MicroRNAs 9a, 9b, 9c and 315 Regulate Expression of a Reporter for the Neuronal Microtubule-Associated Protein Futsch/MAP1B

Leslie M. Rozeboom

University of Denver
Abstract

Fragile X syndrome (FXS) is the most common form of inherited mental retardation in humans. FXS is caused by loss of the Fragile X Mental Retardation Protein (FMRP), an important regulator of neuronal mRNA translation. Patients with FXS display cognitive deficits including memory problems. Protein synthesis-dependent long-term changes in synaptic plasticity are involved in the establishment and maintenance of long-term memory. One prevalent theory of FXS pathology predicts that FMRP is required to negatively regulate the translation of important mRNAs at the synapse. We are investigating microRNAs (miRNAs) as a potential regulator of synaptic FMRP-regulated mRNAs that have previously been described as being crucial to the process of synaptic plasticity.

The general hypothesis underlying this thesis is that FMRP may negatively regulate the expression of futsch (the Drosophila homologue of the microtubule-associated protein gene MAP1B) via the miRNA pathway. The first step we took in testing this hypothesis was to confirm that futsch is subject to miRNA-mediated translational control. Using in silico target analysis, we predicted that several neuronally expressed miRNAs target the futsch mRNA 3'UTR and repress expression of Futsch protein. Then, using an in vitro luciferase reporter system, we showed that miR-315 and members of the miR-9 family selectively down-regulated futsch reporter translation. We have confirmed by site-directed mutagenesis that the miRNA interaction with the futsch 3'UTR is specific to the miRNA seed region binding site. Interestingly, reduction of FMRP levels by RNAi had no effect on futsch 3'UTR reporter expression. Together, these data suggest regulation of futsch expression by the miRNA pathway might be independent of FMRP activity. However, additional experiments need to be completed to confirm these preliminary results.

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Scott A. Barbee, Ph.D.

Second Advisor

Daniel Linseman

Third Advisor

Todd Blankenship

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Leslie M. Rozeboom
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Advisor: Scott A. Barbee
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Chapter One: Introduction

1.1 Overview

Fragile X syndrome (FXS) is the most common form of inherited mental retardation in humans, and is a major genetic cause of autism (Bassell and Warren, 2008). It has an estimated incidence of 1 in 4,000 men and 1 in 8,000 women (Warren et al., 1991). FXS is caused by loss of the Fragile X Mental Retardation Protein (FMRP), an important regulator of neuronal mRNA translation. The absence of FMRP is due to the transcriptional inactivation of the fragile X mental retardation 1 (FMR1) gene, which results from an unstable expansion of a CGG trinucleotide repeat in its 5’ untranslated region (UTR) (Jin and Warren, 2003). Transcriptional repression of the FMR1 gene occurs when the CpG islands and the CGG repeats are hypermethylated. A current model postulates that FMRP regulates mRNA transport and local protein synthesis in response to activated metabotropic glutamate receptors (mGluRs) (Figure 1; Jin et al., 2004b). The activation of group I mGluRs (mGluR1 and mGluR5) stimulates the synthesis of FMRP in synapses (Figure 1; Weiler et al., 1997). It is believed that one major function of FMRP at the cell synapse is to offset local translation of specific mRNAs following mGluR stimulation (Krueger et al., 2011). While a significant amount of progress has been made towards understanding these processes, there are still several important questions that need to be addressed. First, which specific mRNAs are being
FIGURE 1. Model for mRNA regulation by FMRP. After translation, FMRP dimerizes in the cytoplasm then is imported to the nucleus where it forms a messenger ribonucleoprotein (mRNP) complex with specific hairpin structured RNA transcripts and other proteins. The FMRP–mRNP complex is then transported to the cytoplasm where it can either associate directly with the polyribosomes or interact with the RNA-induced silencing complex (RISC). The FMRP-mRNP complexes can regulate protein synthesis in the cell body, or they can be transported to the dendrites to regulate local protein synthesis upon metabotropic glutamate stimulation. In the mGluR theory of FXS pathogenesis, one essential function of FMRP is to repress the expression of specific mRNAs at the synapse. Most of these mRNAs remain uncharacterized. FMRP=green hexagon, Ribosomes = purple ovals, RISC = red star, newly synthesized protein = string of blue circles, mGluR= orange oval. Figure adapted from Jin et al. (2004b).
regulated by FMRP? Second, how does FMRP regulate the expression of these target mRNAs? The second question will be addressed in the hypothesis outlined below.

1.2 Genetics of FXS

FXS has an unusual genetic inheritance pattern referred to as the Sherman Paradox. In the 1980s, Sherman showed that nonpenetrant male carriers exist in fragile X syndrome families, which is an unusual observation for an X-linked disorder. Symptom-free (nonpenetrant) males can transmit their alleles to nonpenetrant daughters who can then bear symptomatic (penetrant) male offspring (Sherman et al., 1985). Affected individuals display a phenotype of a “fragile site” or unstaining gap on the metaphase X chromosome that segregates with the mutant gene (Penagarikano et al., 1991). FMR1, the gene responsible for FXS, has been mapped to the fragile site on the X chromosome. Cloned and first identified in 1991, (Verkerk et al., 1991), it was one of the first trinucleotide repeats linked to a disease. Interestingly, unlike most disease-associated trinucleotide repeats, the expansion responsible for FXS occurs in a non-coding region of the gene (in this case, the 5’UTR of FMR1) (Jin et al., 2004b).

1.3 Polynucleotide Expansions in FMR1

An extreme CGG polynucleotide expansion (>200 repeats) is the causative mutation in 99% of individuals with FXS (Jin et al., 2004b). However, there is a great deal of variation in the number of CGG repeats in the 5’UTR of FMR1 in humans (Figure 2). Three categories of people have been identified. Most people have “normal” alleles
FIGURE 2. Schematic representation of the *FMRI* gene and CGG polynucleotide expansions in the *FMRI* 5’UTR. There are 17 exons in the *FMRI* gene that can undergo alternative splicing. Expansions of the CGG repeat greater than 200 are characterized as a “full mutation”, expansions with fewer than 200 CGG repeats but significantly more than average are characterized as a “premutation”. Premutation alleles can lead to the development of FXTAS (males) or FXPOI (females) later in life. Full mutation alleles are the primary cause of FXS. Figure adapted from Bassell et al. (2008).
containing between 6 and 54 repeats, with 30 repeats being most common. Normal FMR1 alleles are usually transmitted stably, but will occasionally gain or lose a few repeats. This variation leads to a pool of people with elevated repeat numbers that could eventually evolve premutations (Penagarikano et al., 2007). A second class of people has “premutation” alleles with significantly more than average, but fewer than 200, CGG repeats. Premutation alleles are unstable and tend to expand when maternally transmitted. Longer premutation alleles are more unstable than shorter premutation alleles, and more likely to lead to full mutation alleles in offspring than shorter ones. The length of the repeat in a premutation allele in a female is correlative to the likelihood of expansion to a full, symptomatic, mutation in her offspring. As a male does not pass an X chromosome to his sons, the male’s FMR1 allelic status does not affect the likelihood of FXS in his offspring. People carrying premutation alleles are usually asymptomatic, but in some cases will develop problems later in life including fragile X-associated tremor ataxia syndrome (FXTAS) and premature ovarian failure (FXPOI). A third class of people has “full mutation” (>200 and often >500 repeats) alleles. In these people, FXS is always penetrant in males and is 50% penetrant in females.

