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The RNA-Binding Specificity of NT-dFMRP

A Thesis submitted in partial satisfaction of the requirements for the degree

Master of Science

in

Chemistry

by

Lila Mouakkad

Committee in charge:

Professor Simpson Joseph, Chair
Professor Gourisankar Ghosh
Professor Thomas Hermann

2014

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The Thesis of Lila Mouakkad is approved and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2014

DEDICATION

For everyone affected by fragile X syndrome.

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VITA

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ABSTRACT OF THE THESIS

The RNA-Binding Specificity of NT-dFMRP

by

Lila Mouakkad

Master of Science in Chemistry

University of California, San Diego, 2014

Professor Simpson Joseph, Chair

The loss of expression of the fragile X mental retardation protein (FMRP) leads to Fragile X Syndrome, which is a genetically-linked condition resulting in intellectual disability. FMRP is an RNA-binding protein known to repress the translation of target mRNAs that encode pre- and post-synaptic proteins that are directly attributed to the phenotypes of the condition. Although hundreds of mRNAs have been shown to associate with FMRP, very few have actually been validated through biochemical studies. More importantly, the sequence specificities of the KH domains and the RGG box have not carefully been

deciphered. Recently, Tuschl and co-workers proposed that the KH1 and KH2 domains of FMRP bind to WGGA (W=A/U) and ACUK (K=G/U) RNA recognition elements, respectively (Ascano et al., 2012).

We developed a fluorescence anisotropy assay to quantitatively measure the binding affinity of N-terminally truncated *Drosophila* FMRP (NT-dFMRP) and functionally relevant mutants (I244N, I307N, Δ RGG) to PolyG₁₈-FI. Our results show that NT-dFMRP, I244N, and I307N bind PolyG₁₈-FI with similar affinities of 87 ± 18 nM, 105 ± 14 nM, and 52 ± 22 nM, respectively; while the Δ RGG mutant does not. Since circular dichroism spectroscopy demonstrates that PolyG₁₈-FI folds into a standard parallel G-quadruplex structure, we propose that the RGG box is responsible for binding RNA substrates containing this structural feature. Our studies also show that NT-dFMRP does not bind to PolyC₁₈-FI, PolyC₁₈(ACUU)-FI, and PolyC₁₈(UGGA)-FI RNAs, indicating that single sets of ACUK and WGGA sequences are not sufficient enough for NT-dFMRP binding.

Chapter 1: Introduction

1.1 Fragile X Syndrome

Fragile X syndrome (FXS) is an X-linked genetic condition that is one of the most common forms of inherited mental retardation. According to the National Fragile X Foundation, FXS is prevalent in about 1 in 4000 males and 1 in 6000 females in the world population (Coffee B et al., 2009). About 100,000 Americans suffer from the syndrome and the national health care cost of treating individuals diagnosed with FXS runs around \$226 million per year (Ouyang et al., 2010).

Other than suffering from impaired intellectual ability, FXS patients experience symptoms that are on the scope of those found in autism spectrum disorders (ASDs) (Garber et al., 2008). These characteristics generally revolve around cognitive deficits, developmental delays, and behavioral problems. More specifically, they might include difficulties with speech expression, social anxiety/shyness, an increased susceptibility to seizures, and development of attention deficit disorders (Maes et al., 2000). Observed physical phenotypic traits in individuals diagnosed with FXS include elongated facial features and enlarged ears, flat feet, and macroorchidism in males (Lachiewicz et al., 1994, Eliez et al., 2001). Furthermore, postmortem studies have provided evidence that dendritic spines in FXS patients are much denser than normal and exhibit immature morphology (Irwin et al., 2001).

FXS is largely caused by a cytosine-guanine-guanine (CGG) trinucleotide expansion in the 5' untranslated region (UTR) of the fragile X mental retardation gene (FMR1) (Verkerk et al., 1991, Fu et al., 1991). The trinucleotide expansion results in a mutated form of FMR1, which consequently leads to the silencing of the gene by hypermethylation. A recent study has shown that FMR1 mRNA plays a large role in the gene silencing of FMR1 by hybridizing directly with the complementary CGG nucleotides in the 5' UTR of FMR1 DNA and forming a RNA-DNA duplex in order to prevent transcription (Colak et al., 2014). Although more research needs to be done to look into the exact mechanism of how FMR1 gene silencing occurs, the end result is that epigenetic changes of FMR1 leads to decreased expressed levels of the Fragile X mental retardation protein (FMRP). Therefore, FXS is attributed to a loss of FMRP.

The CGG trinucleotide-expansion mutation ranges from 6-54 repeats in normal individuals, 55-200 repeats in premutation carriers, and greater than 200 repeats in individuals carrying the full mutation (Santoro et al., 2012). Unfortunately, pre-mutation carriers can develop fragile X-associated tremor/ataxia syndrome (FXTAS) or fragile X-related primary ovarian insufficiency (FXPOI). FXTAS is characterized as a late onset neurodegenerative disorder in adults that causes involuntary movements, balance problems, and symptoms associated with Parkinson's and Alzheimer's disease (Greco et al., 2006). FXPOI occurs exclusively in females and causes an early onset of

menopause in women under the age of 40 and dysfunctional ovaries that lead to infertility (Sherman et al., 2000). Studies have shown that approximately one-third of male premutation carriers over 50 years old develop mild-to-severe symptoms associated with FXTAS, while one-quarter of female pre-mutation carriers will suffer from FXPOI (Jacquemont et al., 2004).

1.2 FMR1 Encodes an RNA-Binding Protein

The identification of the structural features of FMRP and the various functional domains that it contains has paved the way for decoding its functional properties (Figure 1.1). FMRP has been shown to contain three functional RNA-binding domains, which include two tandem K-homology domains (KH) and an arginine-glycine-glycine (RGG) motif that allow it to bind to nucleic acids and thereby regulate the translation of target messenger RNAs (mRNAs) (Ashley et al., 1993). The KH domains were first identified in the heterogeneous nuclear ribonucleoprotein K and have been since discovered to be found in many prokaryotic and eukaryotic proteins (Siomi et al., 1993). While the KH domains have distinctly different folding arrangements in prokaryotic (Type II) and eukaryotic (Type I) organisms, they are a class of highly conserved residues that could span from one copy to as many as fourteen copies in proteins and bind either DNA or RNA (Valverde et al., 2008).

Although there are currently no solved structures of full-length FMRP from any organism, there is a crystallographic structure available of the KH domains

(KH1-KH2 Δ) of human FMRP (hFMRP) solved at 1.9 Å resolution (Valverde et al., 2007). The KH1 and KH2 domains in FMRP are each about 45 residues long and contain a typical Type I $\beta 1\alpha 1\alpha 2\beta 2\beta'\alpha'$ fold in which two β -strands ($\beta 1$ and $\beta 2$) lie parallel to each other and one β' -strand lies antiparallel in orientation to the other strands. A largely conserved GxxG loop is found in the sequence in between the $\alpha 1$ and $\alpha 2$ helices that has been suggested to be important for specific nucleic acid recognition in the KH-type splicing regulatory protein (KSRP) (Hollingworth et al., 2012). An α' linker also exists between multiple KH domains in proteins (Figure 1.2).

Astonishingly, a single point mutation in the KH2 domain of FMRP that converts an isoleucine residue at position 304 into an asparagine residue (I304N) alone can cause a severe form of FXS (De Boule et al., 1993). This was discovered in a patient that has a number of CGG trinucleotide repeats that is considered to be “normal” in unaffected individuals, but expresses a mutant form of the protein. The I304N mutation has been largely studied as it has been found to abolish polyribosome association and reduce the ability of FMRP to bind to RNA by destabilizing the protein (Feng et al., 1997, Zang et al., 2009). This has been suggested to occur because the isoleucine at position 304 is part of a hydrophobic network of residues that stabilize the core of the KH2 domain. When a positively charged amino acid such as asparagine is introduced into this network, the protein structure becomes disrupted (Valverde et al., 2007).

More than twenty years after the discovery of the I304N mutation, another patient was identified to have a single point mutation in the KH1 domain of

FMRP. In this case, a missense mutation caused a highly conserved glycine residue at position 266 to become mutated into a glutamic acid residue (Myrick et al., 2014). Studies have shown that this mutant does not associate with polyribosomes, has a significantly lower affinity to well-known mRNA targets of FMRP, and also causes increased levels of AMPAR internalization (Myrick et al., 2014). It seems that there is a high probability that FXS is caused by more KH domain point mutations; however, only two have been implicated in FXS thus far because of a lack of clinical screening of the whole FMR1 gene in the diagnostic process.

Unlike the KH domains, the RGG motif is usually present in conjunction with other RNA-binding domains in proteins. RGG “boxes” can occur from one to up to three sets in a polypeptide, but in FMRP it exists as a di-RGG motif in which two sets of RGG sequences are separated by 0-4 residues (Thandapani et al., 2013). The only structural information that has been obtained about the RGG motif in FMRP has come from NMR solution structures of a short RGG peptide interacting with an in-vitro selected *sc1* RNA molecule that folds into a guanine-quadruplex (GQ) structure (Phan et al., 2011). The study showed that the structure of the RGG peptide becomes ordered upon RNA binding, whereas it exists as a random coil when no RNA is present. Furthermore, the stability of the GQ RNA increases upon binding.

The N-terminal domain of FMRP (NDF), which is found upstream of the KH domains, has been shown to form stable dimers and serves as a platform for FMRP-protein interactions (Adinolfi et al., 2003). NMR spectroscopy was used to

determine the solution structure of the NDF and showed that the domain is comprised of a complex organization of two Agenet/Tudor motifs and one C-terminal helix (Ramos et al., 2006). The Agenet/Tudor motifs are part of a “royal” domain family of proteins that are homologous in sequence and structure (Maurer-Stroh et al., 2003). In FMRP, these motifs contain hydrophobic pockets on the surface that bind to trimethylated lysine residues. The Agenet domains in FMRP are similar in structure to the UHFR1 protein, which interacts with histone H3K9 (Ramos et al., 2006). Current research has shown that FMRP plays a role in gametogenesis and DNA damage response, but this function is dependent on FMRP-chromatin binding through the tandem Agenet domains (Alpatov et al., 2014).

