MICRORNA-MEDIATED REGULATION AND THE FRAGILE X FAMILY OF PROTEINS

BY

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DISSESTATION

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ABSTRACT

The main purpose of this work is to understand how two members of the fragile X family of RNA binding proteins, fragile X mental retardation protein (FMRP) and FXR1P, are regulated by post-translational modifications and microRNAs (miRNAs), respectively. Both proteins play key roles in normal development and function. The absence of FMRP leads to the cognitive defects seen in Fragile X syndrome, the leading cause of hereditary mental retardation, while loss of FXR1P expression in mice is fatal after birth, likely due to cardiac and muscle abnormalities.

Small, genomically encoded miRNAs are involved in almost every biological process, specifically in the regulation of mRNA translation. Although their biogenesis is relatively well defined, it is still unclear how they are recruited to their mRNA targets. FMRP and its autosomal paralogs, FXR1P and FXR2P, in addition to the single Drosophila ortholog, dFmrp, associate physically and functionally with the miRNA pathway.

Constitutively phosphorylated FMRP (P-FMRP) is found associated with stalled untranslating polyribosomes and translation of at least one mRNA is downregulated when FMRP is phosphorylated. We hypothesized that translational regulation by P-FMRP is accomplished through association with the miRNA pathway. Accordingly, we developed a phospho-specific antibody to P-FMRP and showed that P-FMRP associates with increased amounts of precursor miRNAs (pre-miRNA) compared to total FMRP.
Furthermore, P-FMRP does not associate with Dicer or Dicer containing complexes in co-immunoprecipitation experiments or in an in vitro capture assay using a P-FMRP peptide sequence bound to agarose beads. These data show that Dicer containing complexes bind FMRP at amino acids 496-503 and that phosphorylation disrupts this association with a consequent increase in association with pre-miRNAs. In sum, we propose that in addition to regulating translation, phosphorylation of FMRP regulates its association with the miRNA pathway by modulating association with Dicer. We present a new model for the effect of phosphorylation on FMRP function, where phosphorylation of FMRP inhibits Dicer binding, leading to the accumulation of precursor miRNAs and possibly a paucity of activating miRNAs.

FMRP’s autosomal paralog, FXR1P, plays an important role in normal muscle development, has been implicated in fascioscapulohumeral muscular dystrophy (FSHD) and its absence or misregulation has been shown to cause cardiac abnormalities in mice and zebrafish. To examine miRNA-mediated regulation of FMRP and FXR1P, we studied their expression in a conditional Dicer knockdown cell line, DT40. We found that FXR1P, but not FMRP, increases upon Dicer knockdown and consequent absence of miRNAs suggesting that FXR1P is regulated by miRNAs, while FMRP is not. Expression of a luciferase reporter bearing the FXRI 3’UTR was significantly increased in the absence of miRNAs, confirming miRNA-mediated regulation of FXR1P. We identified one of the regulatory regions by removing an 8-nucleotide miRNA seed sequence common to miRNAs 25, 32, 92, 363 and 367 in the 3’UTR of FXRI. Accordingly, over expression of a miRNA, miR-367, containing this common seed
sequence decreased endogenous FXR1P expression in HEK-293T and HeLa cell lines. We report for the first time that FXR1P expression is regulated through miRNA binding to the miR-25/32/92/363/367 seed sequence binding site in the FXR1 3’UTR.
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INTRODUCTION

The main goal of my work is to understand how two members of the fragile X family of RNA binding proteins, fragile X mental retardation protein (FMRP) and ‘FMR1 cross reacting relative’ protein (FXR1P), are regulated by post-translational modifications and microRNAs (miRNAs), respectively.

This work was conducted and all data obtained in the lab of Professor Stephanie Ceman and is divided into background information (chapter 1), two data chapters (chapters 2 and 3) and finally, concluding remarks (chapter 4). Much of the background information presented in chapter 1 was published in an abbreviated form as a review. The data presented in chapter 2 has also been published, while chapter 3 is a manuscript that has been submitted for publication.

Chapter 1 highlights key background information on fragile X syndrome and the fragile X family of proteins, progresses into regulation of target mRNAs by FMRP and, after a summary of miRNAs and miRNA processing, outlines what was known about the connection between the miRNA pathway and fragile X proteins prior to my work presented here.

Chapter 2 describes how phosphorylation of FMRP abolishes association with Dicer, a protein that is a crucial component of the miRNA processing pathway, leading to an increase of un-processed precursor miRNAs (pre-miRNAs) associated with
phosphorylated FMRP (P-FMRP). We report a new role for P-FMRP in regulating association with the miRNA pathway and propose a model to explain how a lack of mature miRNAs associated with P-FMRP could lead to translation suppression of target mRNAs.

Chapter 3 asks the question whether miRNAs regulate expression of FMRP and FXR1P, in the process shifting the focus from FMRP to FXR1P, an autosomal paralog of FMRP. Using a conditional Dicer knockdown cell line, DT40, we show that FXR1P, but not FMRP, increases upon Dicer knockdown and consequent absence of miRNAs, suggesting that FXR1P is regulated by miRNAs while FMRP is not. We describe how a regulatory region was mapped in FXR1 by luciferase assay to an 8 nucleotide miRNA seed sequence common to the miR-25/32/92/363/367 group of miRNAs that bind in the 3’ untranslated of FXR1. We confirm this site by over expressing a miRNA, miR-367, containing this common seed sequence and showing that it decreased endogenous FXR1P expression in two human cell lines. We report for the first time that FXR1P expression is regulated through miRNA binding to the miR-25/32/92/363/367 seed sequence binding site in the FXR1 3’UTR.

The dissertation concludes in chapter 4 with a summary of this research, potential future experiments and a discussion of the contributions this work has made to our understanding of the field of fragile X syndrome and miRNA regulation of protein expression.
CHAPTER 1
BACKGROUND
(Originally published in part as a review in RNA Biology, Volume 6, Issue 2, pages 175-178, 2009)

Fragile X syndrome
Fragile X syndrome, the leading cause of hereditary mental retardation, has an occurrence rate of approximately 1 in 4000 males and 1 in 8000 females (Penagarikano et al., 2007). It is caused by the inactivation of the fragile X mental retardation gene, FMR1, due to an expansion of a trinucleotide repeat (CGGn) in the 5’ untranslated region of the gene (O'Donnell and Warren, 2002, Darnell et al., 2005, Garber et al., 2006, Penagarikano et al., 2007). The FMR1 gene product, fragile X mental retardation protein (FMRP) is an RNA binding protein that regulates target mRNAs (Penagarikano et al., 2007, Costa-Mattioli et al., 2009). FMRP associates with approximately 400 mRNAs in the brain (Brown et al., 2001, Miyashiro et al., 2003) and is found nearly ubiquitously throughout the body (Terracciano et al., 2005). High expression levels of FMRP in the brain (Weiler et al., 1997) and its role as a translational regulator (Laggerbauer et al., 2001, Li et al., 2001, Costa-Mattioli et al., 2009) suggests an important role in memory, learning, and normal cognition as suggested by the cognitive impairment exhibited in patients with fragile X syndrome upon the loss of FMR1 gene expression (Terracciano et al., 2005). The most prominent phenotype, mental retardation, also affects spatial skills, information processing, and leads to language and speech deficits (Penagarikano et al., 2007). Other clinical features of fragile X syndrome include facial abnormalities, macroorchidism,
and connective tissue disorders (Penagarikano et al., 2007). There are also behavioral effects attributed to fragile X syndrome. Patients can display hyperactivity, hypersensitivity to sensory stimuli, attention deficits, and 15-50% of patients exhibit autistic-like behaviors such as averted gaze, tactile defensiveness, and repetitive behaviors (Penagarikano et al., 2007).

**The fragile X family of RNA-binding proteins**

FMRP, the protein lacking in people with Fragile X syndrome, is one member of a small family of RNA binding proteins that include two autosomal paralogs, FXR1P and FXR2P (FXRPs) with functional domain sequence similarities to FMRP of 80% and 76% respectively (Penagarikano et al., 2007). In addition to sharing similar functional domains, the FXRPs have the same overall cellular localization patterns as FMRP (Penagarikano et al., 2007) and were validated as FMRP interactors by a yeast two-hybrid screen of a human brain cDNA library for proteins that interacted with FMRP (Siomi et al., 1995, Zhang et al., 1995). FXR2P and FXR1P exhibit an overall sequence similarity to FMRP of 60% (Siomi et al., 1995). However, even though cellular distribution is similar, tissue expression patterns differ greatly between FMRP and the FXRPs. Whereas FMRP is widely expressed in most tissues, particularly brains and testis (Devys et al., 1993, Khandjian et al., 1995, Tamanini et al., 1999), FXR1P is highly expressed in muscle and heart tissues where FMRP is almost absent suggesting different functions for these two proteins (Bakker et al., 2000, Hoogeveen et al., 2002). *In vivo*, it has been demonstrated that FMRP, FXR1P and FXR2P preferentially interact with
themselves to form homo-multimers (Tamanini et al., 1999), however differential tissue distributions indicate that FMRP and the FXRPs probably do not require complex formation for proper function (Hoogeveen et al., 2002).

**Fragile X protein functional domains**

FMRP and the FXRPs share functional protein domains including a nuclear localization signal (NLS), a leucine-rich nuclear export signal (NES), two RNA binding domains with homology to hnRNP K proteins termed KH1 and KH2, and an arginine-glycine rich RNA binding region termed the RGG box (however, the presence of a true RGG box in FXR2P is controversial) (Hoogeveen et al., 2002) (Fig. 1). Although possessing an NLS and NES, FMRP and the FXRPs are localized mainly in the cytoplasm where they form messenger ribonucleoprotein complexes and associate with polyribosomes (Feng et al., 1997, Ceman et al., 1999), but recent data shows that FMRP binds its target mRNAs in the nucleus (Kim et al., 2009). FMRP associates with ribosomes via RNA (Khandjian et al., 1996), but how FMRP or the FXRPs regulate translation of bound mRNAs at the molecular level is still unclear.
Figure 1. Schematic of the FMR and FXR protein domains. Shared domains are indicated. NLS-nuclear localization sequence; KH1, KH2-RNA binding domains with homology to hnRNP K protein; NES-nuclear export sequence, RGG-RNA binding arginine-glycine rich region. SNASETES-phosphorylation site in FMRP with phosphoserines indicated. Amino acid numbering and figure adapted from data in (Ceman et al., 2003).
Alternative splicing

The \textit{FMR1} and \textit{FXR1} transcripts are alternatively spliced. There are several FMRP isoforms ranging from 69-80 kDa with the major species at 69 kDa (Ashley et al., 1993). At least seven FXR1P isoforms (iso-a to iso-g) have been identified (Siomi et al., 1995, Kirkpatrick et al., 2001) with three isoforms showing muscle specificity (Mientjes et al., 2004). In contrast to \textit{FMR1}, the mechanism of \textit{FXR1} alternative splicing is tissue specific and different isoforms are expressed preferentially in certain tissues (Khandjian et al., 1998, Kirkpatrick et al., 1999, Tamanini et al., 2000). Although the functional relevance of these tissue specific isoforms is not known, only FXR1P is expressed in skeletal muscle where it localizes to costameres and Z-lines (Bakker et al., 2000, Dube et al., 2000, Mientjes et al., 2004). The longest FXR1P isoform is highly expressed during myogenesis in adult cardiac and skeletal muscle tissue and exhibits high affinity for mRNAs containing G-quartet structures (Bechara et al., 2007). Some FXR1P isoforms and FXR2P also have a nucleolar-targeting signal (NoS) signal that is lacking in FMRP, again suggesting functional differences among this family of proteins (Tamanini et al., 2000). Although there is very little data to suggest a tissue specific function of \textit{FXR2}, these data suggest that alternative splicing, at least in the case of \textit{FMR1} and \textit{FXR1}, may play an important role in mRNA regulation in different tissues.

\textbf{FMR and FXR animal models}

Since no naturally occurring animal models for trinucleotide repeat disorders such as fragile X syndrome have been described, several groups have generated animal models to better study this disorder. To attempt to study the underlying molecular mechanisms of
trinucleotide repeat amplification, mouse models for CGG repeat instability have been constructed to try to mimic the spontaneous expansion seen in human patients, however artificial insertion of triplet repeats has had little to no effect on CGG\(_n\) stability in these mice (Burright et al., 1995, Goldberg et al., 1996, Lavedan et al., 1998). Transgenic mice created with as many as 120 triplet repeats still did not exhibit the full expansion that occurs over generations in human fragile X patients (Lavedan et al., 1998). This has led to the conclusion that, at least in mice, extra CGG repeats alone are not enough to induce the expansion and full mutation (Lavedan et al., 1998).

To study the physiological and behavioral effects resulting from a lack of FMRP, a FMR1 knockout mouse was first engineered by utilizing homologous recombination to select for a neomycin cassette disruption of exon 5 in mouse genomic FMR1 DNA (Bakker et al., 1994). RT-PCR and western blot analysis confirmed the absence of wild-type FMR1 and FMRP in these mice (Bakker et al., 1994). Absence of FMRP in this knockout mouse led to some of the phenotypic characteristics observed in human fragile X patients such as macroorchidism, hyperactivity, and problems processing spatial information as shown by poor performance in tests such as the Morris water maze (first developed by Richard Morris in 1981), where the mice must locate a hidden platform submerged in water (Bakker et al., 1994).

In Drosophila melanogaster the FMRP homologue, dFmrp, shares a protein domain sequence with human and murine FMRP of 56% overall (Zarnescu et al., 2005), 70% and 71% for the two KH domains and 25% for the RGG box (Gao, 2002, Hoogeveen et al.,
A number of dFmrp mutants have been generated, including dfmr1 containing the I307N mutation which causes a severe form of fragile X in humans (De Boulle et al., 1993, Wan et al., 2000). Although many behavioral tests initially indicated that dfmr1 mutant flies did not differ significantly from wild-type flies, subsequent data show that mutants exhibit some locomotor function deficits as shown by impaired coordinated behavior in flight tests, perhaps linked to the increase of growth and branching at synaptic terminals seen in dfmr1 mutant flies (Zhang et al., 2001). Mutant larvae were shown to exhibit altered crawling behavior with more frequent turns and shorter linear paths (Xu et al., 2004). And, similar to mouse models, dfmr1 mutant flies show little to no rhythmicity in circadian locomotor activity, but can be rescued by an insertion of genomic fragment containing the wild-type dfmr1 gene (Dockendorff et al., 2002, Inoue et al., 2002, Morales et al., 2002). Abnormal spermatogenesis and oogenesis in flies has also been linked to dfmr1 mutation (Zhang et al., 2004, Costa et al., 2005).

