Cells were co-transfected with the FLuc-1xBoxB reporter, RLuc and a plasmid expressing miR-958. Interestingly, miR-958 was unable to repress the reporter when FMRP was depleted by RNAi. Furthermore, in the absence of BoxB motifs, miR-958 could not repress the FLuc reporter. These results suggest a requirement of FMRP (or FMRP binding) for miR-958 to mediate the repression of FLuc-BoxB reporter. Values in columns represent the % of the control activity (*; p<0.05, **; p<0.01). The error bars are ±SEM.

B) List of miRNAs predicted by PITA algorithm for binding to SV40 3’UTR in the FLuc reporter based on the position relative to the first nucleotide in the 3’UTR sequence, seed region, free energy of binding and reads per million (RPM).

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Position</th>
<th>Seed</th>
<th>ΔΔG</th>
<th>RPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-958</td>
<td>80</td>
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<td>-12.27</td>
<td>250</td>
</tr>
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<td>bantam</td>
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<tr>
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<td>6:0:0</td>
<td>-2.61</td>
<td>1.1 x 10^5</td>
</tr>
</tbody>
</table>

C) FMRP containing mRNPs were pulled down and the isolated mRNAs were analyzed by the quantitative real-time PCR.
(qRT-PCR) using probes against miRNAs. Bantam, miR-2b, miR-13b and miR-33 were enriched in FMRP associated RNP fraction vs non-transfected controls. The results are from a single experiment (therefore, no error bars are shown).
3.7 FMRP can repress the translation of FLuc reporters for known target mRNAs that contain BoxB-like sequences in their 3’ UTRs.

Since FMRP represses the translation of a reporter mRNA containing the BoxB motifs, we hypothesized that FMRP may be interacting with endogenous target mRNAs via similar sequences. Previous studies suggest that FMRP directly interacts with mRNAs encoding for the degenerin/sodium channel family protein, Ppk, GTPase activating protein, dRac1, microtubule associated protein Futsch, actin binding protein Chic, activity-regulated cytoskeletal protein, Arc1, protein involved in mRNA localization, Tral, and itself, dFmr1 (Dictenberg et al., 2008; Lee, 2003; Monzo et al., 2006; Reeve et al., 2005; Schaeffer et al., 2001; Xu et al., 2004; Zhang et al., 2001).

We used RNAfold to analyze the secondary structure of the 3’UTRs of the indicated mRNAs and observed stem loop sequences similar in structure to BoxB motifs (Figure 18A). First, we determined if these mRNAs co-immunoprecipitated with FMRP in S2 cell lysates (Figure 18C). We found that dRac, dFmr1 and chic mRNAs were enriched in FMRP associated mRNP complexes. ppk is not expressed in S2 cells. These results suggest a direct association of FMRP with the indicated mRNA targets. Next, we determined whether untagged FMRP could repress the translation of FLuc-3’UTR reporter for all these candidate mRNAs. In each reporter, we removed the SV40 3’UTR sequence including the BoxB sequences and replaced with the 3’UTR of the above-mentioned genes. We observed that untagged FMRP effectively replaced translation of FLuc-ppk 3’UTR, FLuc-chic 3’UTR and FLuc-Arc1 3’UTR in a dosage dependent manner (Figure 18D-E, p < 0.0001). However, FMRP did not repress the translation of the FLuc-dRac1 3’UTR or FLuc-Tral.
3’UTR. (Figure 18E). These results suggest that FMRP mediated repression may not be limited to the BoxB-like stem-loop structures.

We also determined the 3’UTRs of the candidate genes for the most well characterized FMRP-binding motif, G-quartets using QGRS mapper (Kikin et al., 2006). In our analysis, we found the 3’UTR of dRac with 2 and Arc1 with 6 predicted G-quartets while ppk, chic and Tral had none. Therefore, the analysis suggests that structural motifs may not be enough for FMRP to mediate repression and repression may be sequence specific. FMRP is predicted to bind to ACUK or WGGA sequence in the target mRNA for repression. It would be interesting to determine whether the repression is being mediated by the stem loops or specific sequences such as WGGA or ACUK).
Figure 18: Untethered FMRP can repress the translation of FLuc reporters for known target mRNAs that contain BoxB-like sequences in their 3’ UTRs.

A) The secondary structure of the FLuc-1xBoxB structure was folded by RNAfold software. Stable structures were colored by base-pairing probabilities (ranging from red to green). Box B motifs denoted in red color were predicted to be stable structure. B) The 3’UTR of ppk contained Box-B like motifs as predicted by RNAfold. C) FMRP containing
RNPs were pulled down from S2 cells and analyzed for associated mRNAs by Reverse-transcription (RT-PCR). Chic, dRac and dFmr1 were enriched in FMRP-IP fraction compared to the IgG control. Ppk is not expressed in S2 cells. D) The entire 3’UTR region of ppk, chic and tral was cloned downstream of FLuc reporter. The SV40-3’UTR of the FLuc reporter was swapped by the entire 3’UTR of the indicated genes. These reporters were co-transfected with the RLuc, λN-HA (control), and with increasing concentration of untethered FMRP (0.125µg, 0.25µg and 0.50µg). Luciferase data shown here for the FLuc-ppk 3’UTR (****; p < 0.0001). The errors are in ±SEM. E) S2 cells were co-transfected with the FLuc-ppk 3’UTR, FLuc-chic 3’UTR, FLuc-tral 3’UTR reporters, RLuc, λN-HA (control) and 0.50µg of FMRP. Columns indicate the % of the control activity (****; p < 0.0001). The errors are in ±SEM.
3.8 FMRP colocalizes to granules containing AGO1 and GW182 in S2 cells.

Previous studies have demonstrated that FMRP localizes to P-bodies and stress granules along with translationally repressed mRNAs, miRNAs, AGO1, GW182 and several other RBPs, to mediate gene silencing (Parker and Sheth, 2007). The formation of these cytoplasmic granules is linked to the miRNA-mediated repression pathway (Eulalio et al., 2007c). Therefore, we wanted to determine if FMRP colocalizes with Dicer1, AGO1, and GW182 in cytoplasmic RNP granules in S2 cells.

