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Mechanism of Translational Control by the Fragile X Mental Retardation  
Protein

A dissertation submitted in partial satisfaction of the requirements for the  
degree Doctor of Philosophy

in

Chemistry

by

Eileen Chen

Committee in charge:

Professor Simpson Joseph, Chair  
Professor Xiang-Dong Fu  
Professor Gourisankar Ghosh  
Professor Thomas Hermann  
Professor Jing Wang  
Professor Wei Wang

2013

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Chair

University of California, San Diego

2013

## **DEDICATION**

For my dearest parents

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## LIST OF ABBREVIATIONS

|                   |                                      |
|-------------------|--------------------------------------|
| Å                 | Angstrom                             |
| ASF               | A-site finger                        |
| C-terminus        | Carboxy-terminus                     |
| CP                | Central protuberance                 |
| Cryo-EM           | Cryo electron microscopy             |
| DTT               | Dithiothreitol                       |
| <i>E.coli</i>     | <i>Escherichia coli</i>              |
| EDTA              | Ethylenediaminetetraacetic acid      |
| EF                | Elongation factor                    |
| FMRP              | Fragile X Mental Retardation Protein |
| FRET              | Förster Resonance Energy Transfer    |
| FXS               | Fragile X Syndrome                   |
| GTP               | Guanosine triphosphate               |
| kDA               | Kilodaltons                          |
| mg                | Milligram                            |
| µL                | Microliter                           |
| MgCl <sub>2</sub> | Magnesium chloride                   |
| mM                | Millimoles/liter                     |
| mRNA              | Messenger ribonucleic acid           |
| mRNP              | Messenger ribonucleoprotein          |

|            |                                    |
|------------|------------------------------------|
| NaCl       | Sodium chloride                    |
| N-terminus | Amino-terminus                     |
| PAGE       | Polyacrylamide gel electrophoresis |
| RISC       | RNA-induced silencing complex      |
| RNA        | Ribonucleic acid                   |
| SDS        | Sodium dodecyl sulfate             |
| SRL        | Sarcine ricine loop                |
| Tris       | Tris Hydroxymethylaminoethane      |
| tRNA       | Transfer ribonucleic acid          |

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Chapter 3, in full, is currently being prepared for submission for publication of the material. Eileen Chen, Manjuli R. Sharma, Rajendra K. Agrawal, and Simpson Joseph. The dissertation author was the primary investigator and author of this material.

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## **ABSTRACT OF THE DISSERTATION**

Mechanism of Translational Control by the Fragile X Mental Retardation  
Protein

by

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Doctor of Philosophy in Chemistry

University of California, San Diego, 2013

Professor Simpson Joseph, Chair

Fragile X syndrome (FXS) is the most common form of inherited mental retardation and is caused by the functional loss of the Fragile X Mental Retardation Protein (FMRP). The estimated prevalence of FXS is 1:2500 males and 1:5000 females. FMRP is an RNA binding protein that is highly expressed in the brain, particularly at the synapse. FMRP is involved

in mRNA transport and in the translational regulation of several neuronal mRNAs. The current model is that FMRP binds to specific sequences or structural motifs in mRNAs at the synapse and suppresses their translation. However, the precise mechanism of translational repression by FMRP is unknown.

To determine the role of FMRP in FXS and elucidate the mechanism of how FMRP represses translation, we used the *Drosophila* model system, as flies lacking FMRP exhibit phenotypes observed in FXS. First, we showed with *in vitro* translation assays that FMRP represses translation of a *Renilla* luciferase reporter mRNA that does not have G-quadruplex or a pseudoknot forming sequence, which are the previously proposed FMRP-binding elements. Mutational analysis then revealed that the KH1 and RGG domains of FMRP are important for translation repression. Additionally, we developed a fluorescence-based, quantitative assay to monitor the binding of FMRP to the 80S ribosome. We found that FMRP binds with high affinity ( $K_D = 20 \pm 3$  nM) to the *Drosophila* 80S ribosome near the 60S large ribosomal subunit protein L5 and this interaction was mRNA-independent.

Finally, a cryo-electron microscopic reconstruction of the 80S ribosome•FMRP complex showed that FMRP binds within the inter-subunit space of the ribosomes such that it would block the binding regions of tRNA and translation elongation factors on the ribosome. These findings suggest a novel mechanism in which the binding of FMRP to the ribosome inhibits

translation by blocking the docking of essential components of the translational machinery to the ribosome. These studies provide insight into the mechanism of translational control by FMRP and may help in identifying potential therapeutic approaches for treating FXS.