In Drosophila, dFmrp associates with Dicer, Argonaute proteins (Ago1 and Ago2), microRNAs (miRNAs) and other components of the miRNA pathway (Caudy et al., 2002, Ishizuka et al., 2002, Jin et al., 2004b, Xu et al., 2008). Further studies subsequently showed that mammalian FMRP also associates with Dicer, miRNAs, Ago2 and other miRNA pathway components (Jin et al., 2004a, Jin et al., 2004b), suggesting that FMRP utilizes the miRNA pathway to regulate its target mRNAs. This will be discussed in greater detail in subsequent sections.
In contrast to *FMR1* knockouts, *FXR1* knockout neonates die shortly after birth, likely due to cardiac or respiratory failure, indicating that unlike FMRP expression, FXR1P expression is necessary for viability in mice (Mientjes et al., 2004). In other animals such as *Xenopus* and zebrafish, reduction of FXR1P by siRNA or morpholino knockdown technologies results in striated, skeletal and other muscular abnormalities (Huot et al., 2005, Padje et al., 2009), indicating that FXR1P may play an important role in muscle development in multiple species.

Recently, it was observed that humans with facioscapulohumeral muscular dystrophy (FSHD) exhibit abnormal expression patterns of three different FXR1P isoforms in myoblast and myotubes (Davidovic et al., 2008). These altered expression patterns appear to be due to the reduced stability of specific *FXR1* mRNA variants, which leads to a reduced expression of those isoforms in developing muscle (Davidovic et al., 2008).

*FXR2* knockout mice do not exhibit any clear pathology in tissues, however some behavioral phenotypes similar to *FMR1* knockout mice have been observed (Hoogeveen et al., 2002). Additional behavioral abnormalities unique to *FXR2* mutants such as a decrease in fear conditioning, an exaggerated startle response, and a slight decrease in response to painful stimuli have also been reported (Bontekoe et al., 2002). Interestingly, when *FXR2* is knocked out in conjunction with *FMR1* in mice, loss of normal rhythmic activity in a light-dark period is observed (Zhang et al., 2008). These data demonstrate
that both *FMR1* and *FXR2* are required for normal circadian behavior and suggests a dual function of these proteins that could be relevant to the sleep alterations observed in human patients (Zhang et al., 2008).

**Post-translational modifications of fragile X proteins**

Phosphorylation is a mechanism by which protein activity can be modulated by the covalent addition of negatively charged phosphate groups to serine, threonine or tyrosine side chains. This addition changes how the protein interacts with other proteins or nucleic acids and is a rapid, reversible modification that is responsible for many important functions within the cell (Cooper and Hausman, 2006).

Phosphorylation of RNA-binding proteins, such as FMRP, can either up or down-regulate association with RNA (Ceman et al., 2003, Narayanan et al., 2007, Bechara et al., 2009, Cheever and Ceman, 2009a). FMRP is a phospho-protein that is phosphorylated on three serines (496, 499, and 503) between the nuclear export sequence and the RGG box, which is the primary RNA binding domain (Ceman et al., 2003) (Fig. 1). FMRP is also associated with stalled, untranslating polyribosomes as shown by its resistance to ribosomal run-off when constitutively phosphorylated (Ceman et al., 2003) which suggests that phosphorylated FMRP is involved in translation suppression. In *Drosophila*, dFMRP is phosphorylated *in vivo* by dCKII at serine 406 with subsequent RNA-binding activity alteration (Siomi et al., 2002). This, among other evidence, has led to the proposition that phosphorylation of FMRP affects its function as a translational regulator of bound target mRNAs.
Other post-translational modifications such as glycosylation and sumoylation have not been reported with regard to FMRP. However, FMRP has been shown to be affected by ubiquitination where a mGluR-LTD induced transient increase in FMRP is then rapidly degraded by the ubiquitin-proteasome pathway, suggesting a rapid cycle of synthesis and degradation of FMRP (Hou et al., 2006). This cycle could be important to control mRNA binding and hence, synaptic plasticity (Hou et al., 2006).

Arginine methylation, a post-translational modification restricted to eukaryotic cells, often occurs in RGG box containing proteins, including FMRP (Najbauer and Aswad, 1990, Liu and Dreyfuss, 1995, Gary and Clarke, 1998). Arginine methylation was originally shown to affect the ability of FMRP to bind homopolymers and RNAs through the use of an adenosine-2’, 3’-dialdehyde inhibitor (AdOx) (Denman, 2002). Methylation may also play a role in FMRP’s ability to bind to FXR1P in order to co-regulate translation of target mRNAs (Dolzhanskaya et al., 2006). Using double-label fluorescence confocal microscopy to visualize FMRP-containing cytoplasmic granules associating with translational components during oxidative stress, methylation was shown to regulate FMRP-homodimer and FMRP-FXR1P-heterodimer formation by influencing these ratios during translation initiation, suggesting an important role for methylation in normal FMRP function (Dolzhanskaya et al., 2006). Subsequent work showed that FMRP, FXR1P, and dfmr1 are methylated on four arginines in the arginine-glycine-glycine (RGG) box by protein arginine methyl-transferase 1 (PRMT1) in vitro.
Reduced binding to the scl RNA sequence, which contains a stem loop G-quartet structure, upon \textit{in vitro} methylation by PRMT1 suggests that methylation serves to reduce or modulate target mRNA binding by FMRP (Stetler et al., 2006).

**Phosphorylated FMRP and regulation of mRNA translation**

FMRP is phosphorylated on three serines between its nuclear export sequence and the primary RNA binding domain, the RGG box, in both brain and cell lines (Ceman et al., 2003). To determine the role of phosphorylation on FMRP function, amino acid substitutions of the primary phosphorylation site were made, mimicking either constitutive phosphorylation or no phosphorylation (Ceman et al., 2003). Constitutively phosphorylated FMRP (P-FMRP) was relatively resistant to ribosomal run-off, suggesting that it was associated with untranslating polyribosomes (Ceman et al., 2003). In contrast, unphosphorylated FMRP was easily run-off, suggesting that it was associated with actively translating polyribosomes (Ceman et al., 2003). Narayanan and colleagues identified a specific cargo mRNA, SAPAP3, whose translation was modulated by phosphorylation of FMRP (Brown et al., 2001, Narayanan et al., 2007). As predicted by the model, inhibition of phosphatase 2A (PP2A) led to an increase in translation of SAPAP3 (Narayanan et al., 2007). Consequently, it has been suggested that phosphorylation of FMRP functions as a key step in the regulation of bound target mRNAs, suppressing translation through an unknown mechanism.
To verify P-FMRP’s role as a suppressor of target mRNA translation, polyribosome profiles were performed to fractionate the 40S, 60S and 80S subunits from the heavier polyribosomes in cells as described in (Ceman et al., 2003). P-FMRP localization was then visualized by western blot with PSER and the anti-FMRP monoclonal antibody 1C3 (Devys et al., 1993) and P-FMRP was found on polysomes (Fig. 2 ). To determine whether these polysomes are actively translating, we treated stably transfected FLAG-tagged FMRP (WT) cells with sodium azide (NaAz). Although pleotropic, NaAz is an inhibitor of translation initiation that allows visualization of ribosomal run-off of actively translating ribosomes while stalled ribosomes do not change their localization at the dense polusome fractions. More specifically, after translation initiation is blocked, actively translating ribosomes continue to move along the transcript and “run-off” but no new translation is initiated. As published previously with a phospho-mimic transgene in which the serine at 499 was substituted with aspartic acid (Ceman et al., 2003), P-FMRP was resistant to run-off because it is associated with stalled polyribosomes in the denser fractions (Fig. 2-PSER). Densitometry (NIH Image) shows approximately 75% of P-FMRP located in dense polyribosome fractions when translation is blocked compared to 29% of total FMRP. The anti-FMRP (1a) antibody shows FMRP present across all fractions (Fig. 2-1a).
Figure 2. Phospho-FMRP is associated with stalled, untranslating polyribosomes.

Fibroblast cells containing stably transfected flag-tagged FMRP were treated with sodium azide and cycloheximide and harvested. Fractions were centrifuged through a 15-45% sucrose gradient, analyzed with UV to detect optical density, collected and TCA precipitated. P-FMRP was visualized by western blot using PSER and total FMRP with monoclonal antibody 1a.
Nuclear trafficking of FMRP

Recent data show that upon FMRP binding to nuclear mRNAs, exit from the nucleus occurs via the Tap/NXF1 bulk nuclear exporter in combination with FMRP’s own nuclear export sequence (Kim et al., 2009) suggesting a model where once FMRP exits the nucleus with its bound mRNAs, translation could be modulated depending on its phosphorylation state (Fig. 3). Our recent data point toward phosphorylation regulating association of FMRP with the miRNA pathway as a means to regulate translation of target mRNAs (Cheever and Ceman, 2009a) and will be described in more detail in chapter 2.
Figure 3. Model of FMRP localization, target mRNA binding and translation regulation. FMRP enters the nucleus, binds target mRNAs, and exits using a combination of the bulk mRNA exporter Tap/NXF1 and its own NES (Kim et al., 2009). Once in the cytoplasm, translation regulation of target mRNAs occurs based on FMRP’s phosphorylation state.
Non-coding RNAs

Small, non-protein coding RNAs with important gene-regulatory function are a conserved feature of both prokaryotic and eukaryotic genomes. They can perform diverse biological functions in a multitude of cellular processes, including gene silencing at the transcriptional or post-transcriptional level (Bartel, 2004a, Meister, 2007). Classes of non-coding RNAs can be distinguished based on their mechanisms of biogenesis, precursor structure and the genomic region from which they originate (Farazi et al., 2008). Piwi interacting small RNAs (piRNAs), implicated in mRNA cleavage, and repeat-associated siRNAs (rasiRNAs), involved in regulation of chromatin structure, are derived from transcripts that are not double stranded (ds) (Farazi et al., 2008). However, the most well-studied classes of small RNAs are siRNAs and miRNAs that can be grouped together based on their origination from dsRNA precursors. siRNAs, processed from dsRNAs, were the first small RNA discovered to regulate gene expression by binding in a sequence-specific manner in order to initiate target degradation (Fire et al., 1998, Zamore et al., 2000). miRNAs, first characterized in C. elegans (Lee et al., 1993), are the most abundant class of small RNAs in animals, are 19-25 nucleotides in size and are genomically encoded (Ambros, 2004, Bartel, 2004a, Farazi et al., 2008). They can be expressed at extremely high levels, up to ten thousands of copies per cell, potentially regulating many hundreds of mRNA targets (Lim et al., 2003).
**Processing of microRNAs**

miRNAs function by base pairing with sequences in the 3’UTR of their target mRNA sequences. If base-pairing is perfect along their ~22 nucleotide length, the result is mRNA target degradation. In contrast, if base-pairing is imperfect with a bulge in the duplex, the result is translational silencing (Bartel, 2004b). The most recent miRBase update (Release 14.0, Sept. 2009, http://www.mirbase.org/), lists 10,581 mature miRNAs from 115 species. Since each miRNA could potentially interact with multiple mRNAs due to incomplete base pairing to each target, it has been bioinformatically predicted that more than 30% of human genes could be regulated by miRNAs (Ross et al., 2007).

**The role of Dicer in miRNA processing**

In mammals, primary miRNAs are transcribed in the nucleus and processed into 70-80 nt precursor miRNAs by Drosha and DGCR8 (Lee et al., 2003). Precursor miRNAs (pre-miRNAs) are exported to the cytoplasm by exportin 5 (Yi et al., 2003), where they are processed into short, double stranded duplexes by the Dicer pre-miRNA processing complex (Lee et al., 2003, Bartel, 2004a, Ross et al., 2007). Dicer is a key protein in this complex, because it processes the pre-miRNAs into shorter (21-23 nt) double-stranded segments which can then be inserted into the RNA induced silencing complex (RISC) (Perron and Provost, 2008). The RNA induced silencing complex then separates the duplexed strands into a mature miRNA (Bartel, 2004a) (Fig. 4). This guide miRNA associates with Ago2, which is the only human Argonaute family protein with endonuclease activity (Song et al., 2003, Fillipowicz et al., 2005, Hock and Meister, 2008). Incomplete complementarity of the miRNA to its target mRNA leads to
translational repression (or in some cases activation, as discussed later) while complete complementarity triggers Ago2’s endonuclease activity leading to cleavage of the target mRNA, as observed during RNA silencing. Conversely, in *Drosophila*, both Ago1 and Ago2 have cleavage activity, with Ago1 mediating miRNA guided cleavage of RNA and Ago2 mediating siRNA cleavage during RNA silencing (Hock and Meister, 2008).
Figure 4. **microRNA processing diagram.** Primary miRNAs are transcribed in the nucleus and processed into precursor miRNAs by the Drosha/DGCR8 complex. After being exported to the cytoplasm by Exportin 5, the Dicer complex cleaves the precursor miRNA into a double stranded duplex miRNA. One strand is degraded and the other is processed further and inserted into RISC to produce a mature miRNA. This miRNA is now ready to bind a target mRNAs in order to effect degradation, translation suppression, or deadenylation depending on the miRNA’s complementarity to the target.
Although it is unknown exactly how the mature miRNA finds its target mRNA, miRNAs function as part of larger complexes such as the RNA induced silencing complex (RISC) and the miRNA ribonucleoprotein (miRNP) complex, suggesting that miRNAs guide associated proteins to target mRNAs to effect degradation, repression, or in some cases, translation activation (Fillipowicz et al., 2005, Vasudevan and Steitz, 2007, Vasudevan et al., 2007). Although both structure and homology studies of Argonaute explain its “slicing” role, there is no similar evidence explaining its role in translation repression (Fillipowicz et al., 2005). Ago proteins in conjunction with miRNAs bound with incomplete complementarity to mRNA targets can mediate repression by RNA degradation or inhibition of translation (Hock and Meister, 2008). Degradation occurs when an Ago-miRNA complex binds a target and induces deadenylation, decapping, and degradation (Chen and Meister, 2005, Hock and Meister, 2008). Alternatively, an Ago-miRNA complex can bind target mRNAs and suppress translation by blocking ribosome read-through (Chen and Meister, 2005, Hock and Meister, 2008). The mechanism by which Ago proteins mediate translational repression is still under debate; it is not yet clear whether they act on translation initiation (Meister, 2007), or on translation elongation (Olsen and Ambros, 1999, Maroney et al., 2006, Petersen et al., 2006). Neither of these suppressive roles for argonaute can be explained strictly by its structural role as a cleaver of RNA, leading to the assumption that there are other associated proteins within the miRNP complex that enable translational control (Hock and Meister, 2008).
Processing bodies

Once mRNAs have been targeted by miRNAs for degradation (because of complete complementarity) or suppression (incomplete complementarity), they move into cytoplasmic foci. These foci were first observed in yeast and subsequently named processing bodies (PBs) (Kedersha and Anderson, 2007), where mRNPs colocalize with machinery for translation repression and mRNA decay (Parker and Sheth, 2007). Although the environment of PBs is highly dynamic, decapping machinery including Dcp1p/Dcp2p and activators of Dcp proteins have been found there along with, under certain stress conditions, nonsense-mediated decay pathway proteins (Parker and Sheth, 2007). PBs require mRNA to form, as shown by their disruption upon RNase treatment and the observation that overexpression of a nontranslating mRNA increases their size in yeast (Teixeira et al., 2005).