We performed immunostaining in S2 cells for FMRP, Dicer1, AGO1, and GW182 (Figure 19A). Our results show a strong colocalization of FMRP with AGO1 (Mander’s coefficient 0.7± 0.02) and GW182 (0.9± 0.01) in S2 cells (Figure 19B). These results suggest that dFMRP and miRISC proteins are in close proximity in the cell and potentially interact with each other. On the other hand, we did not observe substantial colocalization of FMRP with Dicer1 (0.45±0.02) (Figure 19B). Interestingly, we observed a distinct perinuclear distribution of Dicer1 (Figure 19A). This distinct phenotype for Dicer1 has not been shown in other systems. This observation needs to be explored in future experiments to determine if this is significant for its function as a microRNA processor.
Figure 19: FMRP colocalizes to granules containing AGO1 and GW182 in S2 cells. A) S2 cells were stained with antibodies against FMRP, Dicer1, AGO1, and GW182. Images for FMRP (green), Dicer, AGO1 and GW182 (red) were taken at 100X objective. Cell nuclei were counterstained with DAPI (blue). Arrows point towards the regions with an overlap of green and red fluorescent signals. Scale bar: 5µm. B) Degree of colocalization (Mander’s coefficient) was calculated for the overlap between green and red channels. N=6 independent S2 cells were analyzed for the analysis. (****, p < 0.0001). The error bars are mean ± SEM.
3.9 FMRP is expressed in the same population of neurons as AGO1 and GW182 within the cell bodies in the *Drosophila* larval CNS.

Our data in S2 cells suggest FMRP to colocalize in granules with the miRISC components. Next, we wanted to determine if FMRP also colocalizes with the miRISC components *in vivo*. Previous studies suggest that FMRP can localize to neuronal transport granules in mammalian and *Drosophila* neurons (Cougot et al., 2008). These granules are enriched in translationally repressed mRNAs, along with several miRNAs, miRISC components and various RBPs. Moreover, dendritic P-bodies have also been suggested to contain FMRP, miRNAs, miRISC components with translationally repressed mRNAs (Cougot et al., 2008). The assembly of all the proteins involved in mRNA regulation in distinct granules is speculated to modulate translation in neurons (Barbee et al., 2006). Given the role of FMRP and miRNAs in translation regulation for proper neuronal morphology, we hypothesized FMRP would colocalize with the miRISC components and Dicer1 in neurons within the *Drosophila* larval CNS.

Ventral ganglia of the *Drosophila* CNS contains descending motor neurons that project into the muscles carrying information to control flight, walking and other motor functions (Strausfeld, 2009). Therefore, we counterstained ventral ganglia to examine the intracellular localization of FMRP with Dicer1 and the miRISC components (Figure 20A). To examine the subcellular localization of FMRP and miRISC components in more detail, we imaged neuronal cell bodies of motor neurons along the midline (Figure 20B). We observed a strong overlap of pixels between FMRP, AGO1 (0.6±0.02) and GW182 (0.8±0.01) within the soma of the cell bodies (Figure 20C). However, we do not have the
required resolution to determine if FMRP colocalizes with AGO1 and GW182 within
neuronal granules in motor neurons. This question will require further analysis.

In our analysis, FMRP did not show a as strong of an overlap with Dicer1 (0.5±0.05,
Figure 20C). However, Dicer1 showed the similar perinuclear phenotype within cell bodies
of the ventral ganglia as seen earlier in S2 cells (Figure 20B). Again, since there are no
published studies to indicate that Dicer1 localizes to nuclear membranes, this could be a
novel and potentially interesting finding.
Figure 20: FMRP is expressed with AGO1 and GW182 in the same population of motor neurons within the Drosophila larval CNS.
A) Ventral ganglion of the wandering third instar larvae stained with antibodies for FMRP (green), Dicer1, AGO1, and GW182 (all red). The panels are from a single optical section as acquired by confocal microscopy. The arrows in the merged panels indicate the overlap of FMRP fluorescent intensities between FMRP (green) and the indicated components of the miRNA pathway Scale bar: 50 µM. B) Cell bodies within the ventral ganglion were imaged using 100X for FMRP (green) and the indicated miRISC components (red). Cell nuclei are counterstained for DAPI. The arrows in the merged panels indicate the overlap of FMRP fluorescent intensities between FMRP (green) and the indicated components of
the miRNA pathway (red). C) Degree of colocalization (Mander’s coefficient) was calculated for the overlap between green and red channels. N=3 independent CNS images were analyzed for the analysis. (****; p < 0.0001). The error bars are the mean ±SEM.
3.10  *dFmr1* interacts with GW182 to regulate synaptic growth.

We used *Drosophila* larval NMJs as our model system to further characterize the phenotypic significance of the interaction between FMRP and miRISC components. The *Drosophila* larval NMJ is a well-defined and relatively simple model system to identify genetic interactions between genes by assessing the changes in synaptic structure and function (Budnik, 1996). It has been shown that FMRP interacts genetically with AGO1 to control NMJ growth (Jin et al., 2004b). Flies singly heterozygous for *dAGO1* (*ago1^k00208/+*) and *dFmr1* (*dFmr1^Δ113/+*) have normal NMJ phenotype and a similar number of boutons compared to wild-type larvae. However, removal of a one copy of AGO1 in a phenotypically normal FMRP heterozygous background (an FMRP; AGO1 trans-heterozygote) results in a highly significant overgrowth of the NMJ similar to that seen in a *dFmr1* homozygous null mutant (Jin et al., 2004b). These results suggest that FMRP requires AGO1 to regulate proper synaptic development. Based on the published studies and our results in S2 cells, we speculated that FMRP might also genetically interact with GW182 to regulate NMJ development.