The nuclear localization signal (NLS) and the nuclear export signal (NES) in FMRP also hint at FMRP’s role in nuclear function (Eberhart et al., 1996). The NLS and NES domains allow FMRP to move in and out of the nucleus in order to possibly transport certain mRNAs into the cellular cytoplasm. More research needs to be done to bring further insight into the functional roles of these domains, especially because a single point mutation in the NLS domain in which a conserved arginine residue at position 138 is altered into a glutamine residue (R138Q) was identified to be the underlying cause of developmental delays found in a studied patient (Collins et al., 2010). In recent studies, the R138Q mutant was shown to compromise FMRP’s function in DNA damage response as it fails to bind to nucleosomes, so this interesting aspect of FMRP function will probably be further investigated in the future (Alpatov et al., 2014).

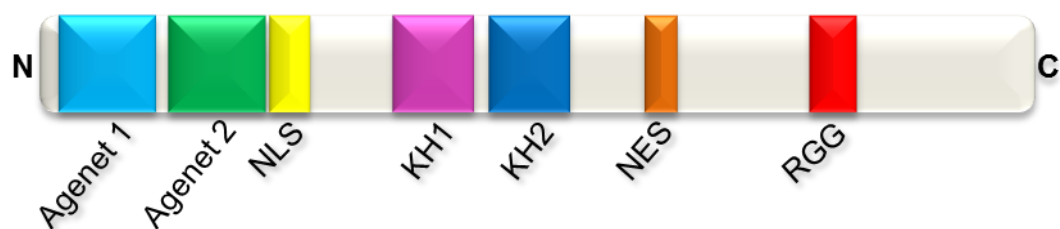


Figure 1.1: A cartoon representation of human FMRP. The relative positions of the functional domains of hFMRP are depicted above.

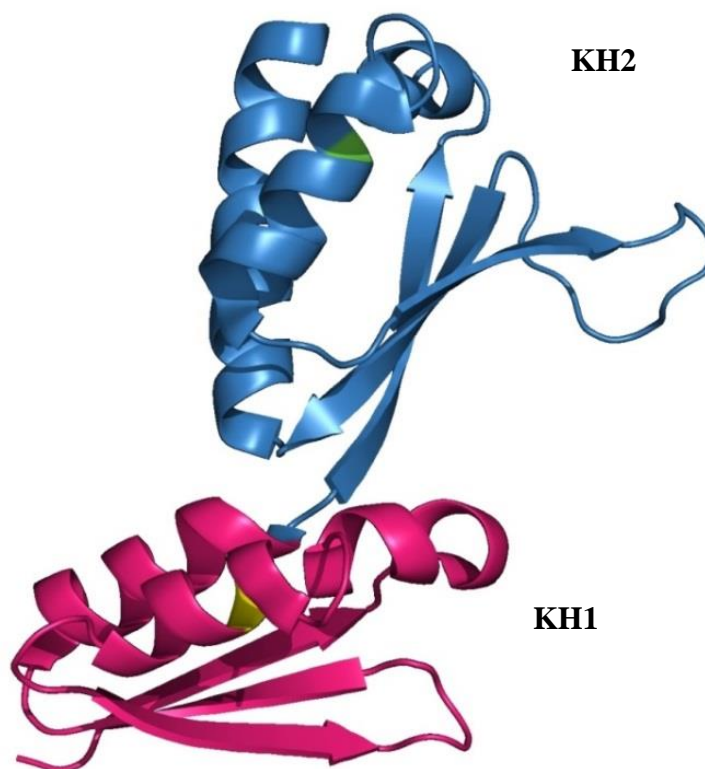


Figure 1.2: The solved X-ray crystallography structure of the KH domains in hFMRP. Amino acid positions at 241 in the KH1 domain and 304 in the KH2 domain are highlighted in yellow and green, respectively. The structure was accessed from the Protein Data Bank with entry code 2QND.

1.3 Models to Study FMRP

The gene coding for FMRP is highly conserved across species from humans to fruit flies (Figure 1.3). Although the pathophysiology of FMRP has been explored in various species such as chimpanzees and zebra fish, researchers have largely focused their efforts on fruit fly and mouse animal models to study FMRP function *in vivo*.

The *D. melanogaster* FMR1 invertebrate ortholog, dFmr1, is 56% similar to FMRP in overall amino acid sequence (Zhang et al., 2001, Santoro et al., 2012). It also contains RNA-binding domains that are 75% identical to the ones found in human FMRP (hFMRP). The fragile X mental retardation protein in *Drosophila* (dFMRP) has been shown to regulate the translation of mRNAs that are also targeted by hFMRP. These mRNAs include futsch (*Drosophila* ortholog of Map1B), chickadee, and Racl (Zhang et al., 2001, Lee et al., 2003, Reeve et al., 2005).

The laboratory of Gideon Dreyfuss first characterized the dFmr1 gene and also implicated the importance of the RNA-binding function of dFMRP through a mutational analysis of the KH domains (Wan et al., 2000). I244N and I307N KH domain mutants were generated that are analogous to the critical I304N mutant that was found to be expressed in a patient with a severe form of FXS (De Boulle et al., 1993). The group found that overexpressing dFmr1 in transgenic fruit fly results in a severe “rough eye” phenotype that leads to cell death by apoptosis. When I244N and I307N mutants were overexpressed, a milder rough eye

phenotype was observed. Interestingly, overexpression of an I244N/I307N double mutant resulted in a nearly complete alleviation of the rough eye phenotype. These results suggest that the overexpression of KH domain mutants is associated with loss-of-function effects possibly related to the disrupted RNA-binding capabilities of dFMRP. Over the years, scientists have moved on to find that a lack of dFMRP expression results in abnormal morphology of axons in mushroom bodies and disrupted courtship behaviors, which is comparable to the symptoms found in human FXS patients (Zhang et al., 2001, Morales et al., 2002, Tessier et al., 2008).

dFMRP models have also been instrumental in helping researchers understand FMRP's role in the mGluR signaling pathway. One astonishing study demonstrated that feeding dFmr1 mutant fruit flies an increased amount of glutamate resulted in death (Chang et al., 2008), which is consistent with the theory that a loss of FMRP results in excess glutamate signaling. This discovery led researchers to screen for small molecules that are able to rescue the lethality of excess glutamate, which can potentially lead to clinical therapeutic applications for FXS patients.

The FMR1 ortholog in mice, Fmr1, shares a 97% sequence similarity to the human version of the gene. In 1994, the first successful Fmr1 knockout mouse model was engineered by creating an allele with a neomycin cassette insert into exon 5 that results in a lack of FMRP expression. This deletion caused the mice under study to exhibit typical symptoms found in FXS patients such as macroorchidism, cognitive deficits involving motor skills, abnormal social

behavior, and immature dendritic spines (Bakker et al., 1994, Comery et al., 1997). Thus far, scientists have been unable to reproduce the same FMR1 gene silencing effects of the full CGG trinucleotide expansion mutation in mice that is found in humans (Brouwer et al., 2007). At 230 CGG repeats, mice still had not experienced transcriptional silencing of Fmr1 due to hypermethylation, even though decreased levels of FMRP were found (Bontekoe et al., 2001).

Years after the first Fmr1 knockout model was discovered, scientists engineered a “conditional” knockout mouse model in which Fmrp expression can be regulated by means of cre-recombinase technology that involves placing bacteriophage lox P sites into the promoter and exon 1 regions of Fmr1 (Mientjes et al., 2006). This allowed researchers to create null Fmr1 alleles and control expression in cell types of choice. A mouse model that expresses the I304N mutation in the KH2 domain was produced in 2009 that has allowed scientists to study the RNA-binding function of FMRP *in vivo* (Zang et al., 2009). Fruit fly and mouse models will inevitably continue to be invaluable tools to study the pathophysiology of FMRP *in vivo*, but induced pluripotent stem (iPS) cell technology is also starting to develop into a useful model to study FMR1 (Eiges et al., 2007).

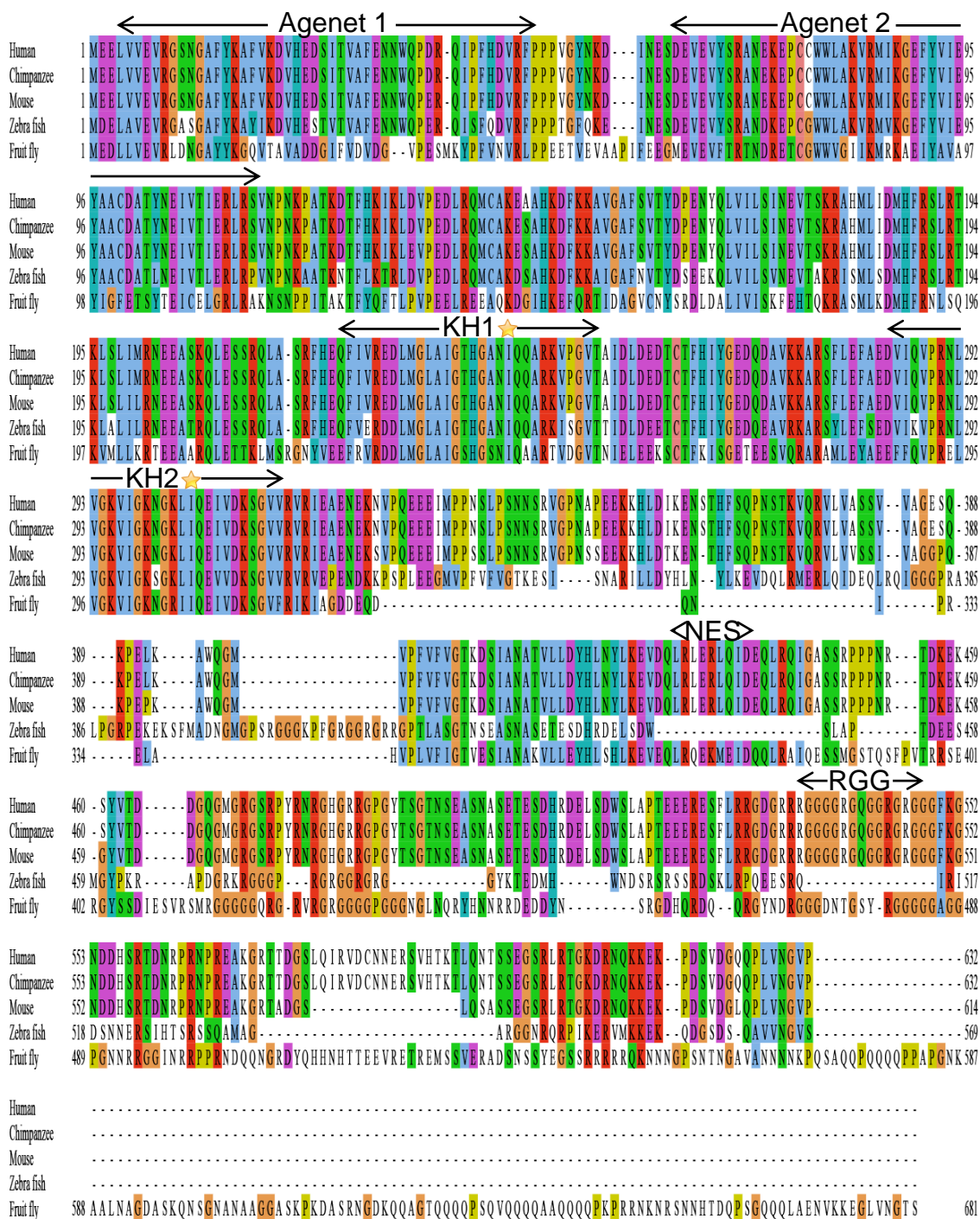


Figure 1.3: The FMR1 gene is highly conserved across various species. Human, chimpanzee, mouse, zebra fish Fmr1 sequences were aligned using COBALT and displayed using Jalview. FMRP functional domains are labeled with respect to the human FMR1 gene. The gold stars highlight the critical I244N and I307N mutants in the KH1 and KH2 domains, respectively.