PBs are also called GW bodies because they contain the protein GW182, an RNA binding protein first observed in discrete foci in HeLa cells (Eystathioy et al., 2002). Knockdown of GW182 with siRNAs results in a loss of GW bodies/PBs, leading to the conclusion that GW182 is a necessary component of PBs, as well as a useful marker for immunofluorescent visualization in cells (Yang et al., 2004, Liu et al., 2005).

The role of microRNAs in muscle development

In addition to FXR1P being implicated in normal muscle development (Mientjes et al., 2004, Huot et al., 2005), miRNAs are also critically important for normal muscle development and function (Wienholds and Plasterk, 2005, Chen et al., 2006, Chen et al.,
Several miRNAs, miR-1, miR-133a and miR-206, are expressed specifically in muscle tissue where they promote myogenesis by targeting transcriptional repressors of muscle gene expression or enhance myoblast proliferation in order to inhibit myogenesis (Baskerville and Bartel, 2005, Chen and Lodish, 2005, Chen et al., 2006, Kim et al., 2006, Rao et al., 2006, Sweetman et al., 2008). Recently, it was shown that alterations in expression of miR-221 and miR-222 in myoblasts and myotubes disrupts the formation of sarcomeres in myotubes (Cardinali et al., 2009). Comprehensive miRNA expression-profiling studies revealed that 185 miRNAs were dysregulated in samples of diseased muscle tissue from ten different muscle disorders (Eisenberg et al., 2009). Specifically, miR-92, one of the candidate miRNAs we found to regulate FXR1P, was shown to be misregulated in Duchennes’ Muscular Dystrophy and in Nemaline Myopathy, a congenital myopathy (Eisenberg et al., 2009). Data showing that FXR1P is regulated by miRNAs will be further discussed in chapter 3.

**FMRP and processing bodies**

Whether FMRP localizes to PBs is controversial. One study reported that FMRP localizes to approximately 50% of granules containing the GW182 marker as shown by immunofluorescence in astrocytoma cells, suggesting that it is present in PBs (Moser et al., 2007). Further, dFmrp colocalizes with Dcp1p and other known PB proteins (Barbee et al., 2006). However, in a study using HeLa cells, the majority of FMRP was found not to colocalize with Dcp1p, but was instead found in stress granules (Didiot et al., 2008). Thus, whether FMRP is localized to PBs may depend on the cell system under study.
**FMRP association with the microRNA pathway**

Since FMRP has been found associated with miRNAs and Ago2 (Jin et al., 2004a, Jin et al., 2004b) and is a known translational regulator which can both activate (Brown et al., 2001, Bechara et al., 2009) and suppress translation (Laggerbauer et al., 2001, Li et al., 2001, Costa-Mattioli et al., 2009), it likely plays an important role in miRNA mediated translation regulation. However, although FMRP associates with components of the miRNA pathway, it is also controversial whether it plays an important or essential role in the function of the miRNA pathway itself. As described above, Didiot and colleagues reported that FMRP localizes separately from RISC machinery components and PBs (Didiot et al., 2008). Further, they showed that FMRP was not required for RISC function in transfection studies with reporter constructs bearing miRNA target sequences (Didiot et al., 2008). In contrast, Plante and colleagues showed that FMRP was required for efficient RNA silencing using reporter constructs expressed in murine fibroblast cells (Plante et al., 2006). They also demonstrated that FMRP directly associates with miRNAs, possibly through the hnRNP K homology domains, to aid assembly of Dicer processed miRNAs onto target mRNAs *in vitro* (Plante et al., 2006). Taking this a step further, Xu and colleagues showed that dFmrp was required for assembly of the Dicer-Ago complex, and in the absence of dFmrp, there were fewer complexes, which may explain the reduced levels of the miRNA 124a (miR-124a) (Xu et al., 2008). They concluded that dFmrp was required for normal neuronal miRNA levels during development, likely because it was necessary for assembly of Dicer with Ago (Xu et al., 2008). Differing conclusions about whether FMRP is required for normal function of the miRNA pathway may be due to the use of different cell systems or the evaluation of
different miRNAs which are developmentally timed and tissue specific (Bartel, 2004a). Regardless of FMRP’s role in RISC function, it is still possible that FMRP utilizes the miRNA pathway for translation regulation of its cargo mRNAs.

The fragile X family proteins and translation activation

Co-expression of the FXR proteins at synapses in patients with Fragile X syndrome demonstrates that the FXRPs cannot completely compensate for lack of FMRP (Weiler and Greenough, 1999, Hoogeveen et al., 2002), suggesting different functions for the paralogs. At the molecular level, recent data show that miRNAs bound to the 3’ untranslated region (3’UTR) of the TNFα gene in quiescent cells recruit both Ago2 and FXR1 proteins, resulting in upregulation of translation (Vasudevan and Steitz, 2007, Vasudevan et al., 2007). The transition between translation upregulation and repression by the AGO2/FXR1 containing miRNPs occurs based on cell cycle (Vasudevan et al., 2007).

Translation activation by fragile X family member FMRP has also been proposed when a subset of mRNA cargoes were found decreased on polysomes in the absence of FMRP (Brown et al., 2001), suggesting that FMRP was required for their translation. Further, a recent study found increased translation of known mRNA cargo (Sod1) through binding by the C terminus of FMRP, revealing the start codon, and leading to translation initiation (Bechara et al., 2009). Thus, fragile X family members FXR1P and FMRP have both been shown to activate translation of some mRNAs.
The role of fragile X proteins in the miRNA pathway

As described above, FMRP associates with many components of the miRNA pathway and localizes to PBs in some systems (Caudy et al., 2002, Ishizuka et al., 2002, Jin et al., 2004b, Barbee et al., 2006, Moser et al., 2007). Morphological data show that loss of FMRP in mouse or Drosophila causes defects in spine and synapse formation (Gao, 2007). Taken together, a role for FMRP in neuronal development mediated through the miRNA pathway seems likely. Phosphorylation of FMRP provides a rapid and reversible way to modulate FMRP’s association with the miRNA pathway. Since FMRP phosphorylation and miRNAs are both mechanisms for translation regulation of target mRNAs, one question addressed in my research and presented in chapter 2 is whether phosphorylation regulates association of FMRP with the miRNA pathway.

In addition, although there is much interest in determining the mechanism by which the fragile X family of RNA binding proteins regulate expression of target mRNAs, the regulation of these translation regulators is not well understood. Chapter 3 details the evidence on how the expression of FXR1P, but not FMRP, is regulated by miRNA binding in the FXR1 3’UTR.
CHAPTER 2

PHOSPHORYLATION OF FMRP INHIBITS ASSOCIATION WITH DICER

(Data presented in this chapter were originally published in *RNA*, Volume 15, Number 3, pages 362-366, 2009)

Summary

Fragile X syndrome is caused by an absence of the protein product of the fragile X mental retardation gene (FMR1). The fragile X mental retardation protein (FMRP) is an RNA binding protein that regulates translation of associated mRNAs, however, the mechanism for this regulation remains unknown. Constitutively phosphorylated FMRP (P-FMRP) is found associated with stalled untranslating polyribosomes and translation of at least one mRNA is downregulated when FMRP is phosphorylated. Based on our hypothesis that translational regulation by P-FMRP is accomplished through association with the microRNA (miRNA) pathway, we developed a phospho-specific antibody to P-FMRP and showed that P-FMRP associates with increased amounts of precursor miRNAs (pre-miRNA) compared to total FMRP. Furthermore, P-FMRP does not associate with Dicer or Dicer containing complexes in co-immunoprecipitation experiments or in an in vitro capture assay using a P-FMRP peptide sequence bound to agarose beads. These data show that Dicer containing complexes bind FMRP at amino acids 496-503 and that phosphorylation disrupts this association with a consequent increase in association with pre-miRNAs. In sum, we propose that in addition to regulating translation, phosphorylation of FMRP regulates its association with the miRNA pathway by modulating association with Dicer.
Introduction

Fragile X Syndrome is the most common form of inherited mental retardation and is caused by the absence of expression of the fragile X mental retardation protein, FMRP. FMRP is an RNA binding protein that binds a subset of mRNAs (Terracciano et al., 2005). FMRP is phosphorylated on three serines between its nuclear export sequence and its primary RNA binding domain, the RGG box, and is associated with stalled, untranslated polyribosomes when constitutively phosphorylated (Ceman et al., 2003). These data suggest that phosphorylated FMRP (P-FMRP) is involved in mRNA translational suppression, although the mechanism by which this occurs remains unclear.

MicroRNAs (miRNAs) are short RNA sequences (21-23 nucleotides) that bind and regulate mRNAs through imperfect base-pairing interactions with the 3’ untranslated region (3’UTR) of target transcripts (Lee et al., 1993, Bartel, 2004b, Perron and Provost, 2008). Although originally described as suppressing translation of all target mRNAs (Ambros & Lee, 2001; Nottrott et al., 2006), miRNAs have recently been shown to be present on actively translating polyribosomes and to activate translation of some target mRNAs (Maroney et al., 2006; Vasudevan & Steitz, 2007; Vasudevan et al., 2007). miRNAs are transcribed in the nucleus by RNA polymerase II and then processed into 80 nucleotide precursor segments by the Drosha/DGCR8 protein complex (Lee et al., 2003; Denli et al., 2004; Gregory et al., 2004; Han et al., 2004). These precursor miRNAs (pre-miRNA) are exported to the cytoplasm by exportin 5 (Yi et al., 2003), where they associate with the pre-miRNA processing complex (Bartel 2004, Jin 2004a) and are
processed by Dicer into shorter (21-23 nucleotide) double-stranded segments that can then be inserted into the RNA induced silencing complex (RISC) (Hammond et al., 2001; Bartel, 2004).

Dicer and other components of the miRNA pathway associate with the Drosophila ortholog of FMRP (Caudy et al., 2002; Ishizuka et al., 2002). Subsequent studies showed that mammalian FMRP also associated with miRNAs, as well as Argonaute 2 (AGO2) (Jin et al., 2004b), the protein necessary for RNAi pathway cleavage and silencing of mRNAs (Hutvagner & Simard, 2008). Recently, the autosomal paralog of FMRP, FXR1, was found to be required for translational activation of the miRNA complex regulating TNFα expression (Vasudevan and Steitz, 2007).

In addition to its RGG box, FMRP has two hnRNP K homology (KH) domains (Terracciano et al., 2005). In vitro, recombinant FMRP aids assembly of Dicer-processed miRNAs onto target mRNAs by acting as an acceptor protein through its KH2 domain (Plante et al., 2006). Further, FMRP is required for efficient RNAi in cells using luciferase reporters in gene silencing assays (Plante et al., 2006). Taken together, these data point toward a mechanism whereby FMRP regulates translation of mRNAs through a miRNA-mediated mechanism. The goal of this study was to investigate the relationship between FMRP and the miRNA pathway as a mechanism by which FMRP can regulate translation of its bound mRNAs.
Results and Discussion

We began this study by creating a phospho-specific antibody to FMRP (PSER) using the eight amino acid sequence that includes three serines identified as the sole phosphorylation sites in FMRP: serine (ser) 499 as the primary phosphorylation site and ser496 and ser503 as secondary sites (Fig. 5A) (Ceman et al., 2003). To determine whether the PSER antibody was specific to P-FMRP, western blots were performed on extracts from cells stably expressing FMRP (WT), an empty vector (VC), or an alanine substitution at serine 499 (S499A) variant of FMRP that cannot be phosphorylated (Fig. 5B) (Ceman et al., 2003; Narayanan et al., 2007). The PSER antibody did not detect unphosphorylated FMRP in the S499A FMRP cells but did detect P-FMRP in WT cells (Fig. 5B). A longer exposure revealed endogenous P-FMRP in the L-M(TK-) cells (data not shown), which express low levels of FMRP (Ceman et al., 1999). As further evidence that PSER is phospho-specific, phosphatase treatment of immunoprecipitated Flag-FMRP completely eliminated reactivity with the PSER antibody (Fig. 5C).