To test the hypothesis, we examined whether *dAGO1* (as a positive control) and *gawky* (fly GW182) could modulate the synaptic growth regulated by *dFmr1* using the loss of function *dFmr1* mutants (*dFmr1^Δ113*). We assessed the morphology of NMJs at muscle 6/7 in abdominal segment 3 of wandering third instar larva (Figure 21A). We stained dissected NMJs with pre-synaptic marker anti-HRP and post-synaptic marker anti Dlg for characterizing synaptic morphology. First, we examined the NMJs from *dFmr1* heterozygotes (*dFmr1^Δ113/+*), *AGO1* heterozygotes (*ago1^k00208/+*) and *gawky*
heterozygotes (gw1/+). In our hands, dFmr1Δ113 nulls did not survive to the third instar stage (data not shown). Counting the number of synaptic boutons for each of these genotypes assessed changes in NMJ development. We normalized the total number of boutons to the corresponding muscle surface area (MSA) to avoid any biases that might arise as the result of variations in the muscle size among different larvae (Figure 21B).

We did not observe any significant increase in the number of synaptic boutons for AGO1 or dFmr1 heterozygotes relative to controls (Figure 21B). The gawky heterozygotes showed a small but significant increase in the number of boutons compared to w1118 controls (25% increase, p < 0.05 Figure 21B). These results suggested that either loss of a single copy of gawky was sufficient to cause synaptic defects or could also stem from the combined effects of the balancer in the gawky heterozygous background.

To address if AGO1 or GW182 act in the same pathway as FMRP, we generated trans-heterozygotes for dAGO1 (ago1k00208/+; dFmr1Δ113/+) and GW182 (gw1/++; dFmr1Δ113/+), respectively. Previous results have shown that dFmr1 causes synaptic overgrowth at the NMJ while dFmr1 heterozygotes appear to have normal NMJ (Jin et al., 2004b). These results suggest that a single copy of FMRP can regulate proper synaptic development at the NMJ. If both the genes interact with dFmr1 in the same pathway, then reducing expression levels of these genes in dFmr1 heterozygous background should disrupt the function of FMRP and cause synaptic overgrowth.

We observed a robust increase in boutons for gawky trans-heterozygotes compared to dFmr1 heterozygotes (80% increase, p<0.0005). The phenotype was stronger than gawky heterozygotes suggesting that the observed phenotype was due to the disruption of
gawky at the NMJ (Figure 21A-B). Taken together, the results suggest that FMRP interacts with GW182 in the same pathway to regulate synaptic growth. However, contrary to published results, the NMJs from AGO1 trans-heterozygotes background did not show any significant changes in the number of boutons (Figure 21A-B). It is possible that the allele for AGO1 used in our studies might have picked up a unique set of mutations or interactions with suppressors may have disrupted its function.

Based on the size, shape glutamatergic type I boutons are classified into 1b (big) and 1s (small) boutons that are derived from two different motor neurons (Hoang and Chiba, 2001). We quantified the number of 1b and 1s boutons at the larval NMJ for the indicated genotypes (Figure 22A-B). The gawky trans-heterozygotes showed an even more pronounced change in the number of 1b boutons compared to mutant dFmr1 heterozygotes (75% increase, p<0.0005) (Figure 22A). Moreover, unlike 1b boutons, we did not observe any significant changes in the number of 1s boutons for AGO1 or gawky trans-heterozygotes (Figure 22B). AGO1 heterozygotes did not show any significant changes in the number of 1b or 1s boutons at the NMJ (Figure 22A-B).

dFmr1 nulls are also characterized by extensive neuronal branching at the NMJ (Zhang et al., 2001). Therefore, we quantified NMJs on the extent of synaptic branching and the number of branch-tips for the AGO1 and gawky trans-heterozygotes (Figure 22C-D). We observed no significant changes in the number of branch points or tips in our analysis. Taken together, our results indicate that FMRP requires GW182 to the proper synaptic structure during development at the Drosophila NMJ.
Figure 21: \(dFmr1\) interacts genetically with \(gawky\) to regulate synaptic growth. 
A) Larvae from \(w^{1118}\) controls, \(dFmr1^{\Delta113}\) heterozygotes, heterozygotes for \(AGO1\), \(gawky\) and trans-heterozygotes for \(AGO1\), \(gawky\) (generated in the \(dFmr1^{\Delta113}\) background) were analyzed for NMJ phenotype. The size of the NMJ (normalized to muscle surface area or...
MSA) was determined at muscle 6/7 in abdominal segment A3 for each genotype as indicated. The NMJs were stained against postsynaptic density marker Dlg (green). Scale bar: 50 µM. B) The total number of synaptic boutons/MSA were quantified for each genotype. Trans-heterozygotes of gawky had a robust increase in the number of boutons /MSA (normalized to control) compared to dFmr1^{Δ113} heterozygotes. (*p < 0.05, **p < 0.01; ****; p < 0.0001). The error bars are the mean ± SEM.
Figure 22: Trans-heterozygotes of *gawky* display an increased number of 1b boutons. NMJs for the indicated genotypes were quantified for various parameters of NMJ morphology and compared to *dFmr1* heterozygotes. A) Total number of 1b boutons/MSA were significantly higher for *gawky* trans-heterozygotes. B) No significant difference was observed for the normalized 1s boutons. C) No significant difference was observed for the tips for the indicated genotypes. D) Branch-points were not affected in the indicated genotypes. (*p* < 0.05, **p** < 0.01; ****; p < 0.0001). The error bars are the mean ±SEM.
3.11 dFmr1 and miRNA pathway components do not interact to control rates of larval locomotion.

It was recently showed that an overgrowth of synaptic boutons at the larval NMJ correlates with locomotion dysfunction in dFmr1 heterozygotes (Kashima et al., 2017). Using a quantitative larval locomotion algorithm, known as “Larvatrack”, the authors used dFmr1 heterozygotes (dFmr1Δ50/+ and dFmr1Δ3/+ and dFmr1Δ113/+), and found that synaptic overgrowth correlated with augmented crawling velocity. Taking a cue from their observations, we used Larvatrack to investigate if dFmr1 heterozygotes (dFmr1Δ113/+ used in our NMJ analysis, exhibits increased velocity (Figure 23 A-B). Based on our NMJ analysis, we speculated that in comparison to dFmr1 heterozygotes, we would observe a higher velocity for gawky heterozygotes and gawky trans-heterozygote larvae and no changes for AGO1 heterozygotes or AGO1 trans-heterozygote larva.