1.4 FXS Pathology

Morphologically, cerebral cortical autopsies from FXS patients show abnormal dendritic spine morphology (Irwin et al., 2001). Behaviorally, the most prominent phenotype of FXS in humans is intellectual disability; patients will have IQ values generally between 20 and 70. Short-term memory for complex information, visuospatial
skills and speech are particularly affected. Speech development is often delayed in FXS children. Some patients also display hyperactivity, hypersensitivity toward sensory stimuli and attention deficit disorder (Penagarikano et al., 2007). Interestingly, between 15% and 50% of FXS patients display autistic behavior (poor visual contact, tactile defensiveness, repetitive behaviors). FXTAS and autism share some symptoms with FXS, which may indicate that related synaptic development problems may underlie all three syndromes. It has been proposed that mutations in FMR1 may be the most common single-gene cause of autism.

1.5 FMRP

In order to understand the pathogenesis of FXS and develop therapeutic strategies, we must understand its underlying cause: the loss of function of FMRP. While FMRP is widely expressed in fetal and adult tissues, its expression is most pronounced in the brain and testis, where major FXS symptoms (mental retardation and macroorchidism) manifest (Warren and Nelson, 1994). FMRP is a selective RNA-binding protein that can form a messenger ribonucleoprotein (mRNP) with polyribosomes (Ashley et al., 1993). FMRP has been shown to associate with actively translating ribosomes (Jin and Warren, 2003). Moreover, a current model postulates that FMRP is involved in synaptic plasticity because it regulates mRNA transport and local synaptic protein synthesis (Figure 1; Jin et al., 2004b). FMRP is thought to bind to approximately 4 percent of fetal human brain mRNAs in vitro (Brown et al., 1998). One theory of how FMRP regulates mRNA translation is that it recognizes its target mRNAs directly. Alternatively, there is
evidence that FMRP may require accessory factors such as a miRNA-containing RISC (miRISC) in order to bind to and regulate target mRNAs (Figure 3; Li and Jin, 2009).

The human \textit{FMR1} gene is approximately 38 kilobases long and contains 17 exons (Figure 2; Ashley et al., 1993). Through alternative splicing, the human \textit{FMR1} gene can generate at least 12 different proteins between 67 and 80 kDa (Devys et al., 1993). The FMRP protein has two sequence motifs characteristic of RNA binding proteins. First, a cluster of arginine and glycine residues known as the RGG box has been shown to bind to a G quartet structure formed by some mRNAs (Darnell et al., 2001). The G quartet is an RNA loop structure with a planar conformation of four guanine residues that is stabilized by Hoodsteen base pairs (Figure 4). Several of these planar quartets can stack to form the characteristic quadruplex recognized by FMRP. Second, FMRP has two heterogeneous nuclear ribonucleoprotein K homology (KH) domains. These are shown to bind a RNA tertiary structure known as a “kissing complex” (Figure 4; Darnell et al., 1993).

FMRP is also believed to interact with target RNAs through several other conserved regulatory elements. FMRP has been found to bind RNA ligands containing U rich sequences of 5-23 bases of repeating U pentamers (Figure 4; Chen et al., 2003). There is also controversial evidence that FMRP can interact with some target mRNAs via the novel noncoding BC1 RNA (Zalfa et al., 2005; Iacoangeli et al., 2007). Of particular interest to work presented in this thesis, FMRP has been found to have both biochemical and genetic interactions with components of the miRNA pathway (Zhang et al., 2001; Ishizuka et al., 2002; Jin et al., 2004a). Thus, FMRP may regulate neuronal translation via or in concert with miRNAs (Menon et al., 2008).
FIGURE 3. Model of miRNA- and FMRP-mediated mRNA regulation. Mature miRNAs are loaded onto the RISC and bind to target mRNAs through complementary base pairing. There are several potential mechanisms of down-regulation of mRNA expression by miRNA expression. The miRISC could bind the mRNA directly and target it for degradation or translational repression. Alternatively, FMRP may associate with the miRISC and facilitate binding to target mRNAs. Cooperative action between FMRP and the miRISC could then direct the mRNA for decay or translational repression. Figure adapted from Jin and Li (2009).
FIGURE 4. Conserved RNA tertiary structures recognized by FMRP. a) G quartet: an RNA loop structure with a planar conformation of four guanine residues that is stabilized by Hoodsteen base pairs. Several of these planar quartets can stack to form a G quadruplex. b) U pentamer: a U rich sequence that forms a characteristic tertiary structure, and c) Kissing complex: a complex “loop-loop pseudoknot” tertiary structure (Penagarikano et al., 2007). Figure adapted from Penagarikano et al. (2007).
1.6 miRNAs

miRNAs are short (~21-22 nucleotides) non-coding regulatory RNAs that modulate gene expression post-transcriptionally (Li and Jin, 2009). miRNAs down-regulate expression of target mRNAs by binding specifically with antisense sequences in the 3’ UTR, although there is evidence that they can bind to sequences in the 5’ UTR and protein coding region (Breving et al., 2010). Briefly, miRNAs are transcribed in the nucleus as primary transcripts (pri-miRNAs) from hundreds to thousands of nucleotides long. The pri-miRNA forms a secondary hairpin structure that is recognized and cleaved by the RNAse III enzyme Drosha yielding a precursor RNA of approximately 80 nucleotides. The pre-miRNA is exported to the cytoplasm where it is cleaved by the RNAse III enzyme Dicer in concert with a dsRNA binding protein into the mature miRNA. One strand of miRNA is then incorporated into the RNA-induced silencing complex (RISC) and the other is typically degraded. It is believed that the miRNA guides the RISC to its target, where a complimentary base pairing between the miRNA and the target mRNA leads to down-regulation of the target’s expression, either by translational repression or by mRNA degradation (Figure 5).
FIGURE 5. **General pathway for miRNA biosynthesis.** 1) Within the nucleus, the miRNA gene is transcribed by Polymerase II resulting in a pri-miRNA precursor averaging from 100’s to 1000’s of nucleotides long. 2) The stem-loop secondary structures of the pri-miRNA are recognized by the Drosha Microprocessor complex and cleaved into a ~70 nucleotide long pre-miRNA hairpin structure which is exported from the nucleus into the cytoplasm by the Exportin-5/Ran-GTP complex. 3) In the cytoplasm, the pre-miRNA associates with the RNase-III type enzyme DICER, The mammalian TAR RNA-binding protein (TRBP; *loquacious in Drosophila*), and possibly an Argonaute protein and is cleaved to yield an ~22 nucleotide miRNA duplex. 4) One strand (termed the “guide” strand) of the mature miRNA associates with the RISC machinery leading either to translational repression or degradation of the target mRNA. Figure adapted from Brevig et al. (2009).
1.7 FMRP Interacts with the miRNA Pathway

A current model for FMRP function is that it binds target mRNAs in the nucleus to form a ribonucleoprotein complex, which is then transported to the dendrites or spines. At the synapse, FMRP is involved in the control of local protein synthesis via general and/or miRNA-mediated translational regulation (Bassell and Warren, 2008; Li and Jin, 2009). Five independent lines of evidence support an interaction between the FMRP and miRNA pathways. First, in vivo, mammalian FMRP interacts with miRNAs and components of the miRNA pathway, including Dicer and the mammalian ortholog of AGO1. Furthermore, RNAs of ~20 and ~80 nucleotides (presumptive miRNAs and premiRNAs) immunoprecipitate with FMRP (Jin et al., 2004). Second, in Drosophila, dFMRP interacts biochemically with the functional RISC proteins including dAGO1, dAGO2 and Dicer (Ishizuka et al., 2002). Moreover, dFmr1 dominantly interacts with dAgo1 to regulate neuronal development and synaptogenesis (Jin et al., 2004). Third, in Drosophila, miR-124 interacts with FMRP and controls sensory neuron structure (Xu et al. 2008). Fourth, rat miR-125b and miR-132 (and others) interact with FMRP and control hippocampal neuron structure and function (Edbaur et al. 2010). miR-125b regulates NMDAR expression (Edbaur et al., 2010). Finally, translational control of the murine postsynaptic density protein 95 (PSD95) by miR-125a has recently been shown to require FMRP activity, although a biochemical interaction has not yet been reported (Muddashetty et al., 2011).
1.8 Futsch/ MAP1B