Together, these results indicate that the PSER antibody is specific for P-FMRP. Further evidence for the specificity of this reagent was also described using neuronal preparations (Narayanan et al., 2007). At the same time, we developed an antibody (NP) that detects the same 8 amino acid region as PSER (SNASETES) but that recognizes FMRP regardless of its phosphorylation state. Although FXR1 and FXR2 contain regions somewhat similar to FMRP, (SNPSETES) and (STASETES), respectively, the NP antibody is specific for FMRP and does not detect FMRP’s autosomal paralogs (Fig. 5D).
Figure 5. PSER is specific for phosphorylated FMRP. (a) Schematic representation of the FMR1 protein. NSL-nuclear localization sequence, NES-nuclear export sequence, phospho-serines 496, 499, and 503 [adapted from (Ceman et al., 2003)]. (b) Western blot of whole cell lysates from VC, WT and S499A FMRP expressing cells was immunoblotted (ib) with the indicated antibody: PSER, 1a (anti-FMRP 1a-1C3 (Devys et al., 1993)) or eIF5. (c) Anti-Flag immunoprecipitations (IP) of Flag-FMRP expressing L-M[TK-] cells were split and mock treated or phosphatase (P-tase) treated (Ceman et al., 2003) before probing with PSER of 1a (anti-FMRP). Ig-immunoprecipitating antibody alone. (d) Flag-FMRP expressing L-M[TK-] cells immunoprecipitated with NP
antibody and probed with 1a (anti-FMRP), FXR1 (Hoogeveen et al., 2002), or FXR2 (Siomi et al., 1995) antibodies. WCL-L-M[TK-] whole cell lysate. Ig-NP antibody alone.
Because P-FMRP has been shown to suppress translation of target mRNAs (Ceman et al., 2003; Narayanan et al., 2007), we hypothesized that the miRNA pathway might play a mechanistic role in the translational regulation of target mRNAs by P-FMRP. To determine if the phosphorylation state of FMRP changes its association with miRNAs, we used the NP antibody, which detects amino acids 496-503, irrespective of the phosphorylation state, and the PSER antibody (Fig. 5) to immunoprecipitate total FMRP and P-FMRP, respectively. Associated RNAs were then extracted and end-labeled with 32P cytidine bisphosphate (Fig. 6A). As shown in Figure 6B, both antibodies capture similar amounts of FMRP. Surprisingly, comparisons between these samples indicated a large amount of 80 nt RNAs associated with P-FMRP (Fig. 6A, top). The size of these RNAs corresponds to the size of precursor miRNAs that have been exported from the nucleus but have not yet been processed by Dicer into mature miRNAs (Jin et al., 2004b).
Figure 6. P-FMRP associates with increased amounts of precursor miRNAs. (a) 2 x 10^9 Flag-FMRP cells were immunoprecipitated with pre-immune serum (pre), NP or PSER. RNA was extracted and end-labeled with P^32 as described (Duan and Jin, 2006), resolved on a 15% acrylamide TBE/urea gel and visualized using autoradiography (8 hrs.). The longer exposure of the gel (16 hrs.-right) shows putative associated miRNAs. Decade marker system (Ambion) indicates RNA size in nucleotides. (b) Equivalent amounts of FMRP were immunoprecipitated by PSER and NP antibodies in (a).
Since P-FMRP associates with large amounts of a precursor miRNA size RNA species, we hypothesized that Dicer does not associate with P-FMRP and was therefore not present to process precursor miRNAs into mature miRNAs. To test this, we immunoprecipitated total FMRP and P-FMRP from HeLa cells, probed with a Dicer antibody that is specific for human Dicer and visualized the co-immunoprecipitated proteins by western analysis. As shown in Fig. 7A, Dicer is associated only with total FMRP and not with P-FMRP. To investigate whether the Dicer-FMRP association was mediated by RNA, FMRP was immunoprecipitated from WT cells and then treated with RNase (+) or not (-), and examined for association with Dicer (Fig. 7B). RNase treatment had no effect on the FMRP-Dicer association, suggesting a protein interaction between Dicer and FMRP.

To test whether Dicer, or a Dicer-containing complex, directly interacts with FMRP at its phosphorylation site, we compared the ability of Dicer to be captured by beads linked to the eight amino acid phospho-peptide sequence of FMRP (SNA[pS]ETES) or the corresponding unphosphorylated FMRP peptide sequence (SNASETES). In Fig. 7C, Dicer is only captured by the non-phosphorylated SNASETES peptide and not by the phosphorylated peptide sequence or a random peptide sequence (Abelson et al., 2005). Thus, Dicer association with FMRP requires unphosphorylated region 496-503. This result also rules out the possibility that PSER binding to FMRP blocks Dicer association.
Figure 7. P-FMRP is unable to associate with Dicer.  (a) Lysate from $2 \times 10^7$ HeLa cells was immunoprecipitated with NP or PSER antibodies and immunoblotted (ib) with Dicer antibody FMRP (1a) antibody.  (WCL)- 50 µg HeLa whole cell lysate.  IP-immunoprecipitations; Ig-immunoprecipitating antibody alone.  (b) Lysate from $10^9$ Flag-FMRP –expressing cells was immunoprecipitated with anti-FMRP antibody 7G1 (Brown et al., 2001), split and incubated with either RNase (+) of mock-treated (-) and immunoblotted (ib) with the Dicer antibody.  (WCL)- 50 µg whole cell lysate.  (c) Lysate from $10^7$ HeLa cells was incubated with matrix-coupled peptide
Figure 7. (cont.)

(NH2-SNA[pS]ETES-CONH2) (P-FMRP), (NH2-SNASETES-CONH2) (FMRP), or a random peptide (RP) (KETAAKFERQHMDS), resolved on a 6% SDS-PAGE gel and immunoblotted with Dicer antibody.
In summary, we originally set out to test the hypothesis that phosphorylation of FMRP inhibits translation of its mRNA cargoes by preferentially associating with miRNAs. In fact, we found that P-FMRP associates with an increased amount of precursor miRNAs, likely due to its inability to bind Dicer. We then demonstrated that Dicer binds unphosphorylated FMRP on residues 496-503. Thus, we propose a new role for phosphorylation in the regulation of FMRP function where phosphorylation inhibits FMRP association with Dicer, a key enzyme in the generation of miRNAs. As a consequence of reduced association with Dicer, we predict a paucity of miRNAs available for association with the mRNAs bound by P-FMRP. If miRNAs are required for translation activation as recently described (Maroney et al., 2006; Vasudevan & Steitz, 2007; Vasudevan et al., 2007), then phosphorylation would suppress translation indirectly by decreasing miRNA production through loss of Dicer binding. Further study is required to investigate whether there is translation activation of mRNAs by miRNAs associated with non-phosphorylated FMRP.

We conclude that Dicer-FMRP association requires FMRP’s unphosphorylated region 496-503 and that phosphorylation of FMRP abolishes this interaction. This is a new role for phosphorylation in regulating FMRP function by modulating association with Dicer and the miRNA pathway. If miRNAs are required for translation activation (Vasudevan and Steitz, 2007, Vasudevan et al., 2007) and FMRP is involved in translation activation (Bechara et al., 2009), phosphorylation of FMRP would indirectly suppress translation by decreasing miRNA production through loss of Dicer binding (Fig. 8).
Figure 8. Potential regulatory role of P-FMRP in translation regulation

Unphosphorylated FMRP associates with Dicer (top left). The Dicer containing complex then processes pre-miRNAs into mature, double-stranded duplex miRNAs (bottom left). After RISC separates the duplex strands, the single-stranded mature miRNA binds FMRP’s target mRNA to induce translation. Conversely, when FMRP is phosphorylated, Dicer cannot bind and pre-miRNAs are not processed into mature miRNAs (top right). Without activating miRNAs, translation of the target mRNA cannot occur and the result is indirect suppression of translation by FMRP due to loss of Dicer binding.
Material and Methods

Cell lines and antibodies. Stably transfected L-M(TK-) cells were created and maintained as described (Ceman et al., 2003). Non-adherent HeLa cells were maintained in Joklik + HEPES + 5% NBCS (HyClone). The rabbit anti-phospho-FMRP specific antibody (phospho-serine or PSER) was raised against the phospho-FMRP peptide NH2-CNSEA(pS)Na(pS)ETE(pS)DHRDE) (Abgent). The rabbit anti-FMRP antibody (NP) was obtained at the same time. Anti-FMRP antibody IAC-1C3, hereafter referred to as monoclonal antibody 1a (Devys et al., 1993) was generously provided by Dr. Jean-Louis Mandel and was used in immunoblots where indicated. The anti-Flag coupled agarose matrix (Sigma) was used to immunoprecipitate Flag-tagged FMRP in the phospho-specific antibody characterization experiments. Anti-eukaryotic initiation factor 5 (eIF5) (Santa Cruz) was used as a loading control. Mouse anti-Dicer (Abcam) was used to detect the Dicer enzyme in co-immunoprecipitation. Rabbit anti-Dicer (Santa Cruz) was used to detect Dicer in the capture assay. Antibody reactivity was visualized using either an anti-mouse HRP conjugate (Jackson Immunoresearch) or an anti-rabbit HRP conjugate (GE Healthcare) and developed with ECL (GE Healthcare).

P-FMRP and NP antibody characterizations. Whole cell extracts of L-M(TK-) cells stably expressing WT, S499A, or vector control (VC) were resolved by 7.5% SDS-PAGE, transferred to Hybond-P PVDF (GE Healthcare) and probed with 1/454 dilution of PSER, 1/10 of 1a hybridoma supernatant or 1/10,000 of anti-eIF5. 107 Flag-FMRP expressing L-M(TK-) cells were lysed in 1ml of lysis buffer (50mM Tris pH 7.6, 300 mM NaCl, 30mM EDTA, 0.5% triton X-100) and immunoprecipitated with the anti-Flag
antibody for 2 hours. After two washes, the immunoprecipitate (IP) was split and either mock-treated or treated with 7.5 ul of shrimp alkaline phosphatase (US Biochemical) for 30 minutes at 37°C (Ceman, et al. 2003). The IPs were resolved by SDS-PAGE and probed with the phospho-FMRP antibody (PSER), as described above. L-M[TK-] cells were lysed and immunoprecipitated as described above except NP antibody was used, resolved by SDS-PAGE, and then probed with either anti-FMRP (1a), FXR1, or FXR2 as described above.

**Precursor miRNA isolation and detection.** 2 x 10^9 Flag-FMRP expressing L-M(TK-) cells were lysed in 40 mls of lysis buffer (10mM HEPES, 150mM NaCl, 30mM EDTA, 0.5% triton X-100) and 500ul was removed for a parallel immunoprecipitation. Lysates were immunoprecipitated with 40 ug NP (total FMRP), 2 ug PSER (phospho-FMRP) antibodies coupled to protein A sepharose (PAS) (Roche) or 100 ul pre-immune serum coupled to PAS. Total RNA was extracted by phenol chloroform/ethanol precipitation of the immunoprecipitations. The RNA was end-labeled with P32 cytidine bis-phosphate (Perkin Elmer), and resolved on a 15% TBE/urea gel as described (Duan & Jin, 2006). The decade size marker system (Ambion) was labeled with P32-CTP and used to size the resulting RNA. Parallel immunoprecipitations were probed for FMRP (1a).

**Dicer immunoprecipitation.** 2 x 10^7 HeLa cells were lysed in lysis buffer (10mM HEPES, 150mM NaCl, 30mM EDTA, 0.5% triton X-100) and 50ug of protein was removed for a whole cell lysate. The extract was then split and immunoprecipitated with
either an NP antibody (total FMRP) or PSER (phospho-FMRP) for 2 hours, washed and resolved on a 6% SDS-PAGE gel. After transfer, blots were probed with antibodies for Dicer and FMRP as described above.

**RNase treatment.** 10^9 WT Flag-FMRP expressing cells were lysed (see above) and 50ug protein removed for a whole cell lysate control. The extract was immunoprecipitated with 7G1 FMRP antibody for 2 hours. After washing, the extract was split and half treated with RNase (Sigma) for 20 minutes at 37°C and resolved on a 6% SDS-PAGE gel. Blot was probed with Dicer antibody as described.

**Capture assay.** 4 x 10^7 HeLa cells were lysed (10mM HEPES, 150mM NaCl, 30mM EDTA, 0.5% triton X-100) and the post-nuclear, cytoplasmic supernatant was incubated at 4°C for 2 hours with 10 ug matrix-coupled non-phosphorylated FMRP peptide sequence (NH2-SNASETES-CONH2) (BioSource), phosphorylated FMRP peptide sequence (NH2-SNA[pS]ETES-CONH2) (BioSource), or a random peptide sequence. After capture, the beads were washed 3 times for 10 minutes with one additional 10 minute wash in 0.3M lysis buffer. The protein was resolved on a 6% SDS-PAGE gel and transferred to PVDF for probing with the Dicer antibody.
CHAPTER 3

FRAGILE X PROTEIN FAMILY MEMBER FXR1P IS REGULATED BY MICRONNAS

(Manuscript submitted for publication, RNA, November, 2009)

Summary

FXR1P is one of two autosomal paralogs of the fragile X mental retardation protein FMRP. Absence of FMRP causes fragile X syndrome, the leading cause of hereditary mental retardation. FXR1P plays an important role in normal muscle development and has been implicated in fascioscapulohumeral muscular dystrophy (FSHD). Its absence also causes cardiac abnormalities in both mice and zebrafish. To examine miRNA-mediated regulation of FMRP and FXR1P, we studied their expression in a conditional Dicer knockdown cell line, DT40. We found that FXR1P, but not FMRP, increased upon Dicer knockdown and the consequent reduction of miRNAs, suggesting that FXR1P is regulated by miRNAs while FMRP is not. Expression of a luciferase reporter bearing the FXR1 3’UTR was significantly increased in the absence of miRNAs, confirming miRNA-mediated regulation of FXR1P. We identified one of the regulatory regions by removing an 8-nucleotide miRNA seed sequence common to miRNAs 25, 32, 92, 363 and 367 in the 3’UTR of FXR1. Accordingly, overexpression of a miRNA mimic containing this common seed sequence decreased endogenous FXR1P expression in HEK293 and HeLa cell lines. We report for the first time that FXR1P is regulated through miRNA binding to the miR-25/32/92/363/367 seed sequence binding site.
Introduction