1.4 FMRP Binds to Target mRNAs

The hunt for identifying *in vivo* mRNA targets of FMRP has been quite challenging, especially because FMRP has been shown to associate with approximately 4% of mRNA found in the brain (Ashley et al., 1993). One of the earliest studies by Darnell and colleagues in 2001 allowed for the identification of mRNAs associated with FMRP in the mouse brain through immunoprecipitation of endogenous FMRP-mRNP complexes and subsequent microarray analysis. Of the 432 mRNAs that associate with FMRP in the mouse brain, 67% were predicted to contain GQ structures and many of them encode for proteins that are important for synaptic function (Brown et al., 2001).

Researchers proceeded to perform *in vitro* selection experiments to select for RNAs that interact with FMRP. Studies found that the RGG domain in FMRP is responsible for binding to a sc1 GQ forming RNA in a sequence-specific manner (Darnell et al., 2001, Phan et al., 2011). A few years later, the same group showed that the KH2 domain in FMRP binds an *in vitro* selected “kissing complex” (kc2) RNA that forms a loop-loop pseudoknot structure in a sequence-specific manner (Darnell et al., 2005). As compared to wild-type FMRP, the I304N KH2 domain mutant showed reduced binding to kc2 RNA, but binding affinity to GQ RNA was equivalent. Since the ability of FMRP to bind to GQ RNA is unaffected by the I304N mutation, this signifies that the RGG domain acts alone in recognizing GQ structures. Although interesting, the pseudoknot

structure formed in the kc2 RNA has not been reported to exist in endogenous mRNA thus far.

In 2011, Darnell and colleagues used high throughput sequencing of RNAs isolated by crosslinking immunoprecipitation (HITS-CLIP) to identify 842 mRNA targets of FMRP found in the mouse brain. (Darnell et al., 2011) Although 24% of the identified mRNAs overlapped with their previous study (Brown et al., 2001) and a significant percentage encoded pre- and post-synaptic proteins, few of them were predicted to form GQ or pseudoknot structures.

Regardless of the fact that hundreds of RNAs have been shown to associate with FMRP, there is still no clear consensus in the field about the RNA-binding specificity of FMRP (Darnell et al., 2005). Thus far, only fourteen mRNA targets of FMRP have been validated through biochemical experiments and overlapping data from several research groups (Table 1.1). Five of the validated mRNA targets encode for proteins that include the amyloid precursor protein (App), Fmr1, microtubule associated protein 1b (Map1b), postsynaptic density protein (Psd95), and semaphorin 3F (Sema3F) fold into structured G-quadruplexes (Santoro et al., 2012).

Other than G-quadruplex structures, there are a few unique cases in which FMRP seems to also have an affinity to either sequence-specific or structure-specific regions in mRNA. The transcription factor human achaete-scute homologue-1 (hASH1) mRNA contains a uracil-rich (U-rich) region that binds to FMRP (Fahling et al., 2009). It has also been shown that FMRP interacts with three unique stem loops termed “superoxide dismutase stem loops

interacting with FMRP” (SoSLIP) present in superoxide dismutase 1 (Sod1) mRNA (Bechara et al., 2009). Interestingly, decreased expressed levels of Sod1 are found in the absence of FMRP, so it has been proposed that FMRP upregulates the translation of Sod1. This suggests that FMRP can also activate the translation of certain mRNAs, but more research needs to be done in this area.

Some groups have proposed that FMRP selectively binds mRNAs with the aid of brain cytoplasmic 1 (BC1), which is a noncoding RNA adapter molecule that binds FMRP and base-pairs with target mRNAs (Zalfa et al., 2003). However, the interactions between FMRP and BC1 are likely to be non-specific as they are both involved in similar neurological pathways. Furthermore, multiple groups have shown that BC1 does not associate with FMRP through *in vivo* or *in vitro* experiments (Iacoangeli et al., 2008). Because there is conflicting data surrounding this topic, it needs to be further investigated before any conclusions can be made.

Most recently, two RNA recognition elements have been proposed to associate with the KH domains of FMRP (Ascano et al., 2012). Through photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) experiments and computational sequence methods, researchers showed that the KH2 domain of hFMRP has a specific affinity for binding ACUK (K=G/U) nucleotide sequences, while the KH1 domain binds WGGA (W=A/U) sequences. Although these findings are groundbreaking, they don't exactly narrow down the RNA-binding specificity of FMRP since the proposed RNA

recognition elements are frequently found within random oligonucleotide sequences.

Table 1.1: A list of 14 confirmed mRNA targets of FMRP. Most of the validated mRNA targets of FMRP encode for proteins that play important roles in synaptic function. The minus signs in the column on the right indicate that the mRNA regions or structural elements that interact with FMRP are not known yet (Santoro et al., 2012).

mRNA Targets	mRNA elements recognized by FMRP
App	G-quadruplex
Arc	-
CamKII α	-
eEF1A	-
Fmr1	G-quadruplex
GABA _{Aδ}	-
GluR _{1/2}	-
hASH1	Uracil-rich region
Map1b	G-quadruplex
Psd95	G-quadruplex
Rgs5	-
Sapap _{3/4}	-
Sema3F	G-quadruplex
Sod1	Superoxide dismutase 1 stem loops interacting with FMRP

1.5 FMRP Represses the Translation of Target mRNAs

FMRP is expressed in neuronal cells in the mammalian brain, but it can also be found in organs within the reproductive system (Abitbol et al., 1993, Devys et al., 1993, Tamanini et al., 1997). FMRP spends most of its time in the cellular cytoplasm, where it largely associates with translating polyribosomes and messenger-ribonucleoprotein (mRNP) complexes (Feng et al., 1997, Khandjian et al., 1996, Khandjian et al., 2004). Therefore, it is not surprising that one of the main features of FMRP function is the regulation of protein synthesis by local translational repression of target mRNAs at dendritic spines (Laggerbauer et al., 2001, Li et al., 2001, Mazroui et al., 2002).

The gene regulating functions of FMRP were first directly investigated through *in vitro* translation experiments where it was found that FMRP inhibited the translation of mRNAs in rabbit reticulocyte lysate and microinjected *X. laevis* oocytes, whereas the I304N KH2 domain mutant did not (Laggerbauer et al., 2001). Other studies established that FMRP inhibits the translation of mRNAs in a dose-dependent manner; however, translation is alleviated as mRNAs compete with each other for FMRP binding (Li et al., 2001).

The mechanism of gene regulation by FMRP is still being deciphered, but several models have been proposed to explain how FMRP inhibits translation of mRNAs. One theory revolves around FMRP interfering with the initiation phase of translation by recruiting a 4E-binding protein, cytoplasmic FMRP-interacting protein (CYFIP1), to block the association of the eIF4F complex with the 5' N⁷-

methyl guanosine caps of mRNAs (Figure 1.4a). Biochemical data has shown that CYFIP1 and eIF4E not only coimmunoprecipitate with FMRP, but are also more likely to form a complex together in the presence of capped mRNA (Napoli et al., 2008).

As the majority of FMRP is associated with polyribosomes, the previously mentioned mechanism probably only represents a small aspect of FMRP function *in vivo*. Some researchers have suggested that FMRP represses the translation of mRNAs on stalled ribosomes during elongation (Figure 1.4b). This comes about from data exhibiting that FMRP is still bound to mRNAs associated with stalled ribosomes even after puromycin treatment (Stefani et al., 2004, Darnell et al., 2011). Furthermore, ribosome “runoff” experiments validated that FMRP still interacts with ribosomes after sodium azide blocks translational initiation, suggesting that FMRP somehow interferes with the elongation step of translation (Ceman et al., 2003).

The most current research on the mechanism of translational inhibition by FMRP also supports the notion that FMRP interferes with elongation by blocking the binding of eEF1A-GTP-aminoacyl-tRNA ternary complex and eEF2 to the ribosome (Chen et al., 2014). Structural cryo-electron microscopy (cryo-EM) data shows that FMRP binds directly to the 80S ribosome near ribosomal protein L5 through its KH domains, while the RGG box sits closer to the A-site on the ribosome where it functions to selectively bind G-quadruplex forming mRNA (Brown et al., 2001, Darnell et al., 2001, Schaeffer et al., 2001, Chen et al., 2014).

dFMRP has been shown to be involved in the RNA interference (RNAi) pathway by associating with Argonaute2 and Dicer proteins, which are essential components of the RNA-induced silencing complex (RISC) (Ishizuka et al., 2002). Since FMRP can also associate with micro RNAs (miRNAs), it has been suggested that FMRP forms a complex with miRNAs and RISC in order to block the translation of mRNAs to which it is bound to (Jin et al., 2004). It is still not clear which part of translation this FMRP/miRNA/RISC complex interferes with (Figure 1.4c).