It has been clearly shown in flies and mammals that FMRP is required for translational regulation of the microtubule-associated protein, MAP1B, however the precise mechanism remains unclear (Zhang et al., 2001; Lu et al., 2004). Microtubule-associated proteins (MAPs) are expressed in eukaryotic cells and bind along the microtubule lattice. Post-translation, MAP1A and MAP1B are cleaved into light and heavy chains. Those chains are then assembled (with the separately encoded light chain 3 subunit LC3) into mature complexes (Halpain et al., 2006). Mammalian genomes usually contain three family members: MAP1A, MAP1B and MAP1S (Halpain et al., 2006). No obvious ortholog of any MAP1-family protein occurs in *Caenorhabditis elegans* or more primitive organisms. The *Drosophila* genome only contains one MAP1-family protein, the homolog of MAP1B, Futsch.

Neurons in FXS patients have more long, thin, immature spines, fewer short spines, and an overall increased spine density, which suggests impaired synapse development (Irwin et al., 2001). Similar synaptic abnormalities are observed in *Fmr1* knockout (mice and *Drosophila*) neurons (Comery et al., 1997; Zang et al., 2001).

1.9 miRNA-Mediated Regulation of *futsch*

The precise details of *futsch* regulation by FMRP remain unknown. As such, MAP1B was considered to be a candidate for regulation by both FMRP and the miRNA pathway. Bearing this in mind, identification of miRNAs that regulate Futsch, and elucidation of the mechanism by which they do so, would be the first logical step towards understanding the Futsch/FMRP regulatory pathway. *In silico* analysis has indicated that
specific miRNAs may regulate Futsch. Here, we investigate the role of candidate miRNAs as a novel regulatory mechanism in the control of Futsch expression.
Chapter Two: Method

2.1 RNA Extraction and Analysis by Quantitative Real-Time PCR (qRT-PCR)

Central Nervous System (CNS) tissues, (including ventral ganglia and optic lobes) were dissected from \textit{w^{1118}} wandering 3\textsuperscript{rd} instar larvae in cold HL-3 buffer then transferred to 700 \( \mu l \) QIAzol Lysis Reagant (Qiagen). CNSs were homogenized with a TissueRuptor (Qiagen) for 30 seconds at high speed. Homogenate was applied to a QIAshredder column (Qiagen). After elution and chloroform precipitation, the aqueous phase was removed and purified using the RNeasy purification kit (Qiagen). RNA was eluted from RNeasy spin columns with 30\( \mu l \) DEPC treated \( H_2O \) then flash frozen with liquid nitrogen and stored at -80\( ^\circ \)C until analysis.

Quantitative real time PCR (qRT-PCR) was performed “in house” to confirm that our miRNAs are expressed in the larval CNS using an iQ5 Real-Time PCR detection system (BioRad). A reverse transcription reaction was performed using the SYBR Green based miScript miRNA RT-qPCR detection system (Qiagen). The purified RNA was used in a reverse transcription reaction using the SYBR Green miScript Kit. Mature miRNAs were converted to cDNA using the miScript Reverse Transcription Kit (Qiagen) and qRT-PCR done using the miScript SYBR Green PCR Kit (Qiagen). The first-strand template was used as the template for the quantitative real-time PCR with the provided universal primer and miScript Primers for mature miR-315, -9a, -9b and -9c (proprietary sequences designed by and obtained from Qiagen).
2.2 miRNA Analysis by miRNA Array

CNS tissues (ventral ganglia and optic lobes) were dissected from \( w^{1118} \) wandering 3\(^{\text{rd}}\) instar larva in cold HL-3 buffer then transferred to lysis buffer. The RNA was then purified (following manufacturer’s protocol) with the miRcury RNA isolation Kit (Exiqon). CNSs were homogenized with a TissueRuptor (Qiagen) for 30 seconds at high speed. Homogenate was applied to a QIAshredder column (Qiagen). After elution and chloroform precipitation, the aqueous phase was removed then purified (following manufacturer’s protocol) with miRCURY RNA isolation kit (RNeasy Mini Spin Kit, Exiqon). The RNA was eluted from RNeasy spin columns with 30\( \mu \)l DEPC- treated \( H_2O \). RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and RNA was shipped to Exiqon on dry ice for quality control and miRNA microarray analysis.

Exiqon employed a chip-based hybridization miRNA microarray based on miRBase 14.0 (http://www.mirbase.org; Kozomara et al., 2011; Griffiths-Jones et al., 2004; Griffiths-Jones et al., 2006; Griffiths-Jones et al., 2008), which at the time represented 99.3\% of known \textit{Drosophila} miRNAs. The most current database release, miRBase 17.0 (http://www.mirbase.org; Kozomara et al., 2011; Griffiths-Jones et al., 2004; Griffiths-Jones et al., 2006; Griffiths-Jones et al., 2008) contains a larger number of miRNAs, so the analysis performed using miRBase 14.0 covers only 62\% coverage of known miRNAs.
2.3 DNA Constructs

In order to confirm that the miRNAs identified by *in silico* analysis actually interact with and down-regulate the target mRNA (*futsch*), we took advantage of an established dual luciferase reporter system in S2 cells. The first step was to construct the necessary vectors to be transfected into the S2 cells.

**Futsch firefly luciferase reporter.**

For expression in S2 cells, a PCR fragment containing the *futsch* 3’UTR was amplified from a BAC vector obtained from the Drosophila Genomics Resource Center (DGRC) with the upstream primer (5’-GCCACGGGTCTGTTTATTG-3’) and the downstream primer (5’-TGACTCTCTGCTCATTCTCGT-3’). The *futsch* 3’UTR was cloned first into pENTR using the Gateway system (Invitrogen) and then into the appropriate destination vector (pAc5.1-FLuc2:dPolyA-RFA) by LR recombination. The expression of the *futsch* reporter in this vector is driven with a constitutively active *Drosophila* actin promoter but is lacking a 3’UTR sequence required for efficient 3’ end formation.

**miRNA expression vectors.**

The primary miR-9a sequence was amplified from *w^{1118}* larval genomic DNA using the forward primer (5’-CACC AATGTCATGGGGCTAGCGA-3’) and the reverse primer (5’-TGGCCGTAAAGCCAAACTGC-3’). The primary miR-9b sequence was amplified from *w^{1118}* larval genomic DNA using the forward primer (5’-CACC GCATTGGCTTCTGCAGGTCA-3’) and the reverse primer (5’-
GCGAGCATATCTCCAGGGCA -3’). The primary miR-9c sequence was amplified from $w^{1118}$ larval genomic DNA using the forward primer (5’-CACC ATTCCAGAGCATCGCCATCG-3’) and the reverse primer (5’-GATCCCGGGCAGCTCTGAA-3’). The primary miR-315 sequence was amplified from $w^{1118}$ larval genomic DNA using the forward primer (5’-CACC TCAGTGCAATTGTGATGCCCA-3’) and the reverse primer (5’- AAATCGTCAGCGTTGAGGGG-3’).