FXR1P is one member of a small family of three RNA binding proteins that include the fragile X mental retardation protein (FMRP) and FXR2P (Siomi et al., 1995, Zhang et al., 1995). Although FXR1P is found throughout the body, it is highly expressed in muscle and heart tissue where FMRP and FXR2P are mostly absent (Devys et al., 1993, Coy et al., 1995, Khandjian et al., 1995). FXR1P has seven isoforms (Kirkpatrick et al., 2001, Mientjes et al., 2004) including one cardiac-specific isoform (Khandjian et al., 1998) and three muscle-specific isoforms (Mientjes et al., 2004). FXR1P expression is essential for postnatal viability; inactivation of FXR1P in mice leads to impaired myogenesis resulting in death of neonates shortly after birth, most likely due to cardiac or respiratory failure (Mientjes et al., 2004). Histochemical analyses of both skeletal and cardiac muscles showed a disruption of cellular architecture and overall structure in the FXR1 knockout mice compared to WT littermates (Mientjes et al., 2004). In addition, reduction of FXR1P was shown to disrupt MyoD expression and somite formation in Xenopus, while re-introduction of long and short FXR1 mRNA variants rescued these muscle-specific effects (Huot et al., 2005). In zebrafish, abnormalities in striated muscle and severe cardiomyopathy resulting in heart failure were observed in embryos after knockdown of FXR1 with a morpholino (Padje et al., 2009). Lastly, altered expression of muscle specific isoforms of FXR1P have been implicated in facioscapulohumeral muscular dystrophy (FSHD) because patients have abnormal expression patterns of three different FXR1P isoforms in myoblasts and myotubes (Davidovic et al., 2008). Collectively, these studies underscore the important role of FXR1P in normal muscle development.
MicroRNAs (miRNAs) are a class of small, genomically encoded RNAs that are ~22 nucleotides in size and regulate translation by base pairing with sequences in the 3’UTR of target mRNA sequences (Bartel, 2004a, Bartel, 2009). If base pairing is perfect along the ~22 nucleotide length, the result is mRNA target degradation (Bartel, 2004a, Bartel, 2009). In contrast, if base-pairing occurs in the “seed” region at the 5’ end of the miRNA but is imperfect to the RNA, leading to a bulge in the duplex, the result is translational silencing (Bartel, 2004a, Bartel, 2009, Jackson et al., 2009). miRNAs are estimated to regulate expression of greater than 1/3 of all expressed genes (Lewis et al., 2005, Nilsen, 2007) but only a fraction of miRNAs have experimentally validated mRNA targets. Although the exact mechanism of how the mature miRNA finds its target mRNA is unclear, miRNAs function as part of larger complexes such as RNA induced silencing complex (RISC) and the miRNA ribonucleoprotein (miRNP) complex, suggesting that miRNAs guide associated proteins to target mRNAs to effect degradation, repression, or in some cases, translation activation (Fillipowicz et al., 2005, Vasudevan and Steitz, 2007, Vasudevan et al., 2007, Cheever and Ceman, 2009a). Once RISC separates the duplexed strands to produce a mature miRNA (Bartel, 2004a), the guide miRNA associates with Argonaute 2 (Ago2), the only human Argonaute family protein with endonuclease activity (Fillipowicz et al., 2005, Hock and Meister, 2008). Incomplete complementarity of the miRNA to its target mRNA leads to translational repression, while complete complementarity triggers Ago2 endonuclease activity, leading to cleavage of the target mRNA, as observed during RNA silencing.
A number of miRNA target prediction programs are available to identify possible miRNA regulatory sites (Doran and Strauss, 2007). Once a miRNA has been bioinformatically identified as a potential gene regulator, *in vitro* experiments using a luciferase reporter bearing the target sequence are used to determine whether the miRNA binds the 3’UTR of the target mRNA (Hurteau et al., 2007, Scott et al., 2007). To address miRNA regulation of endogenous protein expression, researchers have overexpressed the candidate miRNA in the form of synthetic mimics or precursor miRNAs (pre-miRNAs) to examine regulation (Hurteau et al., 2007, Scott et al., 2007). These approaches have been used to identify the mRNA targets of miRNAs such as miR-1, miR133, and miR-206 which are highly expressed in cardiac and skeletal muscle, and has led to insights into normal vertebrate cardiac and skeletal muscle development and function (Chen et al., 2006, Chen et al., 2008, Chen et al., 2009).

Members of the fragile X family of proteins bind target mRNAs to regulate their translation (Terracciano et al., 2005); however, the molecular mechanisms underlying how FMRP or FXR1P regulate the translation of their mRNA cargoes remains unclear. Studies have shown that mammalian FMRP and FXR1P associate with Dicer, miRNAs, Ago2 and other miRNA pathway components (Bartel, 2004a, Jin et al., 2004b), suggesting that they utilize the miRNA pathway to regulate target mRNAs. Recently, phosphorylation of FMRP was demonstrated to abolish association with Dicer, suggesting that phosphorylation regulates interaction of FMRP with the miRNA pathway (Cheever and Ceman, 2009a). FXR1P was implicated in translation regulation when it was shown to be recruited with Ago2 by miRNAs bound to the 3’ untranslated region.
(3’UTR) of TNFα mRNA in quiescent cells. The result is upregulation of translation, suggesting that FXR1P plays a role in translation activation (Vasudevan and Steitz, 2007, Vasudevan et al., 2007).

Although there is much interest in determining the mechanism by which the fragile X family of RNA binding proteins regulate expression of target mRNAs, the regulation of these translation regulators is not well understood. We examined the expression of fragile X family members in a conditional Dicer knockdown cell line and found that FXR1P expression but not FMRP expression is regulated by miRNAs. Further, we identified an 8-nucleotide seed sequence in the 3’UTR of FXR1 that is predicted to bind five different miRNAs and found that one of the miRNAs, miR-367, suppresses expression of FXR1P in HEK-293T and HeLa cell lines. We provide the first evidence that FXR1P is regulated by miRNAs.

Results

Loss of Dicer expression leads to an increase in overall translation.

To examine the role of miRNAs in the regulation of the fragile X family of proteins, we obtained a conditional loss-of-function Dicer cell line created in chicken B cell lymphoma DT40 cells (Fukagawa et al., 2004). Conditional knockdown of Dicer was achieved by addition of tetracycline to the DT40 growth media every 24 hours for 96 hours as previously described (Fukagawa et al., 2004), at which time a significant loss of Dicer expression was observed (Fig. 9A). miRNAs are regulators of translation, primarily acting to suppress translation at the step of initiation (Meister, 2007). To better
understand the effects of Dicer and subsequent miRNA knockdown on translation, we examined polyribosome (polysome) profiles in the presence (-tet) and absence (+tet) of Dicer. Polysomes are mRNAs with one, two, three, four, etc., ribosomes associated with them, reflecting the translational state of the cell, and are visualized by UV absorption of fractionated lysates at 254 nm (Ruan et al., 1997). Examples of polysome profiles from mock treated DT40 cells and cells treated with tetracycline are shown in Fig. 9B. In the presence of Dicer (-tet), none of the polysome peaks were as high as peak 1, which is typical (Khandjian et al., 1996, Feng et al., 1997, Ruan et al., 1997). In contrast, in the absence of Dicer (+tet) and therefore, mature miRNA production, we reproducibly observed an increase in the amount of small polysomes (compare the height of peaks 3-5 to 1), suggesting that there was an increase in translation initiation in the absence of miRNAs (Fig. 9B). When we measured new protein synthesis in similarly treated cells by [3H]-methionine incorporation, we found that the absence of Dicer (+tet) led to an increase in the amount of labeled proteins (40% as measured by TCA incorporation [data not shown] and Fig. 9C), indicating increased protein synthesis in the absence of miRNAs.
Figure 9. Loss of Dicer expression leads to a global increase in translation. (A) DT40 cells were grown in the absence (-) or presence (+) of tetracycline (tet) for 96 hours. Cell extracts were made and 80 µg were examined by immunoblot for the proteins indicated on the right. In the bottom panel, antibody 1C3 (left) was used to detect FXR1P and FMRP. (B) DT40 cells were harvested after 96 hours of growth in the absence (-tet) or presence (+tet) of tetracycline. Polysomes were obtained by protein fractionation through a sucrose gradient and measured at an absorption of 254 nm. (1) indicates mRNAs with one ribosome, (2) indicates mRNAs with two ribosomes, etc. (C) [3H]methyl-methionine, pulse-labeled protein extracts from DT40 cells grown for 96 hours in the absence (-) or presence (+) of tetracycline were resolved on a 4-20% SDS-PAGE gel. Molecular weight markers are shown to the left (kDa).
FXR1P but not FMRP is increased in the absence of Dicer.

Proteins whose syntheses are increased in the absence of Dicer are potential candidates for miRNA-mediated regulation. Thus, we chose to determine whether expression of FMRP and/or FXR1P was increased in the absence of Dicer expression. To explore the regulation of FXR1P and FMRP, we first probed cell extracts for FMRP and FXR1P using monoclonal antibody 1C3 (Devys et al., 1993), which reacts with both proteins in mammalian cells (Mazroui et al., 2003). By immunoblot, we found a large visible increase in the amount of the top band, but very little change in the bottom band (Fig. 1A, bottom panel). Since DT40 is a chicken B cell line, we suspected that FXR1P was the top band and FMRP was the bottom band because both chicken and zebra finch FMRP are missing exons 11 and 12 (Price et al., 1996, Winograd et al., 2008); we therefore predicted that FMRP migrated faster in the gel. To verify the identity of the proteins visualized by antibody 1C3 (Devys et al., 1993) in the DT40 cell line, we immunoprecipitated DT40 cellular extracts with an antibody specific to FXR1P and showed by 1C3 immunoblot that FXR1P was the top band (Fig. 10A). Similarly, immunoprecipitation with two specific FMRP antibodies (Abcam) followed by 1C3 immunoblot revealed that FMRP was the bottom band (Fig. 10B). Thus, the loss of Dicer and miRNA production led to an increase in FXR1P expression but no change in FMRP expression.
Figure 10. FXR1P has a larger size than FMRP in DT40 cells. (A) DT40 cells were grown for 96 hours in the absence (-) or presence (+) of tetracycline, immunoprecipitated with an anti-FXR1P specific antibody (Jin et al., 2004b) or an irrelevant antibody and immunoblotted with antibody 1C3 (Devys et al., 1993). WCL-whole cell lysate. (B) Immunoprecipitation with two independent FMRP specific antibodies (Abcam) and subsequent immunoblot with antibody 1C3 (Devys et al., 1993). Ig-(immunoglobulin) denotes immunoprecipitating antibody alone (B-above blot) or reactivity of HRP detecting antibody to Ig chains (B-right).
We repeated this experiment a number of times and consistently found an increase in FXR1P expression when Dicer was knocked down in DT40 cells (Fig. 11A). On average, FXR1P showed a 4-fold increase (p-value=0.009) in the absence of Dicer, while FMRP showed no significant increase in expression (Fig. 11B). To definitively show that translation of FXR1P was increased in the absence of Dicer and suppressive miRNAs, we labeled cells with [³H]-methionine to visualize newly synthesized FXR1P and FMRP. In the absence of miRNAs, there was more FXR1P synthesized compared to FMRP, whose translation levels remained the same (Fig. 11C). Thus, we provide the first evidence for regulation of FXR1P, likely through the miRNA pathway.
Figure 11. FXR1P but not FMRP is increased in the absence of Dicer. (A) DT40 cells were harvested after 96 hours in the absence or presence of tetracycline (-tet, + tet, respectively) and probed with 1C3 antibody (3). Positions of FXR1P and FMRP are indicated to the right. (B) Quantification of fold-change of FXR1P and FMRP in the presence and absence of tetracycline as indicated. Significance was determined by
Figure 11. (cont.)

Students’s t-test. (C) Immunoprecipitation of DT40 after 96 hours of -/+ tetracycline followed by [³H] methyl-methionine pulse-label. Immunoprecipitating antibodies are indicated above the autoradiograms.
The absence of Dicer has no effect on FXR1 mRNA levels.

miRNAs regulate protein expression by either suppressing translation and/or increasing mRNA degradation (Bartel, 2004a, Nilsen, 2007). To assess FXR1 mRNA levels in the absence of miRNAs, we performed quantitative reverse-transcription PCR on total RNA isolated from DT40 cells that were treated with tetracycline (+tet) or mock treated (-tet) (Fig. 12). ΔCt values were calculated and averaged using triplicate samples from each of three independent trials. FMR1 mRNA levels were used as the internal control for normalization. We found no significant difference in FXR1 mRNA levels in Dicer knockdown cells (Fig. 12). The fact that FXR1 mRNA levels do not significantly change in the absence of Dicer also rules out the possibility that there was an increase in FXR1 transcription due to miRNA-mediated heterochromatin changes. We conclude that the increase in FXR1P expression in the absence of Dicer is not due to increased mRNA levels or transcription, but rather to a release of miRNA-mediated translation suppression.
Figure 12. *FXR1* mRNA levels do not significantly change in the absence of Dicer.

DT40 cells were grown for 96 hours in the presence or absence of tetracycline (-tet, +tet, respectively) and total RNA was extracted and reverse transcribed. Quantitative real-time PCR using triplicate ΔCt values from three independent trials was used to calculate the $2^{-\Delta C_t}$ and normalized to *FMR1* mRNA levels. Lack of significance was assessed by Z-test.
The 3’UTR of FXR1 contains putative miRNA regulatory sites.

To identify potential miRNA regulatory sites in the 3’UTR of FXR1, we used Targetscan 5.1 to predict miRNA binding sites in the 3’UTR (Fig. 13A). Each box represents the seed sequence of a single miRNA or the seed sequence shared by a group of miRNAs. Since functional miRNA regulatory sites have often been found near either the stop codon or the polyadenylation sequence of mRNA (Majoros and Ohler, 2007), we chose to focus on the shared seed sequence of miRNAs 25, 32, 92, 363, and 367 located near the polyadenylation sequence of the FXR1 3’UTR (Fig. 13A, circled miRNAs).