Lastly, post-translational modifications of FMRP can also affect its role as an inhibitor of translation. For example, a highly conserved serine residue at position 500 (S500) in human FMRP needs to be phosphorylated in order for polyribosome association to occur. When S500 is not phosphorylated, FMRP does not associate with stalled ribosomes and translation of mRNAs is not inhibited (Ceman et al., 2003). The same is the case for FMRP found in fruit flies and mice. The RGG box in FMRP contains four critical arginine residues that can be methylated. Methylation of the arginine residues has been shown to result in reduced binding to G-quadruplex forming RNAs (Stetler et al., 2006, Blackwell et al., 2010). Therefore, post-translation modifications of FMRP can potentially be attributed to controlling certain parts of translational inhibition. Since the presented models of the translational repression are all plausible, it could be that FMRP utilizes a variety of methods to regulate protein synthesis under different conditions. This is an exciting aspect of FMRP function that needs to be further investigated.

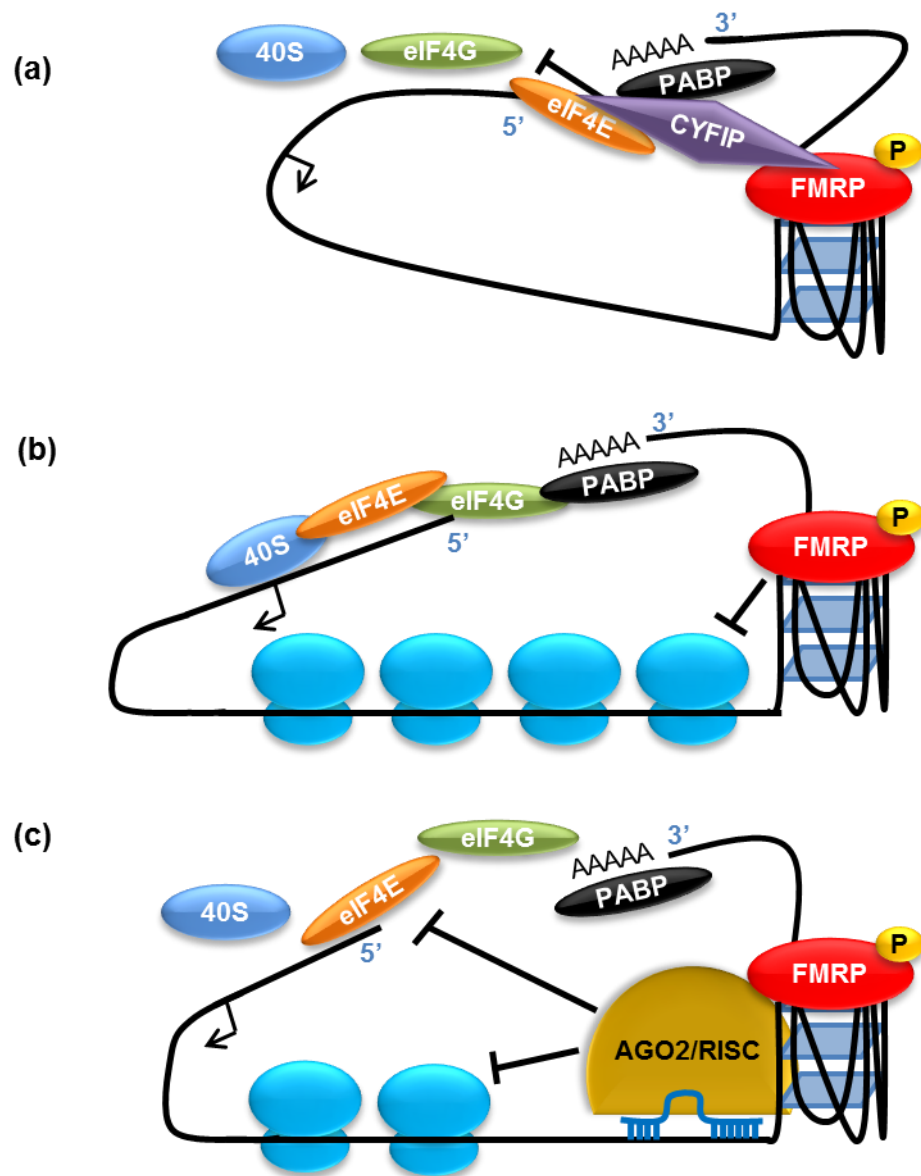


Figure 1.4: Proposed models of translational repression by FMRP.

(a) Phosphorylated FMRP recruits CYFIP1 and eIF4E to block the formation of the eIF4A-eIF4G-eIF4E (eIF4F) complex and interfere with the initiation phase of translation. **(b)** Phosphorylated FMRP stalls ribosomes during translational elongation. **(c)** Phosphorylated FMRP forms a complex with miRNAs and RISC to inhibit the translation of target mRNAs (Santoro et al., 2012).

1.6 A Lack of FMRP Results in Dysregulation of the mGluR-LTD Pathway

The discovery of FMRP's involvement in certain neurological pathways has paved the way for potential therapeutic routes to treat the symptoms of FXS. One of the most extensively studied pathways that FMRP plays a role in is that of Group 1 metabotropic glutamate receptor-long term depression (mGluR-LTD), which influences learning and memory in the brain (Huber et al., 2000). Overtime, changes in long term depression (LTD) and long term potentiation (LTP) result in either weakening or strengthening of synapses, respectively.

Activation of mGluR-LTD requires the synthesis of proteins necessary for synaptic function (Weiler et al., 1997). In *Fmr1* knockout mice, excessive activation of mGluR-LTD and internalization of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) is found (Snyder et al., 2001). This suggests that FMRP acts to repress the translation of synaptic proteins required for mGluR-LTD (Figure 1.5a). Without any regulation by FMRP, proteins are overexpressed and an imbalance between LTD and LTP is created that negatively affects neurological function (Sidorov et al., 2013).

Interestingly, FMRP does not directly affect protein synthesis required for late-phase LTP (L-LTP) (Figure 1.5b), which implies that FMRP specifically and locally regulates the translation of target mRNAs involved in LTD at dendritic spines (Figure 1.5c). However, this does not rule out the possibility that FMRP regulates the synthesis of proteins involved in mGluR-dependent assistance of LTP (Figure 1.5d).

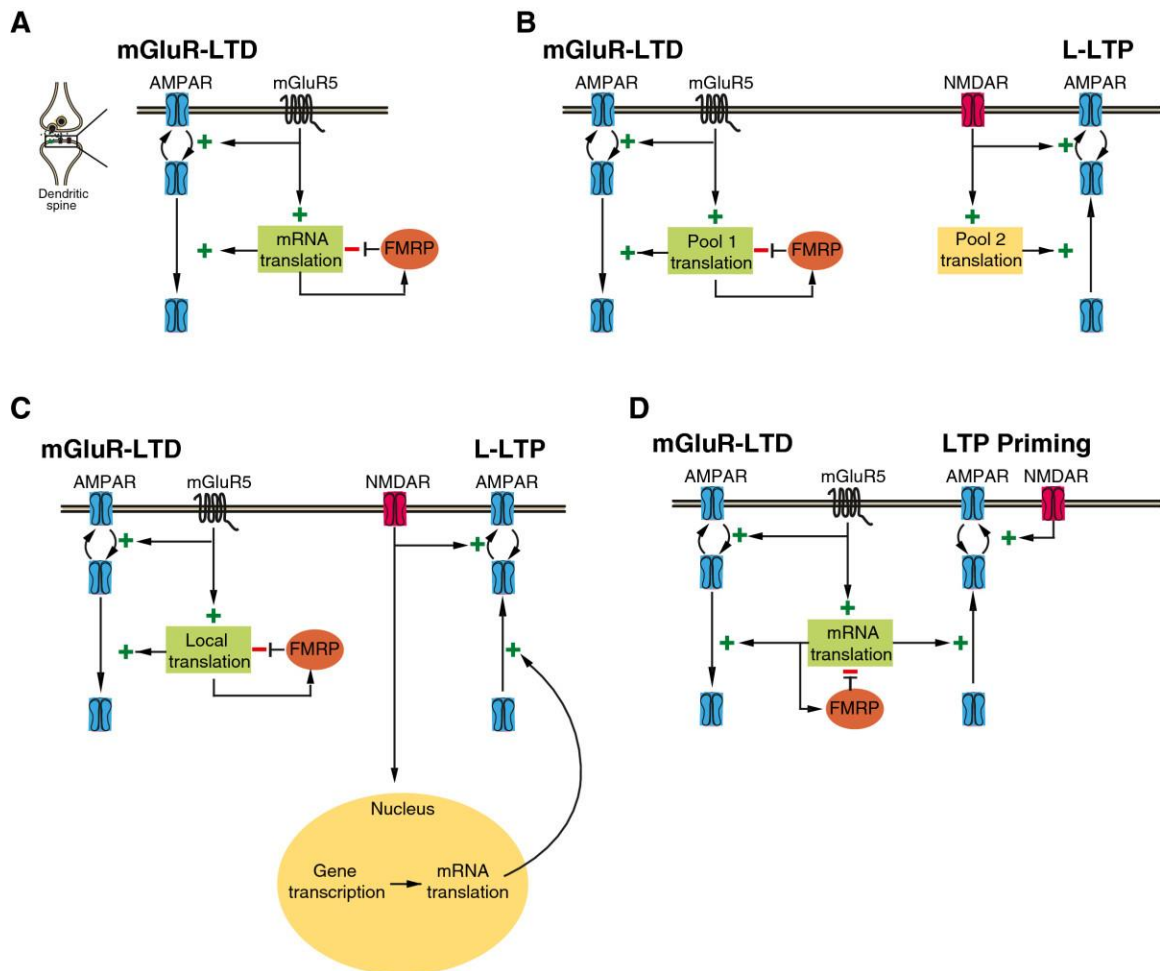


Figure 1.5: FMRP's role in the mGluR-LTD neurological pathway. (a) A lack of FMRP results in the overexpression of proteins implicated in mGluR-LTD. Exaggerated LTD and AMPAR internalization occur. (b) FMRP is probably not directly involved in L-LTP. (c) FMRP is involved in local translational repression at dendritic spines. (d) FMRP regulates protein synthesis involved in mGluR-dependent assistance of LTP. Figure was originally published in Sidorov et al., 2013.