Although we did not observe synaptic overgrowth with dFmr1Δ113 heterozygotes in our NMJ analysis, dFmr1 heterozygote larvae moved 30% faster than w1118 controls (Figure 23B). These results suggest that changes in locomotion dysfunction does not correlate directly with changes in the number of synaptic boutons. Similar to the results observed in dFmr1 heterozygotes, we found that gawky heterozygotes were 22% faster than w1118 controls while AGO1 heterozygotes were 12% faster than the controls. However, neither AGO1 nor gawky trans-heterozygotes displayed any significant alterations in velocity in comparison to the dFmr1 heterozygotes (Figure 23B). Contrary to previously reported observations our results suggest that there is no significant correlation between the observed synaptic overgrowth and aberrant velocity in Drosophila larva. It is also possible
that beyond a certain threshold, larvae cannot move faster, which could explain the lack of any significant changes in the velocity between and $dFmr1$ heterozygotes or trans-heterozygotes for the miRISC components.
Figure 23: *dFmr1* and miRNA pathway components do not interact to control rates of larval locomotion.

Larval locomotion was quantified using the “LarvaTrack” algorithm for indicated genotypes. A) Cumulative distance traveled in millimeters by the larvae over 60 seconds was plotted for comparison between *w*¹¹¹⁸, *dFmr1* heterozygotes, *AGO1*, *gawky* heterozygotes and for the trans-heterozygotes for *AGO1* and *gawky*. No significant differences were observed between the trans-heterozygotes of the indicated genotypes and
*dFmr1* heterozygotes. B) Quantitative histogram showing the velocity of the larvae for the indicated genotypes. No significant difference was observed between the indicated genotypes and the *dFmr1* heterozygotes. The error bars are the mean ±SEM.
3.12 The role of *dFmr1* in regulating synaptic growth is independent of the deadenylase complex (CCR4 and NOT1), PABP or the decapping complex (Dcp1).

We sought to determine the downstream mechanisms by which FMRP-GW182 regulates synaptic morphology. Previous studies suggest that GW182 can act as the mediators of translation repression by interacting with the components of deadenylase machinery, including CCR4 and NOT1 and PABP to mediate deadenylation-dependent and independent mode of translation repression (Braun et al., 2013). Moreover, GW182 also interacts with the decapping enzyme, Dcp1 to mediate gene silencing via the miRNAs (Rehwinkel et al., 2005). GW182 assembles with CCR4, NOT1, PABP, and Dcp1 into mRNP granules along with several miRNAs and associated mRNA targets in P-bodies (Decker and Parker, 2012). Therefore, we hypothesized that FMRP might interact with CCR4, NOT1 or PABP downstream of GW182 to mediate translation repression, which might play a role in proper synaptic development at the larval NMJ.

3.13 FMRP is expressed with CCR4, CAF1, and NOT1 within the same cells in the larval CNS.

We immunostained larval ventral ganglia to investigate if FMRP and the deadenylase components co-localized to the same population of cells. In flies, CCR4 and NOT1 are the main enzymes responsible for the deadenylation of target mRNAs (Temme et al., 2004). CAF1 (fly ortholog of POP2) associates with CCR4 and possesses catalytic deadenylation ability (Temme et al., 2010). CAF1 can replace CCR4 as the main catalytic component of the deadenylase complex and the two proteins may exhibit some functional redundancy. We observed a diffused localization of FMRP and CCR4 in the CNSs (Figure
24A). Compared to CCR4, NOT1 and CAF1 appeared to be more punctate in appearance (Figure 24A). In our analysis, there was strong overlap between FMRP and CCR4 (0.65±0.01) followed by NOT1 (0.6±0.01) and CAF1(0.5±0.01). However, we do not have sufficient optical resolution to conclude if FMRP colocalizes with deadenylase components at the larval NMJ. Imaging at higher resolution is required to determine the extent of colocalization between FMRP and the deadenylase components in granules.
Figure 24: FMRP is expressed with CCR4, CAF1, and NOT1 within the same cells in the larval CNS.
A) Ventral ganglion of the wandering third instar larvae stained with antibodies targeting FMRP (green), CCR4, NOT1, and CAF1 (red). The panels are from a single optical section as acquired by confocal microscopy. The arrows in the merged panels indicate the overlap of FMRP fluorescent intensities between FMRP (green) and the indicated deadenylase components. Scale bar: 50 µM. Cell bodies within the ventral ganglion were imaged using 100X for FMRP (green) and the indicated deadenylase components (red). The arrows in
the merged panels indicate the overlap of FMRP fluorescent intensities between FMRP (green), CCR4, NOT1, and CAF1(red). C) Degree of colocalization (Mander’s coefficient) was calculated for the overlap between green and red channels. N=3 independent CNSs were analyzed for the analysis (****; p < 0.0001). The error bars are the mean ± SEM.
3.14 FMRP shows no genetic interaction with CCR4, NOT1, PABP or Dcp1 at the larval NMJ.

Next, we used the larval NMJ as our in vivo model system to validate the interactions between FMRP and CCR4, NOT1, PABP or Dcp1. Based on the known interactions of GW182 in vitro, we hypothesized that CCR4, NOT1, PABP or Dcp1 may act downstream of FMRP and GW182 in the same genetic pathway to regulate synaptic development. To test this possibility, we asked if CCR4, NOT1, PABP or Dcp1 would show trans-heterozygous interactions with FMRP at the larval NMJ. We examined the NMJs from dFmr1 heterozygotes (dFmr1^A113/+), and heterozygotes for the candidate genes: Twin^GS12209/+, Not1^M107631/+, pAbp^k10109 /+, and Dcp1^442p /+. We assessed the changes at NMJ by counting the total number of synaptic boutons for each of these genotypes. Again, as the number of synaptic boutons can increase proportionally with MSA, we normalized synaptic bouton numbers against MSA for quantification. We did not observe any significant increase in the number of synaptic boutons for the heterozygous larvae of Twin, Not1, PABP or Dcp1 (Figure 26, 27). We observed a 5% increase in Twin Heterozygotes, increase, 0% increase in Not1 heterozygotes, 10% increase in pAbp heterozygotes and a 5% decrease in Dcp1 heterozygotes.