## **Chapter 1: Introduction**

## 1.1 Overview of Fragile X Syndrome

Fragile X syndrome (1, 2) (FXS) is the most common form of inherited mental retardation, with a prevalence of 1:2500 males and 1:5000 females. There is currently no cure for this devastating neurological disease. According to the National Fragile X Foundation, FXS afflicts more than 100,000 Americans. The estimated total national health care cost for taking care of FXS patients is \$226 million per year. Additionally, the annual cost of special education for children with FXS is estimated to be \$60 million. Symptoms of FXS include mild to severe cognitive deficits, autistic-like behaviors, abnormal sleep, increased susceptibility to seizures, hyperactivity, and unusual response to sensory stimuli. Patients with FXS show physical features, such as large ears, elongated face, flat feet, and macroorchidism (3). These features suggest a potential connective tissue disorder that has yet to be elucidated in any detail. Postmortem examination also revealed abnormal neuronal architecture showing dense, immature dendrites in FXS patients (3).

FXS is caused by loss of function of a single gene, the fragile X mental retardation gene (*FMR1*), which was cloned in 1991 (4). *FMR1* is located at cytogenetic position Xq27.3, and encodes the Fragile X Mental Retardation Protein (FMRP). FXS is typically caused by a trinucleotide CGG expansion in the 5' untranslated (5'UTR) region on the *FMR1* gene. Normal

individuals have between 6 and 54 repeats, with 29 or 30 repeats being the most common allele. When there are 60 to 200 repeats, it is referred to as a premutation allele and due to instability can evolve into a full mutation (>200 repeats) during the maternal transmission (5). Although premutation alleles do not produce FXS, they can result in fragile X-related primary ovarian insufficiency (FXPOI) and fragile X-associated tremor/ataxia syndrome (FXTAS). FXPOI patients experience onset of menopause at or before the age of 40 and this affects approximately 20% of female premutation carriers (6). However, very little is known about the underlying molecular pathways behind FXPOI. FXTAS is an adult late onset-neurodegenerative disease that usually occurs in individuals over the age of 50. Studies of FXTAS indicate the CGG repeats in the premutation result in increased levels of *FMR1* messenger RNA (mRNA); however, FMRP levels are lower than levels in normal individuals (7, 8). One hypothesis suggests that the increased *FMR1* transcript levels due to expansion of CGG repeats will cause *FMR1* mRNA transcripts to form intranuclear aggregates (9). These aggregates can then recruit RNA-binding proteins, thus titrating the proteins away from their normal function in other cellular locations and resulting in neurodegeneration. When the number of CGG repeats expands over 200, it is known as a full mutation that causes FXS. This leads to DNA hypermethylation, hypoacetylation of associated histones, and chromatin condensation, which results in epigenetic silencing of *FMR1* and the loss of its protein product,

FMRP.

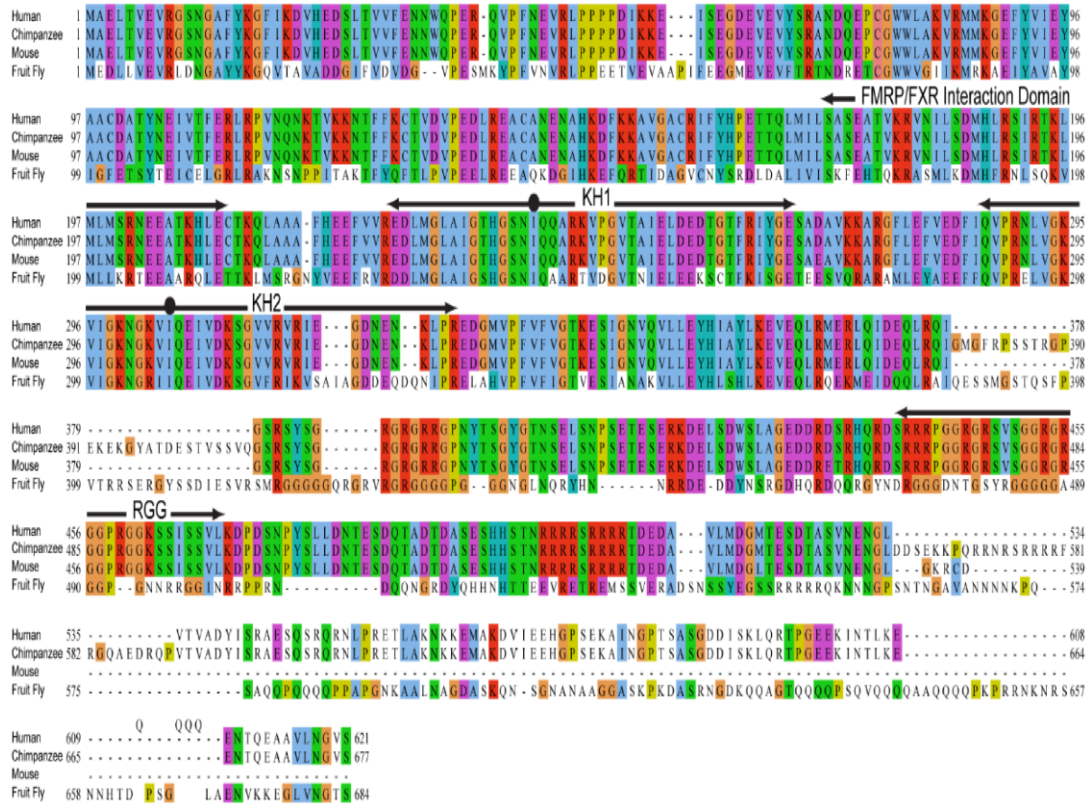
In other cases, missense mutations or deletions of the *FMR1* gene also result in FXS (10-12). In one particular patient, an isoleucine to asparagine point mutation (I304N) in the KH2 domain, one of the RNA-binding domains of FMRP, was identified to cause a severe phenotype of FXS. This point mutation has been meticulously studied and proposed to destabilize FMRP, abolish RNA binding, and abrogate association to polyribosomes (12, 13). However, the exact mechanism of how this I304N point mutation leads to FXS is still unclear. Moreover, recent studies suggest that more comprehensive screening for such mutations may increase the diagnostic yield by 30% compared with screening for CGG repeat length alone (10), which is the standard care for children who exhibit developmental delays, intellectual disability (ID), or autism.