Candidate miRNAs miR-92 and miR-363 are expressed in DT40 cells.

miRNAs exhibit variable expression in cell lines, thus, we examined expression of the candidate miRNAs in DT40 cells. We isolated total RNA from DT40 cells treated with tetracycline (+tet) and mock treated (-tet) DT40 and probed with [32P]-labeled antisense probes to candidate miRNAs (Fig. 13B and 13C). miR-92 and miR-363 were expressed at detectable levels in DT40 cells and were present as pre-miRNAs in the absence of Dicer (+ lanes), as expected. In contrast, we were unable to detect candidate miRNAs 32 or 367 (data not shown) indicating they were either not expressed in DT40 cells or were below the detection limit of northern blotting. miR-25 was not tested. Additionally, miRNAs 30a, 30a-5p, 31, 301, 199b, 200b, 429, and 124a (used as a negative control) were not detected in DT40 cells (data not shown). Thus, bioinformatically-identified miRNAs that bind seed sequences in FXR1’s 3’UTR are present in DT40 cells and are candidates for regulation of FXR1P expression.
Figure 13. miR-92 and miR-363 are expressed in DT40 cells and have putative seed sequence regulatory sites in the \textit{FXR1} 3'UTR. (A) Schematic of the predicted location of all miRNA seed sequence binding sites (miRNAs labeled and seeds represented as boxes) in the \textit{FXR1} 3'UTR based on TargetScan 5.1. Bold boxes represent highly conserved sites. Location of the 8 nucleotide seed sequence common to miRNAs-25/32/92/363/367 is shown on the right and is circled. miR-7 was predicted by TargetScan 5.1 but was not included in the \textit{FXR1} 3'UTR subcloned luciferase constructs. (B) Total RNA from DT40 cells treated with tetracycline for 96 hours (+) or mock treated (-) was extracted. 50 µg of total RNA was resolved on a 15% acrylamide
Figure 13. (cont.)

gel and probed with \[^{32}\text{P}]\text{-labeled antisense oligos to miR-92 and miR-363, as indicated on the top. Size in nucleotides is indicated to the left and positions of pre-miRNAs (upper band) and mature miRNAs (lower band) are indicated to the right.}
The 3’UTR of FXR1 confers miRNA-mediated regulation on a reporter construct.

To test our hypothesis that miRNAs regulate expression of FXR1P through its 3’UTR, we subcloned the FXRI 3’UTR downstream of the luciferase open reading frame in the pGL3 expression vector. We expressed it in DT40 cells treated with tetracycline (+) or mock treated (-) cells and quantified luciferase expression. Introduction of the 3’UTR of FXR1 increased the amount of luciferase expressed compared to the empty vector alone (pGL3) (Fig. 14A). Importantly, we found that in the absence of Dicer (+) and consequently miRNAs, luciferase expression driven by the FXRI 3’UTR was significantly increased compared to its expression in the presence of Dicer (-) (Fig. 14A, compare +FXR1 to -FXR1). (p-values<0.007). Thus, the 3’UTR of FXRI confers miRNA-mediated regulation on the luciferase reporter.

To determine if the miRNA-mediated suppression of luciferase expression occurred through the 8-nucleotide seed sequence circled in Figure 13A, we removed this sequence and examined whether there was an effect on luciferase expression. Deletion of the site increased luciferase expression in the presence of miRNAs compared to expression of the wild-type 3’UTR construct (Fig. 14A, compare −tet/+Dicer SDM to −tet/+Dicer FXR1), but not as much as global miRNA reduction (Fig. 14A, compare −tet/+Dicer SDM and +tet/-Dicer FXR1). Thus, removal of the miRNA binding site significantly abrogated some but not all of the miRNA-mediated suppression. Further, simultaneous removal of the 8-nucleotide seed sequence and reduction of miRNAs significantly increased luciferase expression (+tet/-Dicer SDM), suggesting that the 8-nucleotide seed sequence regulates the 3’UTR of FXR1 through miRNA binding. As a control, we subcloned the
*FMR1* 3’UTR into the pGL3 expression vector and assessed luciferase expression in the presence and absence of Dicer (Fig. 14B). We found that the absence of Dicer and mature miRNAs did not significantly increase expression of the luciferase construct (Fig. 14B, compare –tet/+Dicer FMR1 to +tet/-Dicer FMR1). Thus, the *FXR1* 3’UTR and, specifically, the 8 nucleotide seed sequence shared by miRNAs 25, 32, 92, 363, and 367 confer miRNA-mediated regulation to the luciferase reporter constructs, while the *FMR1* 3’UTR does not. (p-values<0.007).
Figure 14. miR-25/32/92/363/367 seed sequence binding site in the 3’UTR of *FXR1* confers miRNA-mediated regulation on luciferase constructs. A. pGL3 luciferase reporter vector alone (pGL3), containing the 3’UTR of *FXR1* (FXR1) or containing the *FXR1* 3’UTR in which the seed sequence common to miRNAs 25/32/92/363/367 was deleted (SDM) were electroporated into DT40 cells after 72 hours growth in the presence or absence of tetracycline.
Figure 14. (cont.)

24 hours after electroporation, (for 96 total hours in tetracycline), luciferase expression was assessed in DT40 cells grown in the presence (+) or absence (-) of tetracycline (tet). Transfection efficiency was monitored by Renilla expression and all luciferase values were normalized to empty vector (pGL3) in the absence of tetracycline. Values are depicted as expression fold change compared to empty vector. Significance was determined by Student’s t-test ** p-values<0.007 B. pGL3 vector containing the FMR1 3’UTR was electroporated into DT40 cells under the same conditions as described above. No significant difference in luciferase expression was observed in the presence (-) or absence (+) of miRNAs by student’s t-test.
To verify miRNA-mediated regulation of FXR1P in a cell line other than DT40, we overexpressed one of the miRNAs in HeLa and HEK-293T cells (Fig. 15). Since the miR-25, 32, 92, 363, 367-group shares a common seed sequence, we predicted that overexpressing any one of them would repress FXR1P expression. We chose miR-367 because we found it expressed in DT40 cells (Fig. 13B). HeLa and HEK-293T cells were transfected with a double-stranded miR-367 RNA mimic and harvested after 72 or 96 hours (Fig. 15). After assessing FXR1P expression, quantification showed a ~60% reduction (p-value<0.007) in FXR1P expression levels compared to control in HeLa cells (Fig. 15A). In HEK-293T cells, there was an average reduction (p-value<0.05) of greater than 60% compared to the control (Fig. 15B). Transfection of an irrelevant siRNAs did not affect FXR1P expression in either cell line (data not shown). Thus, expression of a miRNA specific to the seed sequence common to miR-25, 32, 92, 363, and 367 is sufficient to significantly down-regulate FXR1P expression in two human cell lines.
**Figure 15. Overexpression of miR-367 reduces FXR1P expression.** HeLa (A) and HEK293 (B) cells were transfected with a miR-367 mimic. 72 or 96 hours post transfection, mimic transfected cells (miR-367) or mock transfected (Cntrl.) were harvested and probed for FXR1P and eIF5, as indicated to the right in top panels (A and B). Densitometry was performed using Image J. FXR1P expression was normalized to the eIF5 loading control before quantifying FXR1P expression on three independent experiments. Significance was calculated by Student’s t-test, HeLa ** p<0.007, HEK-293 * p<0.05.
Discussion

In the present study, we investigated miRNA-mediated regulation of FXR1P and FMRP by utilizing a conditional Dicer knockdown cell line DT40. Reduced Dicer expression was used to effect a global reduction in mature miRNAs due to a lack of Dicer processing. In the absence of miRNAs, we found a four-fold increase in FXR1P, without influencing FMRP expression (Fig. 12). Regulation of FXR1P expression was further characterized by showing that miRNA-mediated regulation could be conferred on a luciferase reporter bearing the 3’UTR of FXRI (Fig. 14). We then showed that removal of an 8-nucleotide seed sequence that is conserved in mammalian and chicken orthologs reduced miRNA-mediated regulation (Fig. 14). Finally, overexpression of miR-367 in two human cell lines resulted in a greater than 50% reduction in FXR1P expression (Fig. 15). Collectively, our data indicate that this site in the 3’UTR is important for regulation of FXR1P expression in multiple cell lines.

FXRI, named ‘FMR-cross-reacting relative’, was first identified by screening a *Xenopus laevis* cDNA library with the human *FMR1* gene (Siomi et al., 1995). *FXRI* has 86% amino acid sequence identity to *FMR1* in the region containing the KH domains and is very similar to *FMR1* over the amino-terminal domain (70% identity). In contrast to *FMR1*, *FXRI* orthologs have highly conserved 3’UTRs (>90%), suggesting an important regulatory function (Siomi et al., 1995). Our data suggest that the 3’UTR of FXR1 is translationally regulated. Further, a recent study showed that the *FXRI* mRNA, but not the *FMR1* mRNA, is found associated with Ago2 in HEK293T cells, suggesting that the *FXRI* mRNA is regulated by miRNAs (Hendrickson et al., 2008). In a separate
study, also with the goal of identifying mRNAs regulated by miRNAs, high-throughput sequencing of mRNAs crosslinked to Ago2 failed to detect the \textit{FMRI} mRNA (\textit{FXR1} was not tested) (Chi et al., 2009). Finally, even though many miRNA binding sites are predicted in the \textit{FMRI} 3’UTR, none of the miRNAs tested in that study bound the 3’UTR, indicating a lack of functional miRNA binding (Chi et al., 2009). Taken together, these findings support our data showing that expression of FXR1P, but not FMRP, is regulated by miRNAs. However, we cannot rule out the possibility that FMRP could be regulated by miRNAs in specific cell types, like neurons.

Although we mapped a regulatory region of the 3’UTR of \textit{FXR1} to the miR-25, 32, 92, 363, 367 seed sequence, other miRNA binding sites may also play a role in regulating FXR1P expression. Removal of the aforementioned seed sequence did not completely abolish miRNA-mediated regulation of the reporter construct and an even greater effect on expression was seen upon global miRNA reduction, suggesting that other miRNAs might regulate expression of FXR1P. Recent data has shown that co-expressed miRNAs influence down-regulation of target genes more than individually expressed miRNAs (Ivanovska and Cleary, 2008). Interestingly, miR-92a and miR-363 are co-expressed on the X chromosome and both were detected in DT40 cells, suggesting that both miRNAs are candidates for regulating FXR1P expression.

miRNAs have been shown to regulate key transcription factors such as MYC, E2Fs and MYB (Xiao et al., 2007, Lal et al., 2009). However, there is very little evidence for miRNA-mediated regulation of RNA binding proteins. HuR is a sequence-specific RNA
binding protein that regulates translation and RNA turnover; which in turn influences the cellular response to stress, proliferative signals, immune triggers and developmental cues (Gorospe, 2003, Lopez de Silanes et al., 2005, Cherry et al., 2006, Abdelmohsen et al., 2007). HuR was recently shown to be regulated by miR-519 (Abdelmohsen et al., 2008). We now add FXR1P as an additional example of one postranscriptional regulatory factor (RNA binding protein) being regulated by another type of postranscriptional regulator (miRNAs).

FXR1P is implicated in normal muscle development and function (Mientjes et al., 2004, Huot et al., 2005). miRNAs are also critically important for normal muscle development and function (Wienholds and Plasterk, 2005, Chen et al., 2006, Chen et al., 2009). In fact, comprehensive miRNA expression-profiling studies revealed that a total of 185 miRNAs were dysregulated in samples of diseased muscle tissue from 10 different muscle disorders (Eisenberg et al., 2009). miR-92, a candidate miRNA for FXR1P regulation, was found to be misregulated in Duchenne’s Muscular Dystrophy and in Nemaline Myopathy, a congenital myopathy that is the most common nondystrophic congenital myopathy (Eisenberg et al., 2009). Thus, it is formally possible that FXR1P, and consequently its target mRNAs, are misregulated in muscle disease states as a result of misregulated miRNA expression. Understanding how FXR1P is regulated by miRNAs will give insight into normal muscle development and function.
Material and Methods

Cell lines and antibodies

The DT40 cell line was maintained in Dulbecco’s modified medium (Sigma D5796) supplemented with 10% tetracycline-free fetal calf serum (Clontech), 1% chicken serum, 1% penicillin/streptomycin (Gibco), and 10 µM β-mercaptoethanol (Fisher) (Buerstedde and Takeda, 1991). Cell suspension concentration was maintained between $10^5$ and $10^6$ per ml. HeLa and HEK293 cell lines were maintained in Dulbecco’s modified medium supplemented with 10% fetal calf serum, 1% penicillin/streptomycin and 1% non-essential amino acids. Anti-FMRP antibody IAC-1C3, hereafter referred to as monoclonal antibody 1C3 (Devys et al., 1993) was used to detect both FMRP and FXR1P in immunoblots where indicated at a 1/10 dilution of the hybridoma supernatant. Anti-FXR1P 2107 was used to detect FXR1P in HeLa and HEK-293T cells by immunoblot as described (Bakker et al., 2000). Anti-FXR1 was used to immunoprecipitate FXR1P as described (Jin et al., 2004b). Anti-eukaryotic initiation factor 5 (eIF5) (Santa Cruz) was used for loading controls. Rabbit anti-Dicer (Santa Cruz) was used to detect Dicer.

Antibody reactivity was visualized using either an anti-mouse HRP conjugate (Jackson Immunoresearch) or an anti-rabbit HRP conjugate (GE Healthcare) and developed with ECL (GE Healthcare).
**Dicer Knockdown**

DT40 cells were cultured as previously described and maintained at $6 \times 10^5$ cells/ml for 2 days before addition of tetracycline dissolved in EtOH at a final concentration of 2ug/ml of media. Freshly made tetracycline (+ tet) or vehicle only (EtOH, - tet) was added to cell media every 24 hours until cells were harvested at 96 hours. Dicer knockdown was confirmed by immunoblot with Dicer antibody.

**Metabolic Labeling**

DT40 cells grown as described above for 96 hours were pre-incubated in methionine-free media [Gibco] supplemented with glutamine and 10% dialyzed FCS for 30 minutes before introducing 50 uCi/ml of 3H methyl-methionine [Amersham] for 45 minutes. The cells were then washed twice with ice-cold PBS and lysed with lysis buffer (50 mM Tris pH 7.6, 300 mM NaCl, 30 mM EDTA, 0.5% triton X-100 and a protease inhibitor tablet [Roche]) for 10 minutes. Cytoplasmic extracts were prepared by spinning to remove the nuclei and either total extract was loaded or the lysate was immunoprecipitated with the anti-FXR1P antibody (Jin 2004) or anti-FMRP antibodies (Abcam) as described (Stetler).
Linear sucrose density gradient analysis of polysomes.