Next, we removed a single copy of the Twin, Not1, pAbp, and Dcp1 in the dFmr1 heterozygous mutant background to generate trans-heterozygotes for the indicated genes. As described previously, an increase in the number of synaptic boutons in trans-heterozygotes for these genes compared to controls would suggest genetic interactions within the FMRP pathway. In comparison to dFmr1 heterozygotes, Twin trans-
heterozygotes ($Twin^GSi2209/+; dFmr1^{G113}/+$) displayed a 10% increase in synaptic boutons. Not1 trans-heterozygotes ($Not1^{M10763}/*; dFmr1^{G113}/+$) exhibited a 15% increase over $dFmr1$ heterozygotes (Figure 26B). The trans-heterozygotes for $pAbp$ ($pAbp^{k10109}/+$; $dFmr1^{G113}/+$) showed a 5% increase, and $Dcp1$ ($Dcp1^{442p}/+$; $dFmr1^{G113}/+$) displayed a 5% decrease in the number of synaptic boutons over $dFmr1$ heterozygotes (Figure 27B). None of these results were statistically significant. These results suggest FMRP does not interact with CCR4, NOT1, PABP or DCP1 in the same pathway to controlling NMJ growth. It is possible that FMRP may regulate NMJ development via the miRISC through a non-deadenylation dependent mechanism. Another possibility for the lack of any observed phenotype at the NMJ could be due to the redundancy between deadenylase enzymes and PABP in Drosophila. It would be interesting to test the genetic interactions between FMRP and CAF1 and other subunits of the NOT1 enzyme complex.
Figure 25: *dFmr1* shows no genetic interactions with *Twin* or Not1 at the larval NMJ. A) Larvae from *w*1118 controls, *dFmr1*Δ113 heterozygotes, heterozygotes for *Twin, Not1* and trans-heterozygotes for *Twin, Not1* (generated in the *dFmr1*Δ113 background) were analyzed for NMJ phenotype. The size of the NMJ was determined at muscle 6/7 in abdominal segment A3 for each genotype as indicated. The NMJs were stained against postsynaptic density marker Dlg (green). Scale bar: 50 μM. B) Total number of synaptic boutons/MSA were quantified for each genotype. No significant difference was observed for the trans-heterozygotes of *Twin and Not1* in the number of boutons/MSA (normalized to control) compared to *dFmr1*Δ113 heterozygotes. The error bars are mean ± SEM.
A) 

![Immunofluorescence images](W118 vs dFmr1^{1112/+} vs pAbp^{10109/+} vs dFmr1^{1112/+} vs Dcp1^{1429/+} vs Dcp1^{1429/+} dFmr1^{1112/+}).

B) 

**Normalized boutons (% control)**

- W^{1118}
- pAbp^{10109/+}
- Dcp1^{1429/+}
- dFmr1^{1112/+}

**Genotypes**
Figure 26: dFmr1 shows no genetic interactions with pAbp or Dcp1 at the larval NMJ.

A) Larvae from w1118 controls, dFmr1Δ113 heterozygotes, heterozygotes for pAbp or Dcp1, and trans-heterozygotes for pAbp or Dcp1 (generated in the dFmr1Δ113 background) were analyzed for NMJ phenotype. The size of the NMJ was determined at muscle 6/7 in abdominal segment A3 for each genotype as indicated. The NMJs were stained against postsynaptic density marker Dlg (green). Scale bar: 50 µM. B) Total number of synaptic boutons/MSA were quantified for pAbp and Dcp1. No significant differences were observed between the indicated trans-heterozygotes and dFmr1Δ113 heterozygotes. The errors bars are mean ± SEM.
3.15 FMRP does not interact with CCR4, NOT1, PABP or Dcp1 to control rates of larval locomotion.

We used the larval crawling assay as a rapid and quantitative approach to screen components of the deadenylase complex including *Twin* (the fly ortholog of CCR4) and the scaffold protein *Not1* as well as *pAbp* and the decapping enhancer *Dcp1* for defects in locomotion. Based on the studies by Kashima et al. we speculated that trans-heterozygotes for these candidate genes might display a faster velocity than controls and might correlate with the synaptic overgrowth phenotype (Kashima et al., 2017).

First, we examined the crawling velocity of the wild-type (*w^{1118}* and *dFmr1* heterozygote (*dFmr1^{Δ113p}/+* ) larvae using “LarvaTrack” algorithm. We observed *dFmr1* heterozygotes to be 30% faster than the wild-type larvae (p< 0.01) (Figure 25). *dFmr1* heterozygotes display a normal NMJ phenotype, therefore, these results do not suggest a correlation between larval locomotion and synaptic overgrowth. Compared to wild-type larvae, *Not1* heterozygotes (*Not1^{M107631}/+*) displayed a higher velocity (p<0.01). However, heterozygotes of *twin* (*Twin^{GS12209}/+*), *pAbp* (*pAbp^{k10109}/+*), or *Dcp1* (*Dcp1^{442p}/+*) did not exhibit a higher velocity than the wild-type larvae. These results suggest that compared to wild type larvae, *Not1* heterozygotes are significantly more hyperactive than the *Twin*, *pAbp*, or *Dcp1* heterozygous larvae (Figure 25).

Next, we compared the locomotion between the *dFmr1* heterozygous control larvae and animals that were trans-heterozygotes for the indicated genes. As with miRNA pathway components, none of the trans-heterozygotes displayed any significant increase in
velocity in comparison to the single heterozygotes genotypes (Figure 25). Together, our results do not support locomotion to be a functional readout for NMJ defects.
Figure 27: *dFmr1* does not interact with *Twin, Not1, pAbp* or *Dcp1* to control rates of larval locomotion.