The CACC sequence was added to the 5’ end of the forward primers to allow directional cloning into pENTR. The primary miRNA sequences were cloned into pENTR using a TOPO Cloning Reaction (Invitrogen kit for cloning blunt-end PCR product into an entry vector for the Gateway System). The primary miRNA sequences were then cloned (using an LR reaction) into pAC5.1-RFA. The expression of the miRNA is driven with a constitutive Drosophila actin promoter and an SV40 3’UTR is cloned downstream of the primary miRNA sequence to ensure robust expression.

**Renilla luciferase transfection control.**

In order to normalize the reporter expression reading results for differences in transfection efficiency between wells, we used a Renilla luciferase vector. The Renilla luciferase is similar to firefly luciferase, but is derived from the *Renilla reniformis* polyp (known as the “sea pansy”). Renilla luciferase cloned into pAc5.1 (Invitrogen) served as a transfection control. The expression of the Renilla luciferase protein is driven with a constitutive actin promoter and contains an SV40 3’UTR cloned downstream of the primary miRNA sequence to ensure robust expression.
Empty expression vector.

In order to control for quantity of DNA input into the S2 co-transfection, the same vector (pAC5.1-RFA) without any primary miRNA sequence is used in place of the miRNA expression vector.

NERFIN reporter vector.

The *nerfin* 3’UTR was amplified from *w^{1118}* genomic DNA using the forward primer (5’–CACC CCATGGCCCCTCGAAATCGAGTGAG–3’) and the reverse primer (5’–CCCTGACAACCCAAAGAGAACCCACAAAGAGTA3’) (Kuzin et al., 2004). The *nerfin* 3’UTR was cloned using an LR reaction into the pAc5.1-FLuc2:dPolyA-RFA vector. All plasmids were maxi-prepped endotoxin-free (Qiagen).

Site directed mutagenesis of futsch reporter.

In order to confirm that the down-regulation observed in the reporter vector is specifically due to the miRNA/mRNA interaction in the predicted seed region binding site, we created versions of the *futsch* reporter plasmid in which the predicted binding site is altered.

The core binding regions of miR-9 and miR-315 on the *futsch* 3’ UTR were mutagenized using the Phusion Site-Directed Mutagenesis Kit by Thermo Scientific (product code: F-541). Using the Firefly luciferase *futsch* 3’UTR reporter vector (“F-Luc-F”) as a starting point, we introduced three nucleotide mutations at the miRNA binding sites. We will refer to these mutagenized plasmids as “FLucF9mut” and “FLucF315mut” respectively.
In order to create the FLucF9mut reporter plasmid, the F-Luc-F plasmid was PCR amplified with the forward 5’ phosphorylated primer (5’-TTAGTAATCCTTTGGGATCAAAATAGTTTT-3’) and the reverse 5’ phosphorylated primer (5’-GCAAGGTAGCTTGGACGTTATGCAA-3’) according to the manufacturer’s protocol. The PCR product was circularized with Quick T4 DNA Ligase. Plasmids were transformed into TOPO10 competent E. coli cells. Mini-prepped DNA was sequenced through the mutation to confirm the mutation was successful.

In order to create the FLucF315mut reporter plasmid, the F-Luc-F plasmid was PCR amplified with the forward 5’ phosphorylated primer (5’-TTAGTAATCCTTTGGGATCAAAATAGTTTT-3’) and the reverse 5’ phosphorylated primer (5’-GCAAGGTAGCTTGGACGTTATGCAA-3’) according to the manufacturer’s protocol. The PCR product was circularized with Quick T4 DNA Ligase. Plasmids were transformed into TOPO10 competent E. coli cells. Mini-prepped DNA was sequenced through the mutation to confirm the mutation was successful.

As a result of the site-directed mutagenesis, in the “FLuc315mut” reporter plasmid three base pairs within miR-315’s binding site were mutated from CAA to AGC. In the other, “FLucF9mut” three base pairs within the seed region binding site (common to all three members, miR-9a, miR-9b and miR-9c) were mutated from AAA to TTG.
2.4 S2 Cells

_Drosophila_ S2 cells were maintained in suspension culture in 250 ml Suspension Culture Flasks with vent caps (Celltreat cat #229520). Cells were maintained and experiments were performed in Complete Schneider’s _Drosophila_ Medium (89% Schneider’s Drosophila Medium (1X), (liquid + L-Glutamine. Gibco cat #11720): 10% Fetal Bovine Serum (Certified, Gibco Cat #10082): 1% Penicillin-Streptomycin, (liquid, prepared with 5,000 units/mL Penicillin G sodium and 5,000 µg/mL Streptomycin sulfate in 0.85% saline, Gibco cat # 15070). Complete media was filter sterilized with Corning 500ml Filter System 0.22µm PES 431097 and stored at 6°Celsius.

Transfections.

S2 cells were transfected in six-well plates (2x10^6 cells/well) using Effectene transfection reagent (Qiagen). All transfections were performed in triplicate (or 3 “biological replicates”). The transfection mixtures contained 0.1µg of firefly luciferase (F-Luc) reporter plasmid, 0.4µg of the _Renilla_ transfection control, and 0.2µg of either plasmid expressing the miRNA primary transcripts or the pAC5.1-RFA (empty) vector (Zekri et al., 2009).

Cells were split the day before transfection to ensure that cells were in an exponential growth phase. On the day of transfection cells were vigorously resuspended, taking care to wash bottom of flask vigorously to dislodge any semi-adherent cells. Concentration of cells was determined using an Improved Neubauer haemocytometer Bright Line Counting Chamber and inverted microscope. Volume of cell suspension to
be used (2x10^6 cells/well to be transfected) was calculated. Cells were spun down, and media decanted. Cells were washed (resuspended in 1xPBS, spun down, PBS decanted) then resuspended in an appropriate volume of Complete media (1.6ml/well to be transfected). 1.6 ml cell suspension was seeded into each well (Costar 6 well, Corning CellBIND Surface cat# 3335). DNA (total of 0.7µg) was diluted in Buffer EC to a volume of 100µl/well. For each well, a mixture of 0.1µg FLuc-F or FLucmiRmut, 0.4µg R-Luc (Renilla luciferase transfection control vector), and 0.2µg miRNA expression or empty vector was used. All plasmids had been maxi prepped endotoxin free (Qiagen Endotoxin Free Maxi Prep Kit). 5.6 µl Enhancer was added to diluted DNA. Mixture was vortexed for 1 second. Mixture was incubated at room temperature for 3 minutes. 25µl of Effectene reagent was added to mixture and mixture was vortexed for 10 seconds then incubated at room temperature for 7 minutes. 1 ml complete media was added to DNA mixture and mixed by pipetting up and down. Solution was added drop-wise to wells containing cells, while gently swirling plate. Cells were incubated for approximately 72 hours at 25°Celsius.