1.7 Potential Therapeutic Treatments for FXS Patients

Although there is currently no cure for FXS, many researchers are devoting their efforts into treating the symptoms of the disorder by targeting the neurological and signaling pathways that FMRP is involved in. In mice, negative allosteric modulators (NAMs) of mGluR5 including fenobam, AFQ056/Mavoglurant (Novartis), CTEP (Hoffmann-La Roche), STX107 (Seaside Therapeutics), and RO4917523 (Hoffmann-La Roche) are able to correct some of the physical and behavioral issues associated with FXS (Braat et al., 2014). Fenobam is able to treat deficits in motor learning and avoidance behavior, as well as change the density of dendritic spines in neurons (de Vrij et al., 2008). AFQ056 can also target abnormal spine morphology and treat behavioral problems (Levenga et al., 2011). Short-term usage of CTEP was shown to decrease the occurrence of seizures due to audiogenics and stabilize mGluR5-LTD, while the effects of chronic treatment included a partial rescue of the macroorchidism phenotype. Antagonists of mGluR1 such as JNJ16259685 and LY367385 also show some promising effects in treating FXS (Thomas et al., 2012).

Astonishingly, mRNA targets of FMRP can also be singled out to pave the way to correct phenotypes of FXS. When expressed levels of amyloid precursor protein (APP) or the striatal-enriched protein tyrosine phosphatase (STEP) are genetically reduced in *Fmr1* knockout mice, a lower susceptibility to audiogenic seizures is exhibited and social anxiety issues are improved (Westmark et al.,

2011, Goebel-Goody et al., 2012). Furthermore, a reduction of APP also resulted in improved spine morphology and normalized levels of mGluR-LTD. Since APP plays a role in the formation and maturation of dendritic spines and STEP dephosphorylates proteins involved in synaptic plasticity, mGluR-LTD proteins that are overexpressed in individuals with FXS can be used as targets to reverse the effects that result from a lack of their translational regulation (Baat et al., 2014). This reinforces the importance of having a clear understanding of the RNA-binding specificity of FMRP.

Chapter 2: Fluorescence Anisotropy Assay to Study FMRP-RNA Binding and Biophysical Characterization of Fluorescent RNAs

Introduction

The fragile X mental retardation protein has an intrinsic property of selectively binding mRNA targets (Siomi et al., 1993, Brown et al., 1998). This feature allows FMRP to regulate the synthesis of proteins important for synaptic function. Since FXS is caused by a loss of expression of the FMR1 gene, a lack of FMRP results in the dysregulation of pre- and post-synaptic proteins that consequently attribute to the phenotypes of the condition. Fairly recent advances in the field have identified hundreds of mRNA transcripts that bind to FMRP (Sung et al., 2000, Brown et al., 2001, Schaeffer et al., 2001, Zhang et al., 2001, Chen et al., 2003, Miyashiro et al., 2003, Zalfa et al., 2003, Dolzhanskaya et al., 2003., Todd et al., 2003, Darnell et al., 2011). Although there is some overlap in the identified mRNA targets, very few have actually been validated through biochemical and biophysical experiments (Santoro et al., 2012). More importantly, there is still no consensus as to which sequences in mRNA targets are recognized by the different RNA-binding domains of FMRP.

One of the few aspects of the RNA-binding function of FMRP that various research groups have agreed upon is that the RGG box in FMRP is likely responsible for recognizing G-quadruplex forming RNA (Brown et al., 2001, Darnell et al., 2001, Schaeffer et al., 2001, Ramos et al., 2003, Zanotti et al.,

2006, Phan et al., 2011). When it comes to the RNA-binding specificity of the KH domains, little is known. One group proposed that the KH2 domain binds to kc2 RNA that forms a loop-loop pseudoknot structure (Darnell et al., 2005). This structure is not found in endogenous mRNA, so the relevance of this interesting finding remains questionable. Tuschl and co-workers proposed that the KH1 and KH2 domains specifically recognize WGGA (W=A/U) and ACUK (K=G/U) sequences, respectively (Ascano et al., 2012). It has recently been proposed by our lab that the KH domains of N-terminally truncated dFMRP (NT-dFMRP) act to dock FMRP onto the ribosome in order to repress the translation of mRNAs (Chen et al., 2014).

With all the exciting new proposals presented, there remains a need for the validation of RNA ligands that interact exclusively with the KH domains. More specifically, the exact roles that the KH domains and the RGG box play in binding mRNAs need to be deciphered. It is important to figure out whether these domains work together to bind RNA or act individually to recognize specific sequences or structural motifs on RNA targets. A thorough understanding of FMRP function relies on this knowledge.

In chapter 2, we established a fluorescence anisotropy assay to quantitatively determine the binding affinity of NT-dFMRP to fluorescently-labeled RNA molecules that include PolyG₁₈-FI, PolyC₁₈-FI, PolyC₁₈(ACUU)-FI, and PolyC₁₈(UGGA)-FI. Through a mutational analysis approach, relevant FMRP mutants allowed us to elucidate which RNA-binding domains are able to bind to

these RNAs. We also used biophysical techniques to characterize the RNAs under study.

Results and Discussion

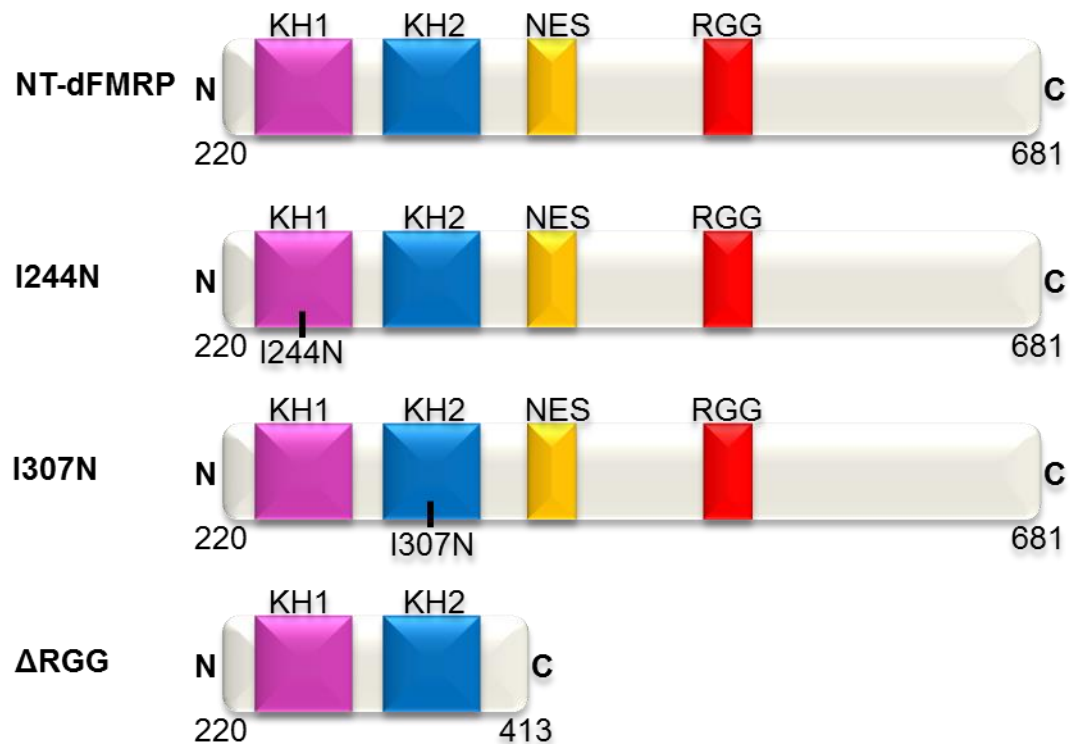
2.1 Purification of NT-dFMRP and Mutants

N-terminally truncated dFMRP (NT-dFMRP) was overexpressed in *E. coli* and purified according to the IMPACT-CN purification system (New England Biolabs). NT-dFMRP was used for the majority of the performed experiments because it is more soluble and less likely to aggregate as compared to the full-length protein (Chen et al., 2014). Not only can this protein be purified to about 95% purity, but it can be concentrated down to levels that are suitable for biochemical experiments. Although it is a truncated protein that is lacking the first 219 amino acids at the N-terminus, it still retains all three RNA-binding domains and has been shown to repress the translation of mRNAs equivalently to full-length dFMRP in an in vitro translation system (Chen et al., 2014).

For our studies, we purified wild-type NT-dFMRP along with I244N, I307N, and Δ RGG mutants (Figure 2.1b). The I244N and I307N mutants are KH1 and KH2 domain mutants, respectively. The Δ RGG mutant is mostly comprised of the KH1 and KH2 domains, as it is lacking the RGG box and the whole C-terminus of the wild-type NT-dFMRP (Figure 2.1a). The I307N mutant in *Drosophila* corresponds to the I304N mutant in humans that was found to cause a severe form of FXS (De Boulle et al., 1993). This mutant has been shown to

abolish polyribosome association and reduce the ability of FMRP to bind to RNA by destabilizing the protein (Feng et al., 1997, Zang et al., 2009). The I244N mutation is analogous to the I307N mutation, but is found in the KH1 domain. When the I244N mutant is overexpressed in transgenic fruit flies; it shows a significantly milder rough eye phenotype as compared to the severe phenotype observed when wild-type dFMRP is expressed. This suggests that the I244N mutant is implicated in a loss-of-function effect in fruit flies possibly associated with disrupted RNA-binding capabilities (Wan et al., 2000, Darnell et al., 2005). Therefore, these proteins are useful candidates for testing the binding of FMRP to target mRNAs through a mutational analysis approach.

(a)



(b)

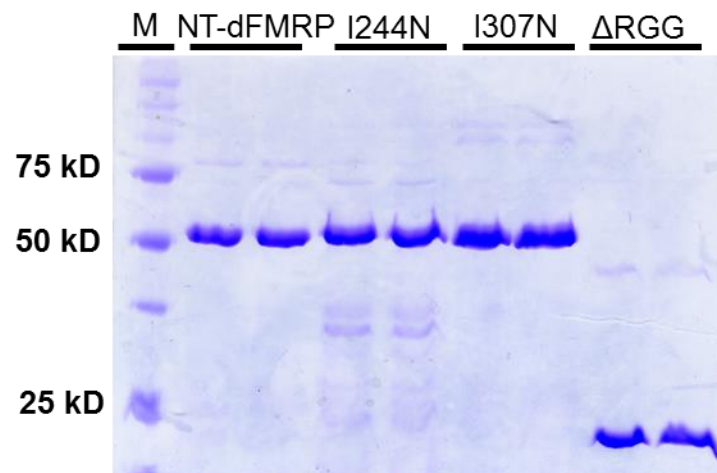


Figure 2.1: Purification of NT-dFMRP and mutant proteins under study. (a) Cartoon representation of the different proteins of interest. NT-dFMRP, I244N, and I307N proteins span from residues 220-681, while the Δ RGG mutant spans from residues 220-413. **(b)** Purified proteins resolved on a 10% SDS-PAGE.