Larval locomotion was quantified using “LarvaTrack” algorithm for the indicated genotypes. Average velocity for the larvae was plotted for comparison between *w*¹¹¹⁸ and *dFmr1* heterozygotes and the trans-heterozygotes for the indicated genotypes. No significant difference was observed between the indicated genotypes and the *dFmr1* heterozygote controls in the rate of velocity. The error bars are mean ± SEM.
CHAPTER FOUR: DISCUSSION

4.1 FMRP requires AGO1 and GW182 to mediate translation repression.

Previous studies suggest that FMRP associates with essential components of the miRNA pathway such as Dicer and AGO proteins, as well as with specific miRNAs (Cheever and Ceman, 2009; Edbauer et al., 2010; Jin et al., 2004b). FMRP associated miR-125b and miR-132 have opposite effects on dendritic spine morphology in hippocampal neurons (Edbauer et al., 2010). Muddashetty et al. suggest that FMRP promotes the formation of an Ago2-miR-125a complex on PSD-95 mRNA and this translation inhibitory complex is regulated by the phosphorylation of FMRP. However, the mechanism by which the association of FMRP regulates translation via the miRNA pathway is not fully understood. Using an in vitro translation reporter assay in S2 cells, we provide the first evidence of a requirement for the downstream effector GW182 in FMRP mediated repression. We speculate that the interaction of FMRP with AGO1 and GW182 may facilitate miRNAs to bind to target mRNAs and repress their translation.

The GW182 proteins are important miRISC effectors, and are recruited by AGO proteins to miRNA-targeted mRNAs (Fabian et al., 2012). FMRP could either interact directly with GW182 or interact with GW182 in an AGO-dependent manner. Our results suggest a weak interaction between FMRP and GW182 in co-IP assays, which could suggest an AGO-dependent interaction at play. However, we do not rule out the possibility
of our assay conditions playing a role in our observations. Additional experiments are required to further clarify the nature of these biochemical interactions.

Why might FMRP interact with GW182? GW182 binds to AGO proteins and act as a platform to recruit several proteins to the target mRNAs for gene silencing (Fabian and Sonenberg, 2012). Some studies suggest that GW182 can bind to PABP and thus interfere with mRNA circularization for target mRNA repression (Fabian and Sonenberg, 2012). However, other studies in cell culture assays suggest that GW182 recruits CCR4-NOT1 to mediate deadenylation independent or deadenylatation dependent mode of translation repression (Fabian and Sonenberg, 2012). We speculate that FMRP could bind to multiple RBPs through GW182 to form an mRNP complex for translation regulation. A growing number of studies suggest that miRNAs and RISC components localize in P-bodies, stress granules and neuronal granules (Buchan, 2014). Moreover, GW182 can act as a scaffold for P-body assembly as their depletion can cause dissolution of P-bodies (Eulalio et al., 2007a). Based on these observations, it is likely that FMRP through its interaction with GW182 could reside simultaneously in different types of mRNP granules, each regulating translation of specific targets in different ways.

4.2 FMRP requires AGO1 and GW182 to control NMJ development.

Previous studies by Jin et al. suggest genetic interactions between FMRP and AGO1 is crucial to regulate larval NMJ phenotypes (Jin et al., 2004b). We investigated genetic interactions between FMRP and both AGO1 and GW182 using Drosophila larval NMJs as our model system. We provide evidence for the first time that FMRP requires GW182 to regulate NMJ development. We observed a robust phenotype of synaptic
overgrowth at the larval NMJ for GW182 trans-heterozygotes. However, we could not detect any effect on the NMJs with AGO1 trans-heterozygotes. We speculate that either the AGO1 allele picked up suppressor mutations that may have disrupted its function. Nevertheless, our results suggest that FMRP requires components of miRNA pathway to control NMJ development. These interactions are crucial to control synaptic plasticity.

How might the interaction control NMJ phenotype? One possibility may be that the interaction between FMRP and miRISC components may be involved in the transport and repression of specific transcripts within neuronal granules in motoneuron axons and that control NMJ development. Thus, disruption of the interaction between FMRP and miRISC components may be linked to defects in mRNP assembly or mRNA transport at the NMJ. However, future experiments are needed to explore the hypothesis in detail.

We also investigated downstream components that might regulate NMJ development. However, none of the trans-heterozygotes for the candidate genes (Twin, Not1, PAbp or Dcp1) tested displayed a synaptic overgrowth phenotype. It may be possible that an alternate pathway might compensate for the function of Twin, Not1, PABP or Dcp1 at the NMJ to control the NMJ phenotype. Therefore, the components by which FMRP through GW182 may regulate the NMJ phenotype still remains unclear. Future experiments will need to identify the downstream components of FMRP and GW182 involved in mediating translation control within the same pathway.

4.3 Cooperative interaction between FMRP and miRNAs regulate translation.

Our results strongly suggest that FMRP binds to the BoxB stem loop sequence in the 3’UTR of the FLuc reporter, which is in close proximity to the binding site for miR-
Both miR-958 and FMRP can cooperatively repress the translation of the FLuc reporter. Interestingly, depletion of FMRP from S2 cells resulted in attenuation of miR-958-mediated repression. These results suggest that FMRP may play a role in recruiting miRNAs to nearby binding sites in target mRNAs. A similar cooperative regulation was reported for the PSD-95 mRNA by the association between FMRP and miR-125a for proper synaptic morphology and function (Muddashetty et al., 2011).

We have identified putative miRNAs that might work in coordination with FMRP to repress translation of the FLuc-BoxB reporter. However, future experiments are required to verify the actual miRNAs involved in FMRP mediated repression in our assay. An obvious question is how might FMRP load miRNAs to the target mRNA? FMRP does not have a canonical miRNA-binding domain for interaction with miRNAs directly. Therefore, we speculate that either FMRP can interact with miRNAs via Dicer1 or through its interactions with AGO1 or GW182. To test this possibility, future experiments will be needed to gain mechanistic insights into this process.