**Passive lysis.**

Cells were washed by very gently removing media from the cells with a transfer pipet. Enough PBS to cover the film of cells adhered to the bottom of the well was gently applied then removed. 500µl of 1x Passive Lysis Buffer (prepared in nanopure-H2O with the 5x Passive Lysis Buffer provided) was applied to washed cells (still adhered to bottom of well). Cells were incubated 15 minutes at room temperature on a shaker set at 140 rpm. Cell lysates were transferred to microfuge tubes.
2.5 Luciferase Assays

In order to quantitatively assay changes in Futsch expression, we utilized a dual-luciferase reporter system (Promega). In each experiment, firefly and Renilla luciferase activities were measured 3 days after transfection. Three samples (technical replicates) were measured from each triplicate (biological replicate) transfection. Firefly and Renilla luciferase luminescence values were measured in a 96 Well, Flat Bottom, Non-treated, White Polystyrene (Corning cat# 3912) assay plate using a BIO-TEK Synergy HT plate reader. Reagents Luciferase Assay Reagent II and Stop and Glow solution were prepared following manufactures instructions. 20µl of cell lysate was added to each well, then 100µl LARII reagent was dispensed into each well. Firefly luminescence measurement was taken with sensitivity set to 100 (s=100). 100µl Stop and Glo reagent was added to each well then Renilla luminescence level was measured (s=100).

2.6 Knock-down of dFMRP

In order to investigate if the down-regulation effect observed is dependent on FMRP, we knocked down FMRP using RNAi.

Creation of dsdFMRI RNA.

A segment of the dFMRI coding sequence was amplified from LD09557 plasmid (obtained from the DGRC) using the forward primer (5′–CGTGCCCGAGAGTATGAAAT-3′) and the reverse primer (5′–ATTGTGCCTGAAACTCCTT-3′). The PCR product was purified using StrataPrep PCR purification kit. The purified PCR product was
ligated into pPCR-Script Amp SK+1 cloning vector and transfected into XL10-Gold cells according to the manufacturer’s protocol. The \textit{dFMR1} pPCR plasmid was amplified and extracted from XL10-Gold cells. Following manufacturer’s protocol, dsRNA was amplified as separate strands from \textit{dFMR1} pPCR using T3 and T7 Megascript kits. Separate RNA strands were annealed by combining equal quantities of each strand in a microfuge tube which was placed in boiling water and allowed to cool.

dsRNA (2 micrograms/well) and reporter vectors were co-transfected into S2 cells according to the protocol published by Rio et al. (2011). Luciferase reporter experiments were conducted essentially as described above in section 2.5. An aliquot of the cell lysate was flash frozen on liquid N\textsubscript{2} for Western analysis of FMRP levels.

\textbf{2.7 Western Blot of \textit{dFMR1} Knock-down}

After 72 hours of incubation, S2 cells were lysed and an aliquot run on a polyacrylamide gel then transferred to a membrane. After blocking, the primary antibodies rabbit anti GAPDH (1:500 concentration) and mouse anti FMRP (6A15) (1:2,500 concentration) were applied. After an over-night incubation and washing, anti-mouse HRP and anti-rabbit HRP secondary antibodies (both at 1:10,000 concentration) were applied for two hours. Membrane was washed, HRP developing solution applied, and membrane was exposed to film for analysis.
Chapter Three: Results

3.1 miR-315 and the miR-9 Family are Predicted to Bind to the futsch 3’UTR

To address the question of whether or not futsch expression is regulated by miRNAs, we first performed in silico analysis of the futsch 3’UTR sequence using the TargetScanFly target prediction algorithm (http://www.targetscan.org; Release 5.1 Friedman, et al. 2009; Grimson, et al. 2007; Lewis, et al. 2005). TargetScanFly predicts targets by analyzing sequence complementation between the miRNA and potential mRNA targets, most importantly sequence conservation in the interaction with the “seed region” of the miRNA (nucleotides 2-9 from the 5’ end). TargetScanFly compares the sequences of potential miRNA/mRNA target interactions amongst 10+ Drosophila species, and ranks those with high conservation to likely be most valid.

Typically, a miRNA binds to a complementary sequence in an mRNA’s 3’UTR, leading to the regulation of that mRNA. For the interaction to be effective, it is necessary for part of the miRNA known as the “seed region” (6-8 nucleotides long, typically close to the miRNA’s 5’UTR) to be a perfect match to the mRNA. The miRNA can still control expression if there are mismatches between it and its target mRNA if those mismatches are outside of the seed region. If the complementary match between the miRNA and its target mRNA are not perfect within the seed region, the over-expression of the miRNA should not down-regulate expression of the target mRNA (Doench and Sharp, 2004).
We queried the futsch 3’UTR and found that six miRNAs were predicted to bind the futsch mRNA 3’UTR, including the three members of the miR-9 family (9a, 9b, 9c), miR-315, miR-963, and miR-976 (Figure 6). A miRNA family consists of several miRNAs with similar sequences. These miRNAs have identical seed regions with identical and overlapping predicted mRNA binding sites. miR-315 has a second predicted binding site in Drosophila that is less conserved between fly species. The sequences of the miRNAs of interest as reported by miRBase (http://www.mirbase.org; Kozomara et al., 2011, Griffiths-Jones et al., 2004; Griffiths-Jones et al., 2006; Griffiths-Jones et al., 2008) of interest are shown in Figure 6.

3.2 miR-315 and the miR-9 Family are Expressed in the Drosophila Larval CNS

We next asked whether these miRNAs of interest are expressed in the larval CNS. We found, both by a miRNA microarray analysis and by quantitative real-time PCR (qRT-PCR), that only miR-9a, -b, -c and miR-315 are expressed in the larval CNS. (Appendix A; data not shown).

First, we purified RNA from control larval Drosophila CNSs (ventral ganglia and optic lobes) then sent samples for locked nucleic acid (LNA) miRNA microarray analysis (Exiqon). At the time of our analysis, we found that of the 148 miRNAs known to be expressed in adult Drosophila melanogaster (miRBase 11.0; 99.3% of known miRNAs) 79 were expressed in the CNS (Appendix A). Since then, updated databases have been released, the most current being miRBase 17.0. With the latest release, our screening
represents 62% of known miRNAs. The microRNAs miR-315, -9a, -9b, and -9c were found to be among the set expressed in the larval CNS (Appendix A).
FIGURE 6. *futsch* is predicted to be regulated by 6 miRNAs. miR-9a, -9b and -9c are from the same “family” meaning that they have identical, overlapping seed regions. miR-315 is predicted to bind to two places on the *Drosophila futsch* 3’UTR, and miR-963 and -976 are each predicted to bind to single sites (TargetScanFly, release 5.1).
in our analysis, miR-976 and miR-963 were not among those expressed in the larval CNS.

Next, we sought to confirm that these miRNAs were expressed in the larval CNS using qRT-PCR. We performed qRT-PCR analysis on control (w^{118} Drosophila larval CNSs. We confirmed that miR-315 as well as the miR-9 family is expressed in the larval CNS (data not shown). Together with the array results, these data demonstrate that miR-315 and the miR-9 family are expressed in the Drosophila larval CNS.

3.3 miR-315 and the miR-9 Family Repress Expression of a futsch Reporter

We next sought to demonstrate that these miRNAs repress Futsch translation as predicted by our in silico target analysis. To determine if the futsch 3’UTR is a true target of miRNAs -9a/b/c and -315, we used a Drosophila in vitro S2 cell system using a dual luciferase reporter assay (Zekri et al., 2009). A firefly luciferase reporter (F-Luc) with the 3’untranslated region (UTR) of the futsch mRNA (F-LucF) was co-expressed with a miRNA expression vector and a Renilla luciferase (R-Luc) transfection control (Figure 7).