DT40 cells grown as described above were treated for 15 minutes with cycloheximide (100 µg/ml). Linear 15%-45% sucrose gradients containing 100 mM KCl, 20 mM Tris (pH 7.5), 5 mM MgCl₂ were prepared using a gradient maker (BioComp). Cells were washed in PBS and lysed in the buffer described above. Postnuclear supernatants were overlayed on the gradient and centrifuged for 75 minutes at 188,000 x g at 4°C. Each gradient was fractionated into 1 ml fractions by bottom displacement using a gradient fractionator (Isco) with the ribosomal profile monitored at OD₂₅₄ nm.

RNA Isolation and Quantitative Reverse Transcription-Polymerase Chain Reaction:

Total RNA was isolated using TriZol (Invitrogen) and reverse transcribed using random primers and superscript II reverse transcriptase (Invitrogen). qRT-PCR was performed and ΔCt values calculated and averaged using triplicate samples from each of three independent trials in a BioRad iCycler using QuantiTect SYBR green (Qiagen). PCR was performed by initial denaturation at 95°C for 15min followed by 40 cycles of 15sec at 94°C, 30sec at 55°C, and 30sec at 72°C. The primers for FXR1 and FMR1 were validated and obtained from Qiagen using the QuantiTect Primer Assay System (Cat. Nos. QT01498000 and QT01501031 respectively). Primer specificity was verified by melt curve analysis and fold change between FMR1 mRNA levels (internal control for mRNA level normalization) and FXR1 mRNA levels was calculated as 2⁻ΔCt. Lack of significance was assessed by Z-test showing no significant difference between the FXR1 mRNA level fold change and 1.
Reporter Gene Assay and Cell Electroporation

DT40 were cultured and tetracycline was added for Dicer knockdown as described above. At 72 hours, $10^7$ cells were transfected by electroporation with 25 µg reporter constructs containing the firefly luciferase gene and either the FXR1 3’UTR, the FMR1 3’UTR, or the mutated FXR1 3’UTR (SDM) (pGL3-promoter vector, Promega) and 10 µg of the Renilla luciferase phRL-Tk vector (Promega). Transfection with empty pGL3-promoter vector served as the control and co-transfection with the Renilla luciferase reporter plasmid was performed for normalization of transfection efficiencies. Following electroporation, cells were immediately transferred to individual T75 cell culture flasks, treated with either tetracycline or vehicle only (EtOH), and grown for an additional 24 hours at which time (96 hours after initial tetracycline/EtOH treatment) $3 \times 10^6$ cells were assayed for luciferase expression and the remaining cells harvested for Dicer expression by immunoblot. The luciferase assay was carried out using the Dual-Luciferase Reporter Assay (Promega) according to the manufacturer’s directions. Luciferase activity was measured in a luminometer (LUMIstar OPTIMA Luminometer) programmed with OPTIMA software version 2.00.

Overexpression of miR-367 mimic

HEK293 and HeLa cells were cultured as described above and then plated at $2 \times 10^5$ in 12-well dishes. 24 hours after plating, cells were transfected with 50ul of 2µM double stranded miR-367 mimic (Dharmacon) was transfected using Lipofectamine2000 transfection reagent (Invitrogen). Control cells were mock transfected using Lipofectamine2000 and each sample was compared to an untreated control. After 72 or
96 hours, cells were harvested and lysed in 0.3M NaCl lysis buffer containing protease inhibitors (Roche) and the lysates run on a 7.5% TBE/acrylamide gel. Blots were probed with anti-FXR1P 2107 to measure FXR1P expression and then eIF5 antibody to control for loading. Reduction in FXR1P expression was quantified using densitometry (Image J) and after normalization to eIF5, calculated as a percent decrease compared to control. Significance of reduction was determined using Student’s t-test.
CHAPTER 4
CONCLUSIONS

The data presented in this dissertation is the culmination of the research I have conducted during my graduate studies in the laboratory of Dr. Stephanie Ceman. The goal of this research was to understand how phosphorylation of FMRP affects association with the miRNA pathway and whether fragile X proteins FMRP and FXR1P are regulated by miRNAs. The research presented here accomplishes these goals by showing that phosphorylation of FMRP modulates an association with the miRNA-mediated regulatory pathway and that FXR1P, but not FMRP, is regulated by miRNAs.

Summary I: Phosphorylation of FMRP inhibits association with Dicer

The results presented in Chapter 2 describe how phosphorylation of FMRP acts as a switch to modulate association with Dicer, a crucial element of the miRNA processing pathway. When we discovered that P-FMRP but not FMRP associated with large amounts of an ~80 nucleotide RNA species, it was hypothesized that phosphorylation interfered with some aspect of miRNA processing, rendering P-FMRP more likely to associate with 70-80 nucleotide precursor miRNAs instead of the 19-23 nucleotide mature miRNAs (Bartel, 2004a). Dicer is the enzyme responsible for cleaving the precursor miRNA hairpin loop, generating the roughly 22 nucleotide miRNA duplex which is then ready for loading into RISC for further processing (Bernstein et al., 2001, Hutvagner et al., 2001, Winter et al., 2009). If the phosphorylation of FMRP inhibited Dicer binding, the 80 nucleotide precursor miRNAs seen associated with P-FMRP could
be explained. This hypothesis was confirmed by co-immunoprecipitation experiments and a capture assay using HeLa cells to show FMRP, but not P-FMRP, associated with Dicer. RNase treatment did not eliminate FMRP’s association with Dicer, suggesting a protein interaction (see Appendix I). Based on our data, we proposed a new role for phosphorylation in the regulation of FMRP function where phosphorylation inhibits FMRP association with Dicer, a key enzyme in the generation of miRNAs. As a consequence of reduced association with Dicer, we predicted that less miRNAs were available for association with target mRNAs bound by P-FMRP.

We concluded that Dicer-FMRP association requires FMRP’s unphosphorylated region 496-503 and that phosphorylation of FMRP abolishes this interaction. If miRNAs are required for translation activation as recently described (Maroney et al., 2006, Vasudevan and Steitz, 2007, Vasudevan et al., 2007) and FMRP is involved in translation activation (Bechara et al., 2009), phosphorylation of FMRP would indirectly suppress translation by decreasing miRNA production through loss of Dicer binding (Fig. 8) (Cheever and Ceman, 2009b).

**Discussion I: Phosphorylation of FMRP inhibits association with Dicer**

We found that there were less miRNAs and a substantial increase in pre-miRNAs associated with P-FMRP. There are two possible ways to reconcile our findings with the reported role of FMRP as a translational suppressor (Ceman et al., 2003, Narayanan et al., 2007). The first is that phosphorylation may suppress translation by reducing the production of miRNAs, which must function as translation activators, as recently
described by Steitz’s group (Maroney et al., 2006, Vasudevan and Steitz, 2007, Vasudevan et al., 2007). Thus, in the absence of activating miRNAs, translation of FMRP mRNA targets is suppressed.

Alternatively, there could be two functions for P-FMRP: one as a translational inhibitor by a molecular mechanism not yet elucidated and the second, described here, as a modulator for association with the miRNA pathway where dephosphorylation would allow FMRP to associate with Dicer which would then process pre-miRNAs in to mature miRNAs for translation of FMRP bound target mRNA transcripts. It is possible that P-FMRP sequesters miRNAs in an inactive form as pre-miRNAs until FMRP is dephosphorylated and pre-miRNAs are processed. At this time, FMRP target mRNA translation would be suppressed by mature miRNAs. Under these conditions, the P-FMRP/pre-miRNA complex is “poised” to effect suppression of target mRNAs in a localized manner. This fits with the Steitz data where miRNAs were shown to recruit specific proteins, including FXR1P, in order to induce activation of translation under specific cell conditions (Vasudevan et al., 2007).

We propose that the complex containing FMRP, mRNAs and pre-miRNAs is present in the cytoplasm and dephosphorylation of FMRP is the signal which allows Dicer to bind and process miRNAs for activation of translation. Although we cannot yet distinguish between these possible scenarios, we can conclude that we have discovered a new role for phosphorylation in the regulation of FMRP function as an inhibitor of its association with Dicer, a key enzyme in the generation of miRNAs.
Future studies I

1. Identify the precursor microRNAs associated with phosphorylated FMRP

Future experiments will need to be done to identify which precursor miRNAs (pre-miRNAs) associate with P-FMRP. It is also formally possible that P-FMRP, as a RNA-binding protein with several RNA binding domains, associates with available pre-miRNAs, perhaps through one of its KH domains. This hypothesis is supported by in vitro data showing that recombinant FMRP aids Dicer processed miRNA assembly onto target mRNAs by acting as an acceptor protein through its KH domains (Plante et al., 2006).

In order to identify at least one of the pre-miRNAs associating with P-FMRP, we used a protocol adapted from Current Protocols in Mol Bio supplement 72 26.4.1-18 in an attempt to clone and sequence the pre-miRNAs. We first extracted RNA associated with P-FMRP and FMRP using the immunoprecipitation and end-labeling protocol described in chapter 2. Then, unlabeled RNA was run on a gel and compared to an identically prepared lane of radio-labeled RNA, along with a size marker. This allowed us to excise the un-labeled RNA aligning to the 70-80 nucleotide region of pre-miRNAs. After elution from the gel, RNA was precipitated and dephosphorylated to prevent circularization during T4 RNA ligation. Phosphorylated 3’ and 5’ adapters were labeled and ligated onto the RNA, which was then ready for reverse transcription, followed by a second PCR step to add a Ban1 restriction site to both ends of the cDNA. The resulting construct was then cloned into a TA cloning vector (Invitrogen) for screening and sequencing.
Unfortunately, the resulting sequence did not match any known precursor or mature miRNA sequences. One confounding factor in this experiment was the starting amount of RNA. While the protocol we used is optimized for a starting amount of RNA in the microgram range, the amount of RNA obtained from our immunoprecipitation and extraction protocol was in the picogram range. It was concluded that the starting amount of RNA was too low to ascertain its identity using this cloning protocol.

Assuming the ~80 nucleotide band seen associating with P-FMRP is precursor miRNAs, an alternative to the above experiment would be to use the NCode™ Multi-Species miRNA Microarray (Invitrogen). This service includes cloning and probing for all miRNA species in human, mouse, rat, Drosophila, C. elegans, and zebrafish. The arrays are done in triplicate, contain controls to monitor hybridization specificity, and have positive and negative controls throughout the array. In order to utilize this method, putative P-FMRP associating pre-miRNAs would need to be processed into mature miRNAs. Treating the extracted RNA with recombinant Dicer (Genlantis) could process pre-miRNAs into miRNAs that could then be detected by this microarray.

2. Does phosphorylation of FMRP modulate miRNA-mediated translation activation of target mRNAs?

Assuming a validated P-FMRP associating pre-miRNA could be identified, future experiments could be done to determine whether, following de-phosphorylation of FMRP, Dicer association could process P-FMRP associated pre-miRNAs into a mature
miRNAs. One hypothesis is that translation activation of specific mRNAs associated with non-phosphorylated FMRP could occur, similar to the reported translational activation function of FXR1P (Vasudevan and Steitz, 2007, Vasudevan et al., 2007). To test whether phosphorylation of FMRP modulates miRNA-mediated translation activation, Stek cells (which contain no endogenous FMRP) could be transfected with a constitutively phosphorylated (aspartic acid substitution at serine 499) FMRP construct (Asp) or a non-phosphorylated (alanine substitution at serine 499) FMRP construct (Ala) (Ceman et al., 2003), along with a putative FMRP-associating miRNA for use in a reporter assay. In this scenario, transfection of Stek cells with Ala and a luciferase construct bearing a complementary miRNA sequence that is known to associate with FMRP would allow Dicer-FMRP binding. The presence of Dicer would allow miRNA processing and lead to luciferase expression. Alternatively, transfection with Asp and the luciferase-miRNA construct would lead to a lack of Dicer binding and, hence, no luciferase expression. Together, these experiments would attach functional relevance to FMRP’s association with the miRNA pathway by showing a direct effect of a miRNA on translation of a target.

**Summary II: Fragile X protein family member FXR1P is regulated by miRNAs**

In addition to FMRP’s utilization of the miRNA pathway to regulate target mRNAs, the close association of both FMRP and FXR1P with the miRNA pathway suggested possible miRNA-mediated regulation of these proteins themselves. The results presented in Chapter 3 addressed the question of whether the fragile X proteins FMRP and FXR1P are regulated by miRNAs.
In order to better understand miRNA-mediated translation regulation, we obtained a Dicer conditional knockdown cell line, DT40. We were able to use this cell line to test whether FMRP and FXR1P were regulated by miRNAs due to the global reduction in mature miRNAs due to a lack of Dicer processing. In the absence of miRNAs, we found a four-fold increase in FXR1P, without influencing FMRP expression (Fig. 10). Collectively, our data indicate that a regulatory site in the 3’UTR of FXR1 is important for regulation of FXR1P expression in multiple cell lines. In addition, it suggests that FMRP is not regulated by miRNAs. Understanding how FXR1P is regulated by miRNAs will give insight into normal muscle development and function. We can now add FXR1P as an additional example of one posttranscriptional regulatory factor (RNA binding protein) being regulated by another type of posttranscriptional regulator (miRNAs).

**Discussion II: Fragile X protein family member FXR1P is regulated by miRNAs**

Although we have identified an important miRNA regulatory site in FXR1, our data does not exclude the possibility of other miRNA binding sites playing a role in FXR1P regulation. The aforementioned seed sequence did not completely abolish miRNA-mediated regulation of the reporter construct because an even greater effect on expression was seen when there was global miRNA reduction. Further experiments to sequentially delete other miRNA binding sites in the FXR1 3’UTR will determine whether other miRNAs could regulate expression of FXR1P and if there are combinatorial effects of multiple miRNA binding sites.
FXR1P is critically important for normal muscle development and function (Mientjes et al., 2004, Huot et al., 2005, Wienholds and Plasterk, 2005, Chen et al., 2006, Chen et al., 2009). miR-92, one candidate miRNA we identified for FXR1P regulation, was found to be misregulated in Duchenne’s Muscular Dystrophy and in Nemaline Myopathy, a congenital myopathy that is the most common nondystrophic congenital myopathy (Eisenberg et al., 2009). Thus, it is formally possible that FXR1P, and consequently its target mRNAs, are misregulated in muscle disease states as a result of misregulated miRNA expression. However, it remains to be seen whether FXR1P levels themselves change throughout normal muscle development and how this relates to miRNA expression levels.