2.2 Characterization of G-quadruplex RNA

We synthesized four RNAs that are each 18 nucleotides long and tagged with fluorescein at the 3' end (Table 2.1). Our intentions were to use a model set of RNAs that have been previously shown to be recognized by FMRP (Brown et al., 1998, Sung et al., 2000, Ascano et al., 2012). The advantage of working with what may seem as simple RNAs is the ability to narrow down factors that affect binding such as sequence, sequence length, and structural features.

Native polyacrylamide gel electrophoresis (PAGE) analysis is commonly used to study the interactions between proteins and nucleic acids as in the case of electrophoretic mobility shift assays (EMSAs), but it can also be used to study the conformation of nucleic acids alone. This is a convenient method to visualize RNA because little amounts of materials are necessary, samples can be studied under a wide range of experimental conditions, and results can be obtained within a few hours (Woodson et al., 2009). After running PolyG₁₈-FI, PolyC₁₈-FI, PolyC₁₈(ACUU)-FI, and PolyC₁₈(UGGA)-FI on a 15% PAGE gel in the presence of 5 mM cacodylic acid pH 7.0 and 100 mM KCl, it was observed that PolyG₁₈-FI's mobility was more obstructed by the gel matrix than the cytosine-rich RNAs (Figure 2.2). Since all the RNAs are equal in length and similar in molecular weight, we hypothesized that PolyG₁₈-FI possibly folds into a G-quadruplex (GQ).

Table 2.1: Sequences of fluorescent RNAs under study.

RNA	RNA Sequence
PolyG ₁₈ -FI	5'-GGGGGGGGGGGGGGGGGGGU-Fluorescein-3'
PolyC ₁₈ -FI	5'-CCCCCCCCCCCCCCCCCU-Fluorescein-3'
PolyC ₁₈ (ACUU)-FI	5'-CCCCCCC ACUU CCCCCCU-Fluorescein-3'
PolyC ₁₈ (UGGA)-FI	5'-CCCCCCC UGG ACCCCCU-Fluorescein-3'

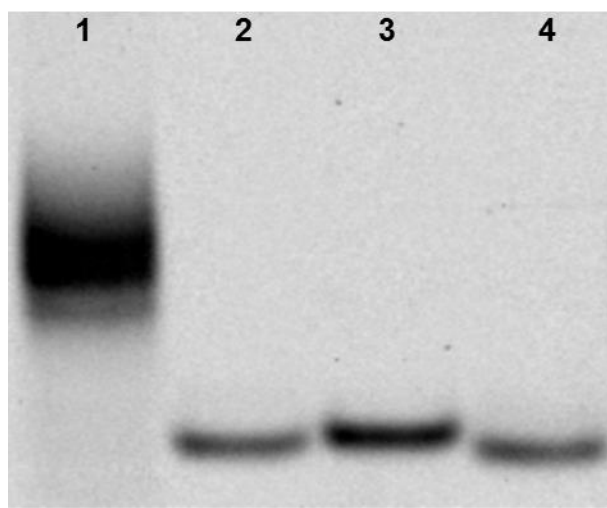


Figure 2.2: Native PAGE analysis of fluorescent RNAs. The visualization of purified PolyG₁₈-FI (1), PolyC₁₈-FI (2), PolyC₁₈(ACUU)-FI (3), and PolyC₁₈(UGGA)-FI (4) RNA in the presence of 5 mM cacodylic acid pH 7.0 and 100 mM KCl. Samples were run on a 15% native PAGE gel containing 40 mM TrisOAc pH 7.5, 12 mM Mg(OAc)₂, and 75 mM KCl. The gel was visualized with the use of a Typhoon scanner (GE Healthcare Life Sciences).

G-quadruplexes take shape when four guanine nucleotides come together to form a square planar tetrad through Hoogsteen base pairing (2.3a). The tetrads are capable of stacking up on each other and the interactions between them are exclusively stabilized by potassium cations (2.3b). In some cases sodium can also stabilize the structure, but lithium has been shown to destabilize GQ structure probably due to atomic size restrictions within the tetrads (Paramasivan et al., 2007).

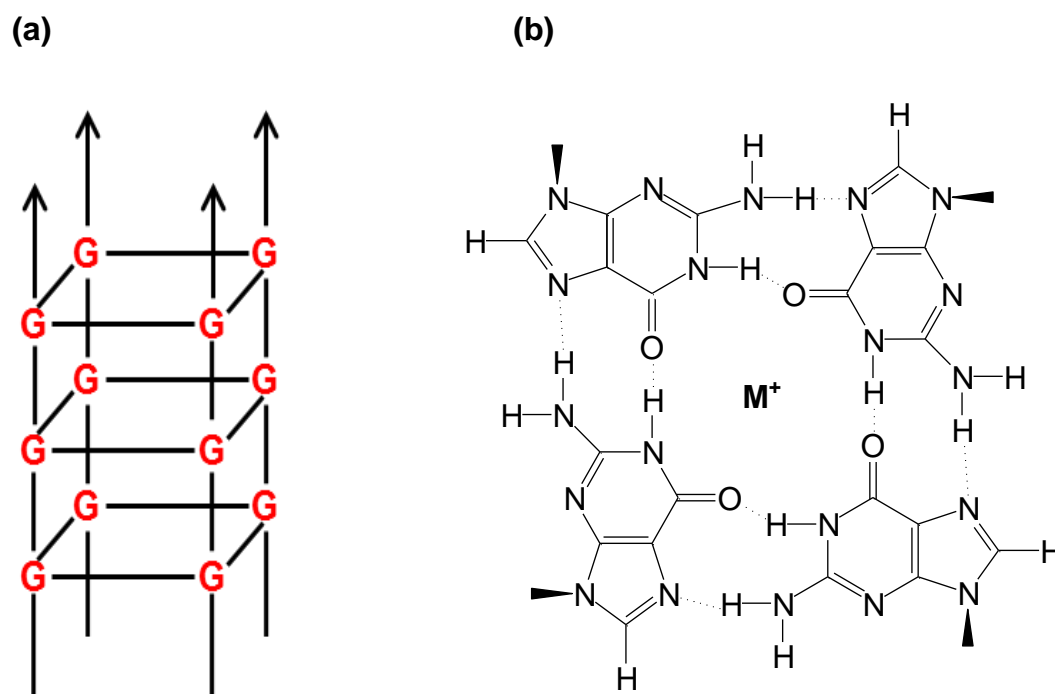


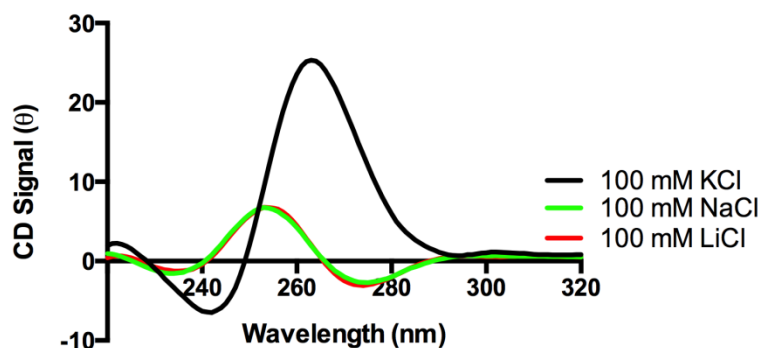
Figure 2.3: Representation of G-quadruplex formation. (a) An example of a parallel GQ conformation displaying three guanine tetrads stacked on top of each other. (b) Four guanine nucleotides come together through Hoogsteen interactions to form a tetrad that can be stabilized by either potassium or sodium cations, but not lithium due to atomic size restrictions (Paramasivan et al., 2007).

We proceeded to use circular dichroism (CD) spectroscopy as a technique to structurally characterize the fluorescent RNAs under study. CD spectroscopy measures the difference in absorption between left circularly polarized light and right circularly polarized light. Since only chiral molecules absorb circularly polarized light, nucleic acids can be studied using this technique. One well-known example is the CD spectroscopy characterization of telomeric repeats as GQs (Paramasivan et al., 2007). Researchers are able to predict structural features of nucleic acids by comparing obtained CD spectra with those that are already established (Bishop et al., 2003). For our experiments, we used a d(TG₄T) DNA oligonucleotide as a positive control as it has been repeatedly shown by CD spectroscopy to fold into a parallel GQ structure under varying buffer conditions. (Figure 2.3a) It has been empirically established that parallel GQs in general exhibit a maximum positive peak at around 265 nm and a minimum negative peak around 240 nm, while antiparallel GQs tend to have a maximum and minimum at 295 nm and 260 nm, respectively. From our results, we showed that d(TG₄T) does indeed form a parallel GQ under buffer conditions that include 10 mM cacodylic acid pH 7.0 and 100 mM KCl (Figure 2.4a). Moreover, GQ structure is perturbed in the presence of both 100 mM NaCl and 100 mM LiCl to a similar extent (Figure 2.4a). This is shown by the drastic decrease in CD signal (>50%) and the apparent shift in the peak from 265 nm to 250 nm and the minimum peak from 240 nm to about 275 nm.

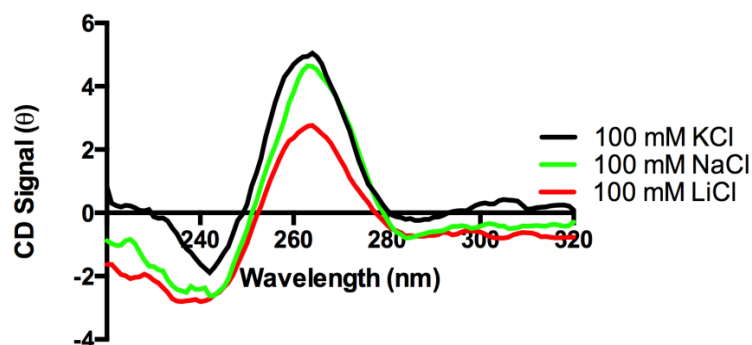
Interestingly, we found that PolyG₁₈-FI also exhibits the same characteristics as parallel GQs with a maximum positive peak at 265 nm and a

minimum peak at 240 nm (Figure 2.4b). The CD signal is seemingly lower than in the case of d(TG₄T) due to the lower concentration of RNA used. Although the structure is only slightly destabilized in the presence of 100 mM NaCl, there is a stark decrease in CD signal in the presence of 100 mM LiCl (Figure 2.4b). This indicates that the GQ structure of PolyG₁₈-FI is exclusively stabilized by potassium. Furthermore, the fluorescein tag on the 3' end of the RNA does not seem to affect GQ formation. When PolyC₁₈-FI, PolyC₁₈(ACUU)-FI, and PolyC₁₈(UGGA)-FI RNAs were tested under buffer conditions that contain 10 mM cacodylic acid pH 7.0 and 100 mM KCl, they all had a maximum CD signal at around 275 nm and a slight minimum peak at 235 nm (Figure 2.4c). This trend is also seen in d(CCCAAT) DNA under neutral pH conditions, however; there is a shift in the CD spectrum when acidic conditions are used (Kypr et al., 2012). Under a pH of 5.0, the maximum CD signal of d(CCCAAT) shifts to 287 nm where it is proposed to form a cytosine-quadruplex (CQ). Therefore, it is very probable that our cytosine-rich RNAs can fold into CQs under more acidic conditions.