**4.4 Role of the KH domains in FMRP mediated translation repression.**

Our results demonstrate that the KH domains of FMRP are involved in FMRP mediated translation repression (Figure 13B). We examined the effects of deleting RNA binding domains from FMRP on the translation of FLuc reporter and found that ΔKH1/2 FMRP rescued the translation repression activity of tethered FMRP. Why might KH domains be involved in mediating this process? The KH domains of FMRP have been suggested to facilitate the direct assembly of miRNAs on some target mRNAs *in vitro* (Plante et al., 2006)). The authors suggested that FMRP could act as an acceptor of mature
miRNAs derived from Dicer processing of pre-miRNAs. Therefore, FMRP through its KH domains may cooperate with Dicer1 in miRNA assembly on target mRNAs for repression. It is possible that in our assays, deleting KH domains from FMRP comprised the ability of FMRP to form miRNA complex on target mRNAs. Additional experiments will be required to determine if KH domains are involved in direct interaction with Dicer1 for miRNA assembly on the FLuc-BoxB reporter.

Another possibility by which KH domains can regulate FMRP mediated translation repression is through its ability to enhance the interaction between mRNA and miRNAs. KH domains of FMRP are known to enhance the formation of the kissing complex in vitro, where a target mRNA is bound to miRNAs in a specific secondary structure conformation (Bassell and Warren, 2008). While we have not directly investigated the secondary structure of the 3’ UTR of the FLuc mRNA when bound to FMRP, it is possible that FMRP mediates interaction between FLuc mRNA and miRNAs through its KH domain. This interaction may alter secondary structure and orient the FLuc mRNA in a favorable conformation for miRNA interaction. Further experiments are required to directly investigate this hypothesis.

A recent study suggests that FMRP can interact directly with ribosomes through its KH domains and the steric conflicts between KH domains and P-site tRNA can cause ribosomal stalling leading to translation repression (Chen et al., 2014). These results suggest a miRNA independent mode of translation repression for FMRP. Since ribosomes are stalled in the CDS region of target mRNAs, we do not consider ribosomal stalling as a potential mechanism for FMRP mediated repression for tethered experiments with the
FLuc-BoxB reporter. However, it is possible that the secondary structure of the mRNA may allow for FMRP (bound at the 3'UTR) to interact with ribosomes within the CDS. So, this mechanism cannot be ruled out completely for endogenous target mRNAs.

4.5 Role of the KH domains in FMRP granule formation.

A recent study by Gareau et al. suggest that FMRP granules assemble via its KH0 domains (Gareau et al., 2013b). KH0 domains are involved in self-interaction of FMRP and may potentially direct multiple protein-protein interactions in RNP granules (Gareau et al., 2013b). KH0 domain of FMRP plays an important role in FMRP’s interaction with FXR1/2, CYFIP1 and FMRP interacting protein (FIP) (Hu et al., 2015). Moreover, lack of a GxxG motif in KH0 provides it the flexibility to interact with multiple mRNAs, including the ncRNA, BC1 (Hu et al., 2015). In consistent with the studies by Gareau et al. using live cell imaging, we observed that deleting KH0 domains from FMRP results in diffused localization of smaller FMRP granules in cells. These results suggest that FMRP requires the KH0 domain for granule assembly. Based on these studies, we speculate that FMRP through its KH0 domains may sequester translationally repressed mRNAs, miRNAs, and core miRISC components to granules. An increased local concentration of miRNAs and miRISC proteins may facilitate efficient translation repression of target mRNA. The observed dissociation of granules due to deletion of the KH0 domain might be due to the removal of the interactions between FMRP, target mRNA, miRISC components and other RBPs. Further, FRAP studies suggest that FMRP requires KH0 domains to shuttle from cytosol to granules. In an absence of shuttling activity, FMRP may not bind to specific proteins for granule formation and translation regulation. It would be interesting to
characterize how the KH0 domain chaperones transcripts and associated proteins to granules from free cytosolic fraction. Another explanation for the diffused localization of the KH0 deleted FMRP protein might be non-specific binding of this mutated FMRP to other proteins in absence of KH0 domain. KH0 domain is required for FMRP to form dimers. Therefore, an absence of self-dimerization of FMRP may lead to non-specific binding with other proteins, leading to loss of function phenotypes.

In contrast to Gareau et al., we observed that ΔKH1/2-FMRP induced the formation of bigger FMRP granules in S2 cells. A possible explanation for the observed aberrant granules may be a loss of translation regulation by FMRP. Previous studies suggest that ΔKH1/2-FMRP has compromised RNA and protein shuttling activity between the granule and the cytosol (Gareau et al., 2013b). Therefore, we speculate that ΔKH1/2-FMRP may be trapped with miRNAs or miRISC components in granules. It would be interesting to characterize whether aberrant granule formation contributes to FXS pathology. Unpublished data from Barbee lab suggests that disease-causing KH1 and two point mutants induce the formation of aberrant granules similar in morphology to ΔKH1/2-FMRP. Further experiments are needed to investigate if difference in size and dynamics are due to mRNAs and miRISC proteins being trapped in these granules.

4.6 RBPs can promote miRISC targeting at the 3’UTR of target mRNA.

Sequences or structures in the 3’UTR of specific mRNAs may influence FMRP's role in the microRNA-mediated translation repression pathway. miRNAs mediate translation repression mainly by binding to seed regions within the 3’UTR. Various other RBPs can also interact with 3’UTRs in a sequence-specific manner. In both cases, this
interaction is necessary to modulate gene expression. An interplay between RBPs and miRNAs in the 3'UTR can either induce a structural switch in the mRNA which could facilitate miRISC assembly on the target mRNA. Conversely, RBP interaction with the 3’UTR could occlude miRNAs from binding to their target mRNAs.