**Future studies II**

1. **Examine effect on miRNA knockdown on endogenous FXR1P expression**

   Although we have shown that absence of the seed sequence common to this group of miRNAs increases expression of a luciferase construct bearing the FXR1 3’UTR and shown a decrease in endogenous FXR1P expression upon miR-367 overexpression, we have not knocked down all of these miRNA in order to show an increase in endogenous FXR1P expression. Our preliminary data shows that knockdown of miR-92 with an antagonim (Ambion) does not increase FXR1P expression to a detectable level in HEK-293T cells. However, since all of these miRNAs share the same seed sequence, if any one of miRNAs-25, 32, 92, 363, or 367 are expressed in HEK-293T cells, they could suppress translation of FXR1P, thereby confounding the results of the miR-92 antagonim experiment. Consequently, it is important to verify which miRNAs are expressed in the
cell line used to examine the effects of miRNA knockdown on FXR1P expression levels.

We obtained this information for HeLa and HEK-293T cells from The Swiss Institute of Bioinformatics (http://www.mirz.unibas.ch/cloningprofiles/) website, showing that miRNAs 25, 32, and 92 are expressed in HeLa cells and miRNAs 32 and 92 in HEK-293T cells. Since these lists may not be comprehensive, knockdown of only these miRNAs may not have an effect on endogenous FXR1P expression. Therefore, antisense locked nucleic acid (LNA) probes specific for miRNAs 25, 32, 92, 363, and 367 could be used to knockdown these miRNAs and examine FXR1P expression levels in these cell lines.

2. Identify other miRNA regulatory sites in the FXR1 3’ untranslated region

It is possible that there are other miRNA binding sites within the FXR1 3’UTR that regulate FXR1P expression. In order to identify these other miRNA regulatory sites, systematic deletion of regions within the FXR1 3’UTR and cloning of these mutants into luciferase vectors for use in reporter assays (as described in chapter 3) could be utilized. Using TargetScanHuman 5.1, the FXR1 3’UTR can be divided into roughly three sections with multiple miRNA binding sites. Region one extends from nucleotides 13-104 and region two from nucleotides 328-629. Region three contains the regulatory site we identified and described here and one other miRNA binding site, miR-7/7ab which was not present in our luciferase constructs and therefore was concluded to have no additional effect on FXR1P expression. Site directed mutagenesis (Stratagene) to delete these regions, insertion into pGL3 luciferase reporter vector and electroporation of these constructs into tetracycline treated Dicer cells to assess luciferase expression could then
be performed. If one region was seen to effect luciferase expression, additional, smaller deletions constructs could be made to narrow the miRNA binding sites down even further. In this way, mapping of the combinatorial effects of multiple regulatory miRNA binding sites could lead to a hierarchy of regulatory sites, with increasing effects on FXR1P expression levels.

3. Utilize a muscle cell line to observe FXR1P expression and regulation

Since FXR1P is implicated in muscle development and disease, examining its expression levels and miRNA regulation in a muscle cell line should be investigated. We have preliminary data showing that FXR1P expression increases substantially over a 24 hour period in C2C12 cells. This change in FXR1P expression level could be miRNA mediated. In order to test this hypothesis, the miR-367 mimic (described in chapter 3) will be used to overexpress miR-367 in the C2C12 cells. If it regulates FXR1P in this cell line and plays a role in mediating the increase in expression seen over a 24 hour period, its overexpression should abolish this increase in FXR1P expression. If correct, this would supply the first evidence for miRNA-mediated regulation of FXR1P in a muscle cell line.

Concluding remarks

FMRP and FXR1P are important regulators of messenger RNAs. The absence of FMRP in fragile X syndrome and the dysregulation of FXR1P in muscle disorders can be attributed to serious human diseases. Unraveling how post-translational modifications and miRNAs modify their ability to regulate cellular processes and how they themselves
are regulated will aid in understanding their complex functions, and ultimately, the
disease states associated with each one. Our work on FMRP and FXR1P in the context
of the miRNA-mediated regulatory pathway provides a framework for a better
comprehension of both these proteins.
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APPENDIX A

Introduction

We have shown that the Dicer enzyme binds unphosphorylated fragile X mental retardation protein (FMRP) on residues 496-503 and phosphorylation of FMRP (P-FMRP) at this site abolishes Dicer association (Chapter 2) (Cheever and Ceman, 2009a). We further showed that this interaction is not RNA mediated by performing co-immunoprecipitation experiments in the presence of RNase and observing that Dicer and FMRP continue to associate. Although FMRP and P-FMRP peptides were used in capture assays to confirm that Dicer association occurred at FMRP’s phosphorylation site (residues 496-503) (Ceman et al., 2003), these experiments do not confirm a direct interaction between FMRP and Dicer. Hence, either Dicer or a Dicer-containing complex binds unphosphorylated FMRP.

We attempted to determine whether there was a direct interaction between FMRP and Dicer and map the interaction site using a yeast-2-hybrid approach. Based on our previous data, if Dicer and FMRP interact, deletion of FMRP’s phosphorylation site should abolish this interaction. We expected a direct interaction between FMRP and Dicer based on our in vitro capture assay and co-IP data. This appendix details the process of cloning Dicer into a yeast vector and outlines the attempt to establish a direct protein interaction between Dicer and FMRP. The end result of the experiment was not informative because co-expression of FMRP and Dicer in yeast was lethal.
Materials and methods

Plasmids and strains

Mouse Dicer cDNA corresponding to Genbank accession number AF408401 (Nicholson and Nicholson, 2002) was a gift from Dr. Allen Nicholson. FMRP subcloned into the two-hybrid bait vector pGBKT7 was obtained from Dr. Edward Khandjian. This construct has been shown to express full length FMRP in yeast (Davidovic et al., 2006).

Clontech protocols, yeast strains, and vectors were used throughout the assay.

*S. cerevisiae* two-hybrid bait strain AH109 (**MAT**a, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1_{UAS}\text{-}GAL2_{TATA}\text{-}ADE2 URA3::MEL1_{UAS}\text{-}MEL1_{TATA}\text{-}lacZ MEL1) was transformed with either the pGADT7-GAL4 activation domain (AD) fusion two-hybrid vector containing full-length Dicer sequence (pGADT7-Dicer), or pGADT7 containing the SV40 large T-antigen sequence (pGADT7-T).

*S. cerevisiae* two-hybrid prey strain Y187 (**MAT**α, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, met-, URA3::GAL1_{UAS}\text{-}GAL1_{TATA}\text{-}lacZ MEL1) was transformed with either the pGBKKT7-GAL4 DNA binding domain (BD) fusion two-hybrid vector containing full-length FMRP sequence (pGBKKT7-FMRP), pGBKKT7 containing p53 sequence (pGBKKT7-53), or pGBKKT7 containing human lamin C sequence (pGBKKT7-LAM).
Results

Two-hybrid assay

Cloning of Dicer into the pGADT7 yeast vector was first attempted by engineering flanking \textit{sfi1} restriction sites into Dicer cDNA (6.5 kb), then ligating this construct into the \textit{sfi1} site of the yeast prey vector, pGADT7, which contains the activation domain that binds the yeast GAL4 UAS of the minimal promoter required to activate the yeast lacZ reporter gene. However, this construct could not be successfully expressed and propagated in bacteria cells, probably due to the large size of the Dicer protein (~220 kDa). Since yeast cells are highly efficient in carrying out homologous recombination, cloning of Dicer into pGADT7 was then attempted in yeast instead of bacteria (Ma et al., 1987). Flanking sequences complementary to ~40 nucleotide overhangs corresponding to the EcoR1 and BAMH1 restriction sites in the pGADT7 yeast vector were added by PCR to the starting and ending sequence of the Dicer cDNA respectively. Since yeast will carry out homologous recombination to “repair” the gap in the vector, digested pGADT7 vector and the Dicer construct containing pGADT7 overhangs were simultaneously transformed into yeast strain AH109. Transformants were selected by growth on YPDA media lacking leucine. Subsequent yeast colony PCR, western blot and sequencing confirmed correct insertion of Dicer into pGADT7 and expression of full-length Dicer in yeast (Fig. 16).
Figure 16. Cloned full-length Dicer in the pGADT7 two-hybrid vector. Four yeast colonies (AH109) were transformed with full-length Dicer containing flanking pGADT7 overhangs and digested pGADT7. Following protein extraction (Clontech), Dicer expression was detected in three of the four colonies (Lanes 1, 3, and 4). Yeast transformed with empty vector (pGADT7- Vector only) served as a negative control for Dicer expression.
The \textit{FMR1} sequence in the yeast bait vector, pGBKT7, containing the binding domain for the yeast GAL4 UAS of the minimal promoter required to activate the yeast lacZ reporter gene (Dr. E. Khandjian) was transformed into the Y187 yeast strain and transformants were selected by growth on YPDA media lacking tryptophan (data not shown).

Once the constructs were successfully transformed into their respective yeast strains, verification by western blot of bait and prey expression was again performed. Both Dicer and full-length FMRP were expressed in yeast (Fig. 17). To determine whether Dicer and FMRP interacted directly, we mated yeast containing the respective FMRP-binding domain (bait) and Dicer-activation domain (prey). A direct interaction would have been indicated by colonies able to grow on triple dropout (-LEU2, -TRP1, -HIS) media.

The SV40 large T-antigen (pGADT7-T) with murine p53 (pGBK7T-53) has been shown to interact in a yeast-2-hybrid and was used as the positive control. The Human lamin C (pGBK7-LAM), has been shown to neither form complexes nor interact with SV40 large T-antigen (pGADT7-T) and was used as a negative control.
Figure 17. Verification of FMRP and Dicer expression in yeast transformants.

Yeast lysate probed with either anti-HA antibody (Covance) (Blot #1, a and b) or anti-myc antibody (Blot #2, c) in order to verify Dicer, FMRP, large T antigen, and p53 expression prior to mating. (a.) Blot #1 was probed with anti-HA and exposed for 10 minutes on film. Histidine tagged Dicer was detected, clone 3-5 (lane 1) and clone 7-4 (lane 2). Lane 3 is described in part (b). Lane 4- negative control: untransformed AH109 whole cell lysate without Dicer or large T-antigen expression. (b.) Blot #1 was again exposed for 2 minutes on film in order to observe large T-antigen expression in Lane 3. (c.) Blot #2 was probed with anti-myc. Lane 1- p53 expression. Lane 2- myc-tagged full-length FMRP. Lane 3 was loaded with Human lamin C expressing yeast lysate but was not detectable on the western blot. Lane 4- negative control: untransformed AH109 whole cell lysate without FMRP of p53 expression.
Table 1. Mating crosses: Yeast two-hybrid.

<table>
<thead>
<tr>
<th>AH109:</th>
<th>pGADT7-Dicer</th>
<th>pGADT7-T</th>
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</thead>
<tbody>
<tr>
<td>Y187 X AH109</td>
<td></td>
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</tr>
<tr>
<td>-LEU, -TRP, -HIS</td>
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<tr>
<td>pGBK7-FMRP</td>
<td>-</td>
<td>-</td>
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<tr>
<td>pGBK7-53</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>pGBK7-LAM</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

- Denotes zero yeast colonies

++ Denotes greater than 50 yeast colonies

Yeast strain Y187 containing the constructs shown on the left were crossed with yeast strain AH109 containing the constructs shown across the top. No colonies were observed where indicated (-); yeast colonies were observed when p53 interacted with large T-antigen where indicated (++). Crosses were screened on triple dropout media (-LEU, -TRP, -HIS).
Discussion

We could not verify a direct protein interaction between FMRP and Dicer using a yeast two-hybrid assay. Although both FMRP and Dicer could be expressed independently in the two separate yeast strains, mating and co-expression of these proteins proved to be lethal in yeast (table 1).

If a direct interaction had been established, FMRP deletion constructs (Fig. 18) which have already been cloned into the yeast bait vector pGBKT7 would have been transformed into yeast and mated with the Dicer construct. This would have allowed mapping of where the direct interaction between FMRP and Dicer occurs on the FMRP protein. Since our previous data indicate there is an interaction between Dicer and FMRP at residues 496-503 between exons 14 and 15, a positive result using these constructs in a yeast two-hybrid would depend on whether each FMR1 construct contained this intact, non-phosphorylated region. As shown in Figure 18, only when there is a constitutively phosphorylated mutant (Asp) (Ceman et al., 2003) or a lack of this region (only exons 16 and 17 present) would a lack of Dicer binding be expected.
Figure 18. Predicted results of yeast two-hybrid interaction between Dicer and FMRP deletion constructs. The FMR1 exon map (above) shows the exons present in the FMR1 gene, the location of the functional domains with respect to the exons and the location of the phosphorylation site. 1. An FMR1 construct containing exons 14, 15, 16, and 17 would be expected to interact with Dicer. 2. An FMR1 construct containing exons 14-17 but with an alanine (Ala) or aspartic acid (Asp) substitutions at serine 499, the primary FMRP phosphorylation site, would be expected to interact with Dicer as shown to the right. 3. An FMR1 construct containing only exons 16 and 17 would not be expected to interact with Dicer. 4. An RGG box deletion would not be expected to affect Dicer association.
It is possible that an intermediate protein provides a link between FMRP and Dicer in vivo. Had the yeast two-hybrid technically worked and we had seen no interaction, we would have assumed this to be the case. Bridging proteins could be identified by immunoprecipitating FMRP and probing with antibodies to candidate proteins. Two likely candidates would be Ago2 based on previous studies showing it is present in miRNP complexes (Hutvagner and Simard, 2008) containing FMRP (Jin et al., 2004a), and FXR1P, which has been shown to be in miRNP complexes involved in translation activation (Vasudevan et al., 2007). Alternatively, one could determine whether phosphorylation of FMRP disrupts the FMRP-bridging protein association by immunoprecipitating with PSER and probing for the candidate bridging proteins. Mass spectrometry of proteins that co-immunoprecipitate with P-FMRP compared to FMRP would also reveal whether Dicer is differentially bound and if there are additional associating proteins based on FMRP’s phosphorylation state.
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