(a)



(b)



(c)

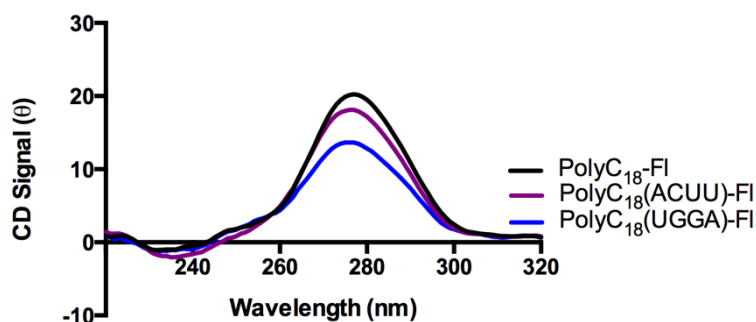


Figure 2.4: Circular dichroism spectroscopy. (a) CD spectra of 10 μM d(TG₄T) in the presence of 10 mM cacodylic acid pH 7.0 and 100 mM KCl (black), 100 mM NaCl (green), or 100 mM LiCl (red). (b) CD spectra of 1 μM PolyG₁₈-FI tested in the previously mentioned buffer conditions. (c) CD spectra of 1 μM PolyC₁₈-FI (black), 1 μM PolyC₁₈(ACUU)-FI (purple), and PolyC₁₈(UGGA)-FI (blue) in the presence of 10 mM cacodylic acid pH 7.0 and 100 mM KCl.

2.3 Fluorescence Anisotropy Assay to Study FMRP-RNA Binding

In order to study the binding affinity of NT-dFMRP to PolyG₁₈-FI, PolyC₁₈-FI, PolyC₁₈(ACUU)-FI, and PolyC₁₈(UGGA)-FI RNAs, we established a fluorescence anisotropy assay that would aid us in quantitatively determining the equilibrium dissociation constant (K_D) of the binding partners. Fluorescence polarization or anisotropy is a tool for the direct measurement of the ratio of the bound-to-free state of a fluorescently-labeled molecule. Unlike electrophoretic mobility shift assays, fluorescence anisotropy allows for the study of molecular binding events in solution under conditions where true equilibrium can be achieved (Invitrogen 2006).

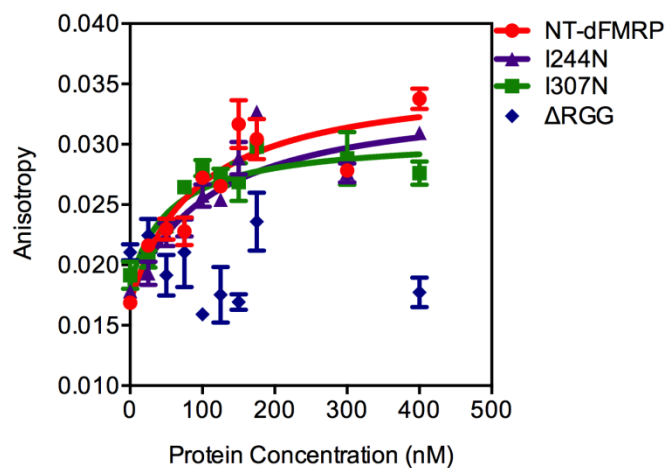
Fluorescence anisotropy takes advantage of the tumbling properties of fluorescently-labeled molecules as they bind to larger macromolecules. Exciting a free fluorophore such as an RNA molecule with plane-polarized light will result in a relatively rapid tumbling motion due to the size of the molecule. In contrast, slow tumbling occurs when two molecules are bound due to the overall increase in size of the complex (Lea et al., 2011). Rapid tumbling causes a depolarization of emitted light while slow tumbling causes polarization of emitted light. One of the very few limitations of fluorescence anisotropy is the size of the ligand as compared to its binding partner, which shouldn't be too large or else anisotropy values will be too minor to observe sufficient changes (Invitrogen 2006).

For our experiments, we set up a series of titrations with increasing concentrations of NT-dFMRP, while maintaining a constant concentration of 5 nM

fluorescein-labeled RNA. In each case, the binding buffer contained 20 mM Tris-HCl pH 7.65, 5 mM MgCl₂, 300 mM KCl, 1 mM DTT, 0.1 mg/mL BSA, and 100 ng/μL transfer RNA (tRNA) to prevent nonspecific binding (Ascano et al., 2012). The binding of NT-dFMRP to PolyG₁₈-FI resulted in a K_D of 87 ± 18 nM (Figure 2.5a). The KH1 domain I244N and the KH2 domain I307N mutants bound PolyG₁₈-FI with an affinity of 105 ± 14 nM and 52 ± 22 nM, respectively (Figure 2.5a). In all three cases, saturation was reached by 400 nM of protein. The ΔRGG mutant exhibited no apparent binding to PolyG₁₈-FI as up to 400 nM of protein was titrated into the samples. This is indicated by a lack of an increase of anisotropy and relatively scattered data points. The same trend is observed when increasing concentrations of NT-dFMRP were titrated into solutions containing 5 nM PolyC₁₈-FI, PolyC₁₈(ACUU)-FI, and PolyC₁₈(UGGA)-FI (Figure 2.5b).

Since NT-dFMRP, I244N, and I307N bind PolyG₁₈-FI with a similar affinity, our results demonstrate that the critical mutations in the KH domains have no significant effect on binding GQ RNA. Furthermore, our results hint that the RGG box in NT-dFMRP is likely responsible for binding GQ RNA, especially since the ΔRGG mutant that is lacking the RGG box does not seem to bind to PolyG₁₈-FI. For our experiments, PolyC₁₈-FI served as a negative control and we wanted to see if adding the ACUK and WGGA RNA recognition elements proposed by Tuschl's group would allow for binding. It seems that single ACUU and UGGA nucleotide insertions into the PolyC₁₈-FI RNA are not enough for NT-dFMRP association. The KH domains in FMRP theoretically should be capable of

(a)



(b)

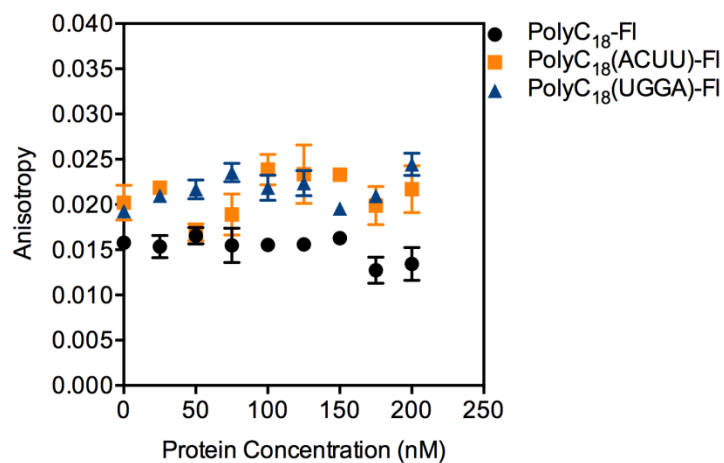


Figure 2.5: Fluorescence anisotropy assay. (a) Binding curves of NT-dFMRP, I244N, and I307N to 5 nM PolyG₁₈-FI. ΔRGG exhibits no apparent binding to 5 nM PolyG₁₈-FI. **(b)** NT-dFMRP exhibits no apparent binding to 5 nM PolyC₁₈-FI, PolyC₁₈(ACUU)-FI, and PolyC₁₈(UGGA)-FI.

specifically binding a four nucleotide sequence just like the KH domains of the neuro-oncological ventral antigen (NOVA) family of proteins, but probably to a weaker extent than oligonucleotides that contain multiple ACUK/WGGA sequences (Lewis et al., 2000, Ascano et al., 2012). Therefore, we have current efforts in our lab to synthesize fluorescently-labeled RNAs that contain multiple ACUK/WGGA sequence repeats in order to truly decipher the specificity of the KH domains in FMRP.

Other groups have demonstrated that FMRP shows preferential binding to homopolymer RNAs in the order of poly(G)>>poly(U)>>poly(A)>>poly(C), but have not given a biophysical explanation as to why FMRP is more likely to bind to poly(G) than poly(C) (Siomi et al., 1993, Brown et al., 1998, Sung et al., 2000, Ascano et al., 2012). Since a good number of mRNAs that encode pre- and post-synaptic protein implicated in FXS phenotypes have been shown to contain GQ structures, we propose that FMRP binds to these targets through the RGG box. This supports our lab's most recent proposal that FMRP acts as a general repressor of translation by binding directly to the 80S ribosome through its KH domains, while the RGG box is responsible for selectively binding GQ RNA since it sits near the A-site on the ribosome. (Chen et al., 2014) This however does not rule out the possibility that FMRP also targets ACUK/WGGA sequences in mRNAs for other functional reasons. More research in this area needs to be done in order to clarify the specificity of the KH domains.