Since FXS is a monogenic and relatively common cause of autism, it has been a useful model for dissecting the pathophysiology that may apply to genetically heterogeneous autisms and ID. As expected, research into FXS has revealed many of the pathways that are crucial to learning and memory formation. Knowledge of these pathways has enabled the rational design of potential therapeutics for FXS. This knowledge can then be applied to our general understanding of other forms of complex disorders, such as Alzheimer's disease and autism.

## 1.2 Model organisms and stem cells for the study of FXS

The *FMR1* gene is highly conserved across species (4) (Figure 1.1), and this has allowed researchers to develop murine (14, 15), *Drosophila* (16, 17), and zebrafish (18, 19) animal models to study the cellular, molecular, and behavioral phenotypes of the disease. The *Drosophila* and mouse models are the most widely used models in the FXS field.

*Mouse models.* *Fmr1*, the *FMR1* ortholog in the mouse, is located on the murine X chromosome, and it shares 97% amino acid similarity with FMRP (20). Efforts to generate a full CGG expansion mouse model were unsuccessful as these mice failed to recapitulate the hypermethylation and transcriptional silencing found in humans (8). Nevertheless, in 1994, the first mouse model was produced by insertion of a neomycin cassette in exon 5 of the *Fmr1* gene, creating a *Fmr1* knockout (KO) which is the functional equivalent of the full mutation in humans. This mouse model presents an array of anatomical, neurological, and behavioral similarities to the phenotype of FXS individuals (14). For example, *Fmr1* KO mice also suffer from cognitive deficiency, although milder than that seen in FXS patients (21-23). Abundance of dense, long, and tortuous immature cortical dendritic spines, demonstrating a delay in maturation, are also encountered in the knockout mice (24). Moreover, macroorchidism, which is a hallmark of FXS patients, is also seen in the knockout mice (14). More recently, a conditional



**Figure 1.1: Amino acid sequence alignment of FMRP.** The amino acid sequence of human, chimpanzee, mouse, and fruit fly FMRP were aligned using COBALT and displayed using JALVIEW. Previously identified functional domains of FMRP are indicated by the arrows and include the FMRP/FXR interaction domain, KH1, KH2, and RGG domains. The highly conserved isoleucine residues that are important for the function of the KH domains are indicated by the filled circles.

*Fmr1* KO (15) (*Fmr1* CKO) and a second generation *Fmr1* KO null for *Fmr1* mRNA (*Fmr1* KO2) have been engineered by flanking the promoter and exon1 of *Fmr1* with loxP sites (15). These latest mouse models will provide innovative ways to explore FMRP's function *in vivo* by facilitating the creation of null alleles in specific cell types and at particular developmental stages. Moreover, another mouse model recently created mimics the mutation I304N that leads to severe manifestation of FXS (25). This mouse model phenocopies the behavioral and electrophysiological deficits observed in *Fmr1* KO mice, thereby allowing an enhanced understanding of the effects of this unusual mutation. Interestingly, I304N FMRP levels are lower than wild-type (WT) FMRP levels, and I304N *Fmr1* mRNA and WT *Fmr1* mRNA have similar distribution profiles on polyribosomes. This suggested that the lower I304N FMRP levels do not relate to translational control, but may relate to increased turnover of the mutant protein, particularly in younger mice. Polyribosome analysis by sucrose density centrifugation also revealed that global translation status was normal in the mutant mice. However, I304N FMRP was largely dissociated from polyribosomes in mouse brain, indicating this mutation impacts the normal function of FMRP on polyribosomes (25).