Before we could test our primary hypothesis, we needed to confirm that the S2 reporter system worked in our hands. To do this, we co-transfected S2 cells with a nerfin reporter vector, the Renilla luciferase control vector, and the miR-9b expression vector. nerfin has been previously shown by others to be down-regulated by miR-9b using a similar assay (Zekri et al., 2009). We observed a 75.7% down regulation of the nerfin F-
FIGURE 7. Diagrams of experimental plasmid constructs. *Drosophila* S2 cells were co-transfected with three vectors: a primary miRNA expression vector, a *Renilla* luciferase (R-Luc) transfection control vector and a firefly luciferase (F-Luc) reporter vector in which the firefly luciferase gene’s expression is under regulation of the 3’UTR sequence of interest. An endogenous actin promoter drives all three plasmids. The SV40 3’UTR attached to R-Luc and the primary miRNA sequences provides a strong poly (A) signal to ensure high levels of translation. The reporter vector includes the endogenous poly (A) signal within the reporter 3’UTR.
Luc reporter (Figure 8), consistent with published results. This indicates that the S2 reporter expression assay works in our hands.

In order to address our primary question of whether expression of our miRNAs of interest down-regulates futsch expression, we then co-transfected S2 cells with three vectors: the luciferase futsch expression reporter vector, the Renilla transfection control, and each of the miRNA (or empty) expression vector individually (Figure 7).

As we hypothesized, we found that co-expression of these miRNAs does in fact decrease expression of the futsch reporter (Figure 9). miR-315 over-expression demonstrated the most profound repression of futsch reporter expression (82.2%, p<0.0006). Each of the miR-9 family miRNAs down-regulated the futsch reporter expression to a lesser, but still statistically significant, extent. miR-9a over-expression reduced futsch reporter levels by 62.3% (p<0.002). miR-9b over-expression reduced futsch reporter levels by 58.3% (p<0.0025). miR-9c over-expression reduced futsch reporter levels by 41.6% (p<0.011). These experiments have each been repeated, with consistent results. While these data indicate that futsch reporter expression is controlled by each of these miRNAs, it is unclear whether this regulation is due to translational repression or targeting of the mRNA for degradation. This question needs to be addressed in future experiments.
FIGURE 8. Over-expression of miR-9b suppresses expression of the F-Luc-Nerfin reporter. S2 cells were transfected with a mixture of three plasmids: the F-Luc-Nerfin reporter, another expressing the miR-9b primary transcript, and the third expressing Renilla luciferase. Firefly luciferase activity was normalized to that of Renilla Luciferase. Mean values of 3 technical replicates for a single biological replicate.
FIGURE 9. Over-expression of miR-9a, -9b and -9c and -315 suppress expression of the F-Luc-Futsch reporter. S2 cells were transfected with a mixture of three plasmids: the F-Luc reporter, another expressing the miRNA primary transcript or the empty vector, and the third expressing Renilla luciferase. Firefly luciferase activity was normalized to that of Renilla luciferase and then to the empty vector control. Mean values ± standard error from three biological replicates. * = p<0.05; ** = p<0.005
FIGURE 10. Two new futsch reporter vectors created using site directed mutagenesis. (A) In order to create “FLuc315mut” we mutated three base pairs within miR-315’s binding site from CAA to AGC. (B) In order to create “FLuc9mut” we mutated three base pairs within the seed region-binding site (common to all three members, miR-9a, miR-9b and miR-9c) from AAA to TTG.
3.4 Repression of futsch Reporter Expression is Specific to the miRNA Binding Sites in its 3’UTR

We next sought to confirm that the repression response was caused specifically by the miRNA/futsch 3’UTR interaction. To address this issue, we used site-directed mutagenesis to mutate the central seed region binding sites for each of the miRNAs in the futsch 3’UTR (Figure 10).

We identified the seed region binding sites for the miR-9 family and miR-315 using TargetScanFly (Figure 10; http://www.targetscan.org; Release 5.1 Friedman, et al., 2009; Grimson, et al., 2007; Lewis, et al., 2005). Members of the miR-9 family share the same seed region binding site. While the entire predicted binding sites for miRNAs -9a/b/c and -315 partially overlap, they do not share the same seed regions. Bearing this in mind, we then generated two new futsch reporter vectors using site-directed mutagenesis. In one, “FLuc315mut”, we mutated three base pairs within miR-315 binding site from CAA to AGC (Figure 10A). In the other, “FLuc9mut” we mutated three base pairs within the seed region binding site from AAA to TTG (Figure 10B).

We essentially repeated the experiments outlined above in Figure 9 except used the appropriate mutagenized futsch 3’UTR reporter vector. In each case, we found that the miRNA-mediated repression of Futsch expression is relieved when the miRNA binding site on the futsch 3’UTR is mutated to prevent miRNA binding (Figures 11-14). Interestingly, while examining miRNA repression, we observed several instances where miRNA-9a and miR-9c over-expression actually up-regulated the expression of the mutated futsch reporter (Figures 11 and 13).
FIGURE 11. Over-expression of miR-9a suppresses expression of the F-Luc-Futsch reporter, but does not suppress expression of the miR-9 binding site mutagenized F-Luc-Futsch reporter. S2 cells were transfected with a mixture of three plasmids: the F-Luc reporter, another expressing the miRNA primary transcript or the empty vector, and the third expressing Renilla luciferase. Firefly luciferase activity was normalized to that of Renilla luciferase, and normalized values of F-Luc were then normalized to the reporter plasmid without miRNA. Mean values ± standard error from three biological replicates. * = p< 0.05 ** p= <0.005
FIGURE 12. Over-expression of miR-9b suppresses expression of the F-Luc-Futsch reporter, but does not suppress expression of the miR-9 binding site mutagenized F-Luc-Futsch reporter. S2 cells were transfected with a mixture of three plasmids: the F-Luc-Futsch reporter, another expressing the miRNA primary transcript or the empty vector, and the third expressing Renilla luciferase. Firefly luciferase activity was normalized to that of Renilla luciferase, and normalized values of F-Luc were then normalized to the reporter plasmid without miRNA. Mean values ± standard deviations from three biological replicates. * = p<0.005.
FIGURE 13. Over-expression of miR-9c suppresses expression of the F-Luc-Futsch reporter, but does not suppress expression of the miR-9 binding site mutagenized F-Luc-Futsch reporter. S2 cells were transfected with a mixture of three plasmids: the F-Luc-Futsch reporter, another expressing the miRNA primary transcript or the empty vector, and the third expressing Renilla luciferase. Firefly luciferase activity was normalized to that of Renilla luciferase, and normalized values of F-Luc were then normalized to the reporter plasmid without miRNA. Mean values ± standard error from three biological replicates. * = p<0.005
FIGURE 14. Over-expression of miR-315 suppresses expression of the F-Luc-Futsch reporter, but does not suppress expression of the miR-315 binding site mutagenized F-Luc-Futsch reporter. S2 cells were transfected with a mixture of three plasmids: the F-Luc-Futsch reporter, another expressing the miRNA primary transcript or the empty vector, and the third expressing Renilla luciferase. Firefly luciferase activity was normalized to that of Renilla luciferase, and normalized values of F-Luc were then normalized to the reporter plasmid without miRNA. Mean values ± standard error from three biological replicates. * = p<0.005
3.5 FMRP May Not Regulate \textit{futsch} Expression Through Elements in the 3’UTR