Chapter 3: Conclusion and Future Directions

In conclusion, we have contributed a fluorescent anisotropy assay to the field to carefully validate targets of FMRP in an effort to gain more insight of the RNA-binding specificity of NT-dFMRP. To our knowledge, this is the first time fluorescence anisotropy has been used to quantitatively study the binding affinity of FMRP to fluorescently-labeled RNAs. The results obtained from our fluorescence anisotropy assay and biophysical experiments indicate that NT-dFMRP is a specific RNA-binding protein in that it preferentially binds the G-quadruplex forming PolyG₁₈-FI RNA over PolyC₁₈-FI, PolyC₁₈(ACUU)-FI, and PolyC₁₈(UGGA)-FI RNAs. Furthermore, we have reaffirmed that the RGG box is responsible for recognizing and binding GQ RNA since the KH domain mutants bind PolyG₁₈-FI with a similar affinity as wild-type NT-dFMRP. In contrast, NT-dFMRP that is lacking the RGG motif does not bind to PolyG₁₈-FI.

We are currently in the process of synthesizing fluorescently-labeled RNA molecules that contain multiple ACUK/WGGA sequences (Table 3.1). We hope to use our new fluorescence anisotropy assay to test the binding of these RNAs to NT-dFMRP, I244N, and I307N in order to clarify the specificity of the KH domains. In the future, it would of interest to further validate our fluorescence anisotropy assay by a mutational analysis of the RGG box. Since there are certain residues in the RGG box that have been shown to be critical for binding G-quadruplex RNA (Arginine 533 and 538 in mouse FMRP), it would be interesting to mutate these residues in NT-dFMRP to see if binding to G-

quadruplex RNA is disrupted (Phan et al., 2011). Lastly, we also have ongoing efforts in the lab to express and purify full-length human FMRP. If we are able to obtain hFMRP to concentrations that are suitable for biochemical experiments, we should be in the position to study the RNA-binding specificity of human FMRP.

Table 3.1: Sequences and lengths of NF1 and NF1 ACUK, WGGA(-) fluorescent RNAs.

NF1	5'-Fluorescein-GAAUUC GGA UCAUUU UGUUGGACU CAAUUUCAACUCUAACUUUAACUUUGCAUUGGU UGGACACCUUCGGAUC-3'
NF1 ACUK, WGGA(-)	5'-Fluorescein-GAAUUC aa AUCAUUUU aaUaaAaaCA AUUUCAaaaUAAAAUAAAAUUGCAUUAAUAAA CACCUUCGGAUC-3'

Chapter 4: Materials and Methods

4.1 Purification of NT-dFMRP, I307N, I244N, and Δ RGG Proteins

PTXB1 constructs transformed into either DH5 α or Rosetta (DE3) competent cells coding for NT-dFMRP, I307N, I244N, and Δ RGG proteins with C-terminal intein tags were obtained from Eileen Chen (Chen et al., 2014) and purified according to the IMPACT-CN manual (New England Biolabs). In brief, two 1L bacterial cultures grown in Luria Broth (LB) media originally treated with ampicillin and chloramphenicol were each inoculated with 10 mL of starter culture and grown for approximately 3.5 hours until the optical density (OD₆₀₀) was about 0.6. After the appropriate OD was reached, cultures were treated with 0.8 mM IPTG and incubated in a shaker overnight at 15°C. The cells were spun down the next morning in a 4°C centrifuge at 5,000 rpm for 15 minutes.

The cells were resuspended in 75 mL of cell lysis buffer (24 mM HEPES pH 7.5, 500 mM NaCl, 1 mM EDTA pH 8.0, and 0.1% Triton X-100), sonicated, and spun down twice at 20,000 x g for 30 minutes at 4°C. Working in parallel, about 7 mL of chitin bead slurry was loaded onto a column and equilibrated with column buffer (24 mM HEPES pH7.5, 500 mM NaCl, 1 mM EDTA, and 10% glycerol). The supernatant containing the protein was then passed through the column at a rate of 0.8 mL/min. The beads were then washed with at least 250 mL of column buffer at a rate of 2.0 mL/min.

Following elution, the proteins were placed in 6-8 kD Spectra/Por molecularporous membrane tubing (Spectrum Laboratories) and dialyzed overnight directly into storage buffer (24 mM HEPES pH 7.5, 375 mM NaCl, 1 mM DTT and 10% glycerol). Lastly, the proteins were concentrated down to a volume of at least 500 μ L through the use of 10K Amicon Ultra-15 centrifugal filter units (Millipore), flash-frozen using dry ice and ethanol, and stored in a -80°C freezer.

4.2 Purification of Fluorescent RNAs

PolyG₁₈-FI, PolyC₁₈-FI, PolyC₁₈(ACUU)-FI, and PolyC₁₈(UGGA)-FI RNAs were ordered on a 0.2 μ mol scale with a fluorescein tag attached at the 3' end from Thermo Scientific/Dharmacon. In order to prepare the RNA for gel purification, 400 μ L of the provided 2'-deprotection buffer (100 mM acetic acid-TEMED pH 3.8) was added to each tube. The RNA was subsequently resuspended in deprotection buffer by pipetting, vortexed for 10 seconds, and centrifuged for 10 seconds at 13,000 rpm. This was followed by a 30 minute incubation time in a 60°C water bath. Lastly, a SpeedVac was used to completely dry down the RNA before resuspension in 80-100 μ L of H₂O.

A 10% denaturing polyacrylamide gel (1.5 mm thick) was prepared ahead of time and pre-run in 1X TBE running buffer for 30 minutes at 35 Watts before the samples were loaded onto it. The RNA was mixed with gel-loading dye, heated at 95°C for 1 minute in a heat block, and immediately placed on ice to

cool down. The gel was run for at least 1.5 hours at room temperature and the RNA was visualized by UV-shadowing.

After the purified RNA was cut out from the gel, it was placed in a 1.5 mL microfuge tube and 500 μ L of RNA elution buffer (0.5 M sodium acetate pH 5.2, 0.1 mM EDTA pH 8.0, 0.1% SDS) was added to it. The tubes were immediately placed in an Eppendorf 5432 mixer in a 4°C refrigerator and were left shaking overnight. The RNA was recovered from the gel pieces by three rounds of chloroform extraction followed by ethanol precipitation. After incubating in a -80°C freezer for at least 3 hours, samples were spun down at 13,000 rpm for 30 minutes. Lastly, the RNA pellet was dried down for 2 minutes using a SpeedVac and resuspended in a small volume of H₂O. The concentration of the RNA was taken using a spectrophotometer and purified RNA samples were stored in small aliquot volumes in a -80°C freezer.

4.3 Native Gel Analysis of Fluorescent RNAs

A 15% native polyacrylamide gel (1.5 mm thick) containing 40 mM TrisOAc pH 7.5, 12 mM Mg(OAc)₂, and 75 mM KCl was made using a 40% w/v acrylamide: bisacrylamide (19:1) solution (Omnipur, Calbiochem). The gel was then allowed to polymerize for at least an hour before use. 20 μ L total samples composed of 0.25 pmoles of PolyC₁₈-FI, PolyC₁₈(ACUU)-FI, or PolyC₁₈(UGGA)-FI and a final concentration of 5 mM cacodylic acid and 100 mM KCl were prepared. In the case of PolyG₁₈-FI, 6 pmol of RNA was used. The gel was pre-

run at 11 Watts in 1X buffer (40 mM TrisOAc pH 7.5, 12 mM Mg(OAc)₂, 75 mM KCl) for at least 1.5 hours in a cold room. The samples were heated at 95°C for 5 minutes in a heat block and slow-cooled to room temperature in a water bath to ensure proper folding. A gel-loading buffer containing only 30% glycerol was subsequently added to the samples. Dyes such as xylene cyanol and bromophenol blue weren't added to the gel-loading buffer because they fluoresce under certain conditions. Lastly, the gel was run for 14 hours overnight at 4°C and scanned using a Typhoon 9410 instrument the next morning.

4.4 Fluorescence Anisotropy Assay

In order to study the binding of fluorescently-labeled RNAs to NT-dFMRP, I307N, I244N, and Δ RRG mutant proteins, a series of titrations were set up in which samples contained increasing concentrations of protein but an equal concentration of fluorescent RNA. Each sample contained a final concentration of 5 nM PolyG₁₈-FI, PolyC₁₈-FI, PolyC₁₈(ACUU)-FI, or PolyC₁₈(UGGA)-FI while a range between 0-400 nM of final protein concentration was used. Each sample was composed of a total volume of 800 μ L containing a binding buffer with a final concentration of 20 mM Tris-HCl pH 7.65, 300 mM KCl, 5 mM MgCl₂, 1 mM DTT, 100 ng/ μ L tRNA, and 0.1 mg/mL BSA that was freshly made the day of testing (Ascano et al., 2012).

Samples were prepared on ice and allowed to come to room temperature in a drawer for exactly 30 minutes before being placed in a 10 mm path-length

clear quartz cuvette and scanned using a Fluoromax-P (HORIBA Jobin Yvon) instrument. Under the “Fluormax-3 with Autopolarizers” option, the instrument was set to measure and display polarization, anisotropy, VV, VH, HH, and HV values. The excitation wavelength was set to 494 nm and the emission wavelength was set to 520 nm. The band pass was set to 5 nm and the integration time on the instrument was set to 1 second with a standard error of 1%. Using the constant wavelength analysis (CWA) feature on the fluorometer, each sample was consecutively scanned three times and the average was used to calculate the final anisotropy value. The K_D was determined by fitting the binding curves to the following equation (Daum et al., 2009):

$$r([R]_t) = r_0 + c \left[\frac{K_D + [L]_t + [R]_t}{2} - \sqrt{\frac{(K_D + [L]_t + [R]_t)^2}{4} - [L]_t[R]_t} \right]$$

4.5 Circular Dichroism Spectroscopy

A d(TG₄T) oligonucleotide was ordered from IDT on a 1 μmole scale and resuspended in 100 uL of H₂O. 500 μL total samples were prepared with a final concentration of 10 μM d(TG₄T) in a buffer containing 10 mM cacodylic acid pH 7.0 and 100 mM KCl/100 mM NaCl/100 mM LiCl, depending on the sample. Previously purified homopolymer fluorescent RNAs (Section 4.2) were prepared

at a final concentration of 1 μM in the same buffer. Buffer control samples were identical, but did not contain any DNA or RNA.

The samples were heated at 95°C in a heat block for 5 minutes and slow-cooled to room temperature in a water bath in order to allow proper folding of oligonucleotides. The samples were placed in a clear 10 mm path-length quartz cuvette and scanned every 1 nm from 220-320 nm with a 1 second averaging time on an Aviv CD spectrometer. Each sample was scanned three times with zero waiting time between scans. An average of the three scans was used to calculate the final CD signal for each sample. Samples containing only buffer were used as controls and were subtracted out from each CD signal calculation.

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