As an example of RBP-mediated modulation of miRNA pathway, the 3'UTR of the p27 mRNA is regulated by miR-221, miR-222, and Pumilio (Kenny and Ceman, 2016). Pumilio is a ubiquitously expressed sequence-specific RBP. However, binding site for these miRNAs in the 3’UTR of p27 mRNA are inaccessible to miRISC for binding, because they are found within regions of complex secondary structure. There is evidence that Pumilio1 interacts with p27-3'UTR and induces a local change in mRNA structure. As a consequence of this structural change, the seed sequence is exposed, which allows for the interaction of miRISC components with the target mRNA. Another example of RBP-mediated miRISC modulation is the role of dead end 1 (Dnd1) protein in inhibiting miRNA binding to target mRNAs. Dnd1 has been shown to compete with miRNAs to inhibit miRNAs from associating with their target sites (Kedde et al., 2007).

Other RBPs have been demonstrated to have both an agonizing or antagonizing function in miRNA-mediated translation regulation. For example, the polypyrimidine tract binding protein (PTB) and human antigen R (HuR) can suppress or enhance miRNA binding in the 3'UTR of a subset of target mRNAs (Kenny and Ceman, 2016; Kim et al.). Therefore, we speculate that FMRP may serve a similar function in promoting miRNA binding to the 3’UTRs of some target mRNAs. FMRP may facilitate or inhibit miRNA mediated repression by modification of secondary structure in target mRNA, either by
making miRNA binding site accessible, or by masking miRNA binding sites. The factors that modulate secondary structure in the target mRNA require further investigation.

4.7 Future directions

Our data suggests that FMRP interacts with the components of the miRNA pathway to mediate translation repression. Based on our data, we propose a model where FMRP binds to 3’UTRs of target mRNAs and recruits the miRISC to a nearby miRNA binding site for translation repression (Figure 28). It would be interesting to perform a CLIP-Seq for FMRP to identify its mRNA targets in neuronal cell lines or in motor neurons and investigate whether these targets are co-regulated by miRNAs and FMRP. Future experiments will also be required to identify other RBPs that may interact with FMRP and miRNAs for coupled control of mRNA translation in neurons.

Our results also suggest a role of KH domains in FMRP translation in a mRNA independent way. It would be interesting to generate stable cell lines for ΔKH FMRP, pull down associated RBPs with FMRP, and compare these RBPs with the cell lines expressing wild-type FMRP to identify KH domain mediated FMRP-RBP interactions. The experiments will help identify if the absence of KH domains can lead to non-specific interactions of FMRP with other RBPs which leads to aberrant granules. Further, FMRP lacking KH domains and RGG domain should be investigated for their interactions with components of the miRNA pathway. We could also track the movement of miRISC components or other RBPs in FMRP granules in motor neurons using super-resolution microscopy to measure the dynamics of their interactions within FMRP granules.
Taken together, that work described in this thesis (and experiments that will logically follow) could help shed some light on the mechanisms by which FMRP regulates translation of target mRNAs with miRNAs, as well as identify key neuronal transcripts whose translation is affected by the loss of FMRP. An understanding of these processes will help in the development of therapeutic approaches to find a cure for FXS.
Figure 28: Proposed model of how FMRP interacts with the miRNA pathway to regulate translation.

We propose that FMRP first recognizes structural elements in target mRNA and then recruits the miRISC components to mediate translation repression. FMRP potentially interacts with the miRISC complex through AGO1 which then recruits GW182 to mediate repression. GW182 could potentially interact with the components of the deadenylase complex (CCR4, NOT1) to mediate either deadenylation dependent or an independent mode of degradation. GW182 could also interact with other RBPs to mediate RNA trafficking to P bodies.
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APPENDIX: LIST OF ABBREVIATIONS

1. AGO protein - Argonaute protein
2. APP - Amyloid Precursor Protein
3. ApppN - An unmethylated cap analog
4. ARC - Activity-regulated cytoskeleton-associated protein
5. BC1 - Brain Cytoplasmic RNA1
6. CaMKII - Calcium/Calmodulin-Dependent Protein Kinase II
7. CDS - Coding Sequence
8. CYFIP1 - Cytoplasmic FMRP Interacting Protein
9. Dlg - Discs large
10. Dnd1 - Dead end 1
11. 4E-BP - eIF4E-binding proteins
12. FMRP - Fragile X mental retardation protein
13. FXPOI - Fragile X-related primary ovarian insufficiency
14. FXTAS - Fragile X tremor ataxia syndrome
15. GW - Glycine-Tryptophan
16. HITS-CLIP - High-throughput sequencing of RNA isolated by crosslinking immunoprecipitation
17. HRP - Horseradish peroxidase
18. HuR - Human antigen R
19. IRES - Internal Ribosome entry site
20. KH domains - Ribonucleoprotein K homology domains
21. LCD - Low complexity domain
22. MNK - MAP kinase-interacting serine/threonine-protein
23. mGluR 5 - Metabotropic glutamate receptor 5
24. MAP1B - Microtubule associated protein
25. NES - Nuclear export signal
26. NLS - Nuclear localization signal
27. MSA - Muscle surface area
28. NR2A - N-methyl-D-aspartate receptor subunit
29. NXF - Nuclear export factor
30. PABP - Poly-A binding protein
31. PACT - Protein activator of the interferon induced protein kinase
32. PAIP2 - Polyadenylate-binding protein-interacting protein 2
33. P-bodies - Processing bodies
34. PSD 95 - Post-synaptic protein 95
35. Rac1 - Ras-related C3 botulinum toxin substrate 1
36. RBP - RNA binding protein
37. RBD - RNA binding domain
38. RGG box - Arginine Glycine Glycine box
39. RISC - RNA induced silencing complex
40. RNP - Ribonucleoprotein complex
41. SIX3 - Sine oculis homeobox homolog transcription factor
42. SMN-Survival of motor neuron
43. Sod1 - Superoxide Dismutase
44. SoSLIP - Sod1 mRNA Stem Loops Interacting with FMRP
45. TNRC - Trinucleotide repeat-containing protein
46. TRAP - Translating Ribosome Affinity Purification
47. TRBP - TAR RNA binding protein
48. UTR - Untranslated Region
49. ZC3H14-Zinc-finger CysCysCysHis [CCCH]-type 14