Next, we wanted to determine whether miRNA-mediated repression of the \textit{futsch} reporter might be mediated by FMRP. However, we first wanted to determine if \textit{Drosophila FMRI} could alone regulate reporter expression. In order to address this question, we knocked-down \textit{dFMRI} expression in S2 cells using RNA interference (RNAi) and confirmed the success of the knock-down by Western Blot (Figure 15). We assayed the same S2 cell lysates from the \textit{FMRI} RNAi cells using the dual luciferase system. Interestingly, we found that knock down of FMR1 does not affect levels of our \textit{futsch} expression reporter (Figure 16). Based on these observations, we elected to not pursue additional experiments addressing FMRP- and miRNA-mediated control of the expression of this \textit{futsch} reporter at this point in time.
FIGURE 15. RNAi reduces the expression of FMRP in S2 cells. Introduction of double stranded RNA that is complementary to the coding sequence of $dFMR1$ reduces expression of $FMR1$ by 58% in S2 cells.
FIGURE 16. Knock-down of FMRP does not change levels of *futsch* reporter expression. S2 cells were transfected with a mixture of the F-Luc reporter plasmid, the *Renilla* luciferase transfection control plasmid, and dFMR1 dsRNA. Firefly luciferase activity was normalized to that of *Renilla* luciferase. Mean values ± standard error from three biological replicates.
Chapter Four: Discussion

4.1 *In silico* Analysis Shows That There Are Predicted Binding Sites for miRNAs in the *futsch* 3’UTR

The miRNAs revealed by our *in silico* analysis are interesting for several reasons. Notably, the sequence of *Drosophila* miR-9a is perfectly identical to human miR-9_1, and each targets Futsch/MAP1B respectively (TargetScanFly5.2; TargetScanHuman 5.2 (miRBase; [http://www.targetscanmirbase.org](http://www.targetscanmirbase.org); Friedman, et al., 2009; Grimson, et al., 2007; Lewis, et al., 2005). Moreover, murine miR-9 has been shown to co-immunopurify with FMRP and is involved in the control of synapse structure (Edbauer et al., 2010). Together, these data suggest that miR-9a is a strong candidate for co-regulation of FMRP-mediated control of MAP1B/Futsch expression. Second, miR-963 and miR-976 are predicted to bind to *futsch* 3’UTR, but are not expressed in the larval CNS (Figure 8; Appendix A). Because they are not expressed in the larval CNS, we have chosen not to pursue them as potential regulatory factors in larval synapse function. However, it would be very interesting to see if they are involved in the control of *futsch* expression at embryonic or adult stages of development.

4.2 miR-315 and the miR-9 Family are Expressed in the *Drosophila* CNS

Both the miRNA microarray and qRT-PCR demonstrate that our miRNAs of interest are expressed in the Drosophila larval CNS. The binding sites of the miRNAs we
have chosen to pursue (the miR-9 family and miR-315) have important properties. In order for a miRNA to recognize and bind its target, a specific portion of the miRNA known as the seed region (underlined in Figure 17) must match the target mRNAs 3’UTR with perfect complementation. Our in silico analysis showed the miR-9 family and miR-315 seed regions to potentially bind to the futsch mRNA. Interestingly, the predicted binding sites for the miR-9 family and miR-315 partially overlap. There are several implications to these observations. First, the binding sites for each of these miRNAs are mutually exclusive. For example, a miR-9a containing RISC cannot physically occupy the same site as a miR-315 containing RISC. It is unclear what the implications of this would be on futsch regulation. Second, the seed regions for the miR-9 family and miR-315 overlap by a single nucleotide. This raises the possibility that the FLuc9mut reporter plasmid may also disrupt regulation by miR-315. Finally, the presence of a second poorly conserved miR-315 binding site (found in Drosophila melanogaster) raises the possibility that futsch may be coordinately regulated by multiple miR-315 containing RISCs. Perhaps this is why the repression of the futsch reporter upon co-expression of miR-315 with the wild type FLuc-F reporter is more robust than any member of the miR-9 family (Figure 9).

4.3 miR-315 and the miR-9 Family Specifically Repress the Expression of futsch

While in silico analysis is a powerful tool, even with a perfect seed region match, the miR-9 family and miR-315 interaction with futsch is merely a prediction and requires
FIGURE 17. The miRNA (miR-9a, miR-9b, miR-9c (the “miR-9 family”), and miR-315) binding sites on the futsch 3’UTR. (As predicted by TargetScanFly algorithm (http://www.targetscan.org; Release 5.1 Friedman, et al., 2009; Grimson, et al., 2007; Lewis et al., 2005) and the sequences of the miRNAs which we decided to investigate. The underlined sequences indicate the miRNA seed regions.

5’…AUACAGUCACCUUAGCUAGUAAUCUCAAGUUUAGUUUACAAAC..3’

AGUAUGUCGAUCUU-UUUUCU
UAUGUCGAUUVUAGUUUUCU
AGAUGUCGAUCUUUUAU-UUUUCU
CCGAAAGACUGUGUUUAUUU

miR9a
miR9b
miR9c
miR315
validation. It is not unprecedented for predicted binding partners to have no interaction in vivo. Using the S2 luciferase reporter system, we were able to conclusively demonstrate that our miRNAs of interest actually down-regulate expression of our futsch reporter (Figure 10). We found that the site-directed mutagenesis of the futsch 3’UTR binding sites for our miRNAs of interest relieve the repression caused by miRNA over-expression, though to different extents. Again, a possible explanation for the observation that miR-315 has the strongest repression effect is that there are two miR-315 binding sites on the futsch 3’UTR compared to the miR-9 family, which each have only one. We only mutated the miR-315 site that has the highest predicted binding potential. It would be interesting to mutate the second site to see if it changes reporter levels. Also, the TargetScanFly algorithm (http://www.targetscan.org; Release 5.1 Friedman, et al., 2009; Grimson et al., 2007; Lewis et al., 2005) predicts the miR-315 binding interaction to be especially strong because it has a precise 8 base pair match to the futsch 3’UTR mRNA, as opposed to the perfect 7 base pair match for the miR-9 family miRNAs.

4.4 Unexpected Findings: miRNAs miR-9a and miR-9c Can Up-regulate the Translation of a Mutagenized futsch Reporter

Our primary goal in conducting the site-directed mutagenesis experiments was to demonstrate that the reduction in F-Luc observed upon miRNA over-expression was specifically due to an interaction at the miRNA core binding site. The site-directed mutagenesis experiments clearly show that this is the case.
Unexpectedly, in the case of miR-9a and miR-9c, we observed that miRNA over-expression actually increased the mutated F-Luc reporter expression above the levels of the mutated reporter without miRNA over-expression. We had expected site-directed mutation of the reporter to relieve the down-regulation caused by miRNA over-expression, but it appears that in some cases miRNA over-expression actually enhances mutated reporter expression. We theorize that this unexpected observation of increase in translation in the mutated futsch reporter plasmid upon over-expression of a miRNA might represent a novel regulatory mechanism. Perhaps miR-9a and -9c are negative regulators of futsch and some other protein which itself regulates Futsch. These two miRNAs share nearly 100% sequence identity outside of the seed region while miR-9b is considerably more divergent (Figure 17). One interesting possibility based on in silico data from TargetScanFly suggests that FMRP itself could be this protein. FMRP is predicted to be a very strong candidate for regulation by both miR-9a and -9c. Based on this observation, we propose the following working model (Figure 18). In this model, miRNAs independently down-regulate expression of Futsch and FMRP. Our data are consistent with this model. If we increase miRNA expression while blocking their binding to futsch 3’UTR, it would follow that the repression of FMRP would continue. The repression of FMRP, itself a repressor of futsch, would result in an increase in futsch expression.
FIGURE 18. Model for miRNA repression of Futsch and FMRP. We have found that miRNA over-expression down-regulates futsch expression. We have observed that over-expression of miRNAs sometimes increases expression of the futsch reporter in which the miRNA binding site has been mutated. A possible model to explain this phenomenon is that miRNA over-expression (specifically miR-9a and -9c) down-regulates another protein (perhaps FMRP) which itself is a negative regulator of futsch.
4.5 Down-Regulation of FMRP Does Not Increase Expression of the *futsch* Reporter

It has been demonstrated that FMRP is a negative regulator of *futsch* (Zhang et al., 2001). It follows that down-regulation of FMRP would result in higher levels of *futsch* expression. Thus, we hypothesized that we would observe increased levels of our *futsch* reporter with our (successful) down-regulation of FMRP by RNAi. Interestingly, we did not find this to be the case (Figure 16). We did not observe any significant change in the levels of expression of the *futsch* reporter. There are several explanations for this observation. First, it could be that the pathway by which these phenomena occur is more complicated than what has been predicted, thus down-regulation of FMRP in isolation from the rest of the system does not result in lower levels of *futsch* expression. Another possibility is that FMRP down-regulates expression of *futsch* via a direct interaction, but not through the 3’UTR mRNA region. Our reporter is only sensitive to effects of 3’UTR *futsch* regulation. If FMRP down regulates *futsch* by interacting with a different portion of the *futsch* mRNA (perhaps the 5’UTR or the coding sequence), levels of our reporter would not be affected by FMRP knock-down, as our reporter only contains the 3’UTR of the *futsch* gene. A third possibility is that partial knockdown of FMRP is not sufficient to down-regulate *futsch* expression. It is unknown whether or not heterozygous null FMRP flies have normal *futsch* expression levels. We were not able to prove or disprove the hypothesis, but our experiments suggest a potential model for the regulation of Futsch expression that we had not considered.
4.6 Potential Caveats

In closing, one important fact to consider in the interpretation of all of our dual-luciferase results is that the firefly luciferase reporter indicates what Futsch levels should be, but does not actually measure endogenous changes in expression of the Futsch protein. The firefly luciferase reporter is under the control of putative regulatory elements in the *futsch* 3’UTR, but is not connected to the actual Futsch protein. Therefore, this reporter system has an additional caveat in that the reporter is only sensitive to regulation imposed on the 3’UTR of *futsch*. In reality, Futsch may be under regulation of many factors (perhaps including FMRP) due to interactions with the coding sequence or 5’UTR of the *futsch* mRNA.

4.7 Potential Future Directions

miRNAs are believed to bind to and target mRNAs for either mRNA decay or translational repression. We were not able to conclude whether RNA stability (or instability) is the cause of observed changes in expression, as opposed to translational repression. To test this, we could perform qRT-PCR on the S2 cell lysates to determine if the levels of *futsch* mRNA are reduced upon over-expression. A finding that *futsch* mRNA levels are lower after over-expression of miRNAs would indicate that the miRNAs are targeting the *futsch* mRNA for degradation. If *futsch* mRNA levels were not changed by miRNA over-expression, it would indicate that the miRNAs are repressing the translation of Futsch, but that the *futsch* mRNA remains intact.
There are several additional steps that could be taken to address our unanswered hypothesis. It is possible that we might observe a change in *futsch* reporter expression if we were able to achieve a complete FMRP knock down, vs. the 58% knock-down we observed. It is possible that a longer incubation of the S2 cells (on the order of a week or more) or repeated applications of *dFMR1* dsRNA might achieve a greater or even complete reduction of FMRP expression, and perhaps an observable change in our futsch reporter levels. Further, it is possible that *dFMR1* dsRNA complementary to a different portion of the *dFMR1* gene might provide more effective repression.

Another option is to perform essentially the opposite experiment: co-express the *futsch* reporter plasmid with a plasmid that over-expresses FMRP. If we still did not observe any change in *futsch* reporter levels, we would have additional evidence indicating FMRP is not involved in regulating *futsch* (at least via the 3’UTR). We could investigate the possibility that FMRP regulates *futsch* via a sequence not in the 3’UTR by creating a reporter under the control of a 5’regulatory sequence.

It would also be interesting to perform genetic epistasis experiments between miRNA and FMRP in S2 cells. We could perform the experiment shown in figure 9 while knocking-down FMR1. If we could accomplish a 100% knock-down of FMR1, and we observed identical repression of the futsch reporter, that would provide additional evidence that miRNAs regulate *futsch* independently of FMRP.

Finally, we have produced fly lines that over-express the miRNAs -9a, -9b, -9c and -315 with which we could perform many *in vivo* assays, including, but not limited to, genetic interactions affecting NMJ synaptic structure and development.
miRNAs could play an important role in long-term synaptic plasticity underlying learning and memory as well as in dynamic regulation of synaptic protein synthesis in response to activation of neurotransmitter receptors, such as mGluRs (Muddashetty et al. 2011). It has been theorized that exaggerated protein synthesis (due to the lack of the protein synthesis inhibitor, FMRP) downstream of the mGluRs is a main cause of FXS pathology (Krueger and Bear, 2011). While a treatment that blocks mGluR stimulation could potentially alleviate the symptoms of FXS (Figure 19), it would likely create many unintended side effects because the mGluR regulatory system affects a diverse array of functions.
FIGURE 19. Model of FXS pathogenesis and potential pharmacological interventions. The activation of mGluR receptors stimulates translation of mRNAs at the synapse. Normally, FMRP represses translation of those mRNAs. In absence of FMRP, the mRNAs are over-expressed, which may cause pathology. Pharmacological interventions that block mGluR stimulation may alleviate FXS pathology. Figure adapted from Penagarikano et al., 2007.
Chapter Five: Summary

We have hypothesized that FMRP and miRNAs co-regulate expression of \textit{futsch} (the \textit{Drosophila} homologue of \textit{MAP1B}). Using an \textit{in vitro} luciferase reporter system, we have shown that miR-315 and members of the miR-9 family selectively down-regulate \textit{futsch} reporter translation. We have confirmed by site-directed mutagenesis that the miRNA interaction with the \textit{futsch} 3’UTR is specific to the miRNA seed region binding site. Reporter plasmids with mutated miRNA binding sites did not display down-regulation upon over-expression of the applicable miRNAs. These data strongly suggest that miRNAs-9a/b/c and -315 regulate \textit{futsch}. We have not been able to determine whether or not miRNAs and FMRP co-regulate \textit{futsch}. The course of our experiments however, has suggested potential previously unknown regulatory mechanisms.
References


APPENDIX 1. 79 miRNAs are expressed in the *Drosophila melanogaster* 3rd instar larval CNS as identified by miRNA microarray analysis (Exiqon). The database used to construct the profile above covered 62% of miRNAs currently known to be present in *Drosophila melanogaster* (miRBase 17.0). However, at the time that the array was performed, our results covered 99.3% of known miRNAs (miRBase 14.0). Analysis was conducted in collaboration with Robert Sand.
Appendix B

List of abbreviations/definitions

CNS = Central Nervous System

dFMR1 = Drosophila FMR1

FMRP = Fragile X mental retardation protein

FMR1 = fragile X mental retardation 1, the gene responsible for FXS.

FXPOI = fragile X-associated premature ovarian failure

FXS = Fragile X syndrome

FXTAS = fragile X-associated tremor ataxia syndrome

MAP1B = microtubule associated protein 1B, the mammalian homolog of Futsch

miRISC = miRNA-containing RISC

miRNA = microRNAs

NMJ = Neuromuscular Junction

RISC = RNA-Induced Silencing Complex

RNAi = RNA interference

qRT-PCR = quantitative real-time PCR