*Drosophila models.* The fly ortholog of *FMR1*, *dFMR1*, shares ~56% amino acid similarity with mammalian FMRP. Null mutations have been created in *dFMR1* and such models display abnormal neuronal architecture and synaptic function (16, 26, 27). For example, *dFMR1*-null mushroom

bodies exhibit over-branching and increased axonal growth. Other phenotypes demonstrated in these null mutation models are reduced courtship interest (a model of behavioral abnormalities) (28) and long-term memory deficiency (29). The *Drosophila* model provides a more amenable alternative for investigations that are challenging and difficult to study in mice models. For example, *dFMR1* mutant flies were discovered to die during development when reared on food containing increased levels of glutamate, strongly suggesting that *FMR1* loss results in excess glutamate signaling. This observation created the fundamental basis for a small-molecule screen that identified several compounds that could rescue the lethality and phenotypes, such as mushroom body defects, excess *Futsch* translation, and abnormal courtship behavior. These compounds, including  $\gamma$ -aminobutyric acid (GABA) and acetylcholine receptor agonists, point to potential therapeutic approaches for treating FXS and provide beneficial insights into the pathways involved in disease pathogenesis (30).

*Zebrafish Models.* In 2006, the first attempt to produce a model for FXS in *Danio rerio* was done by microinjecting morpholinos in early embryos to create a knockdown of the *FMR1* gene (19). Although the authors observed deficiencies in craniofacial development and abnormal neuronal branching in embryos, further studies could not reproduce this phenotype in two *FMR1* KO lines (18).

*Embryonic and Induced Pluripotent Stem Cells.* One critical missing

piece for a clearer understanding of the FXS disease etiology is the knowledge of the timing and mechanism behind the methylation and gene silencing observed in full mutation alleles. Moreover, as previously described, mouse models with a CGG expansion could not recapitulate the hypermethylation or gene silencing found in humans. Cutting-edge advances in embryonic and induced pluripotent stem (iPS) cell technology are allowing explorations that are difficult to study in animal models. Using human embryonic stem cells (hESCs) and chorionic villi (CV) samples from FXS pregnancies, researchers have found that, at early embryogenesis, when extra embryonic tissue separates from the embryo proper, the FXS full mutation alleles are still active. Therefore, in early development, *FMR1* is expressed, presumably producing transcripts with long CGG tracts, and is silenced between 10 and 12.5 weeks of gestational development (31, 32). However, controversial ethical challenges revolve around continuous usage and generation of hESCs. iPS cells, on the other hand, are a type of pluripotent stem cell artificially derived from non-pluripotent cells such as adult somatic cells. iPS cells bypass controversial ethical problems and recent studies reported the derivation of the first FXS-iPS lines from FXS patients. In contrast, iPS cells generated from the fibroblasts of FXS individuals show that the *FMR1* CGG repeat region remained hypermethylated and transcriptionally silenced, which differed from findings in FXS hESC (33, 34). A conceivable explanation for the discrepancy in

*FMR1* methylation between iPSs and hESCs could be that human iPS cells represent a later stage of development in which silencing of the full mutation has already occurred; hence, FXS-iPS cells may not have all the full characteristics of early pluripotency. Nonetheless, generation of FXS-iPS cells that represent an earlier developmental stage, the ground state/naïve pluripotent stage, is underway, and should unlock important aspects of the epigenetic status of the full mutation (35, 36). Thorough understanding of the differences between hESCs and iPSs could lead to innovative approaches to demethylate the full mutation allele in the FXS patients.

### **1.3 FMRP Function**

From the time when the *FMR1* gene was cloned in 1991 (4), tremendous progress in understanding FMRP's functionality has been made. The combined efforts of basic neuroscience and biochemical characterization has informed and provided key insights into the pathophysiology of FXS. It is now well established that FMRP plays an essential role in synaptic plasticity and dendritic spine development. Loss of FMRP impairs normal synaptic plasticity which results in intellectual disability, and postmortem examination also revealed abnormal dense and immature dendrites in FXS patients (3).

FMRP is widely expressed in mammalian tissues, but is particularly abundant in the brain and testes (37, 38). FMRP is mainly expressed in

neurons, where it is predominantly cytoplasmic, being found in the cell body, dendrites, and synapses (37, 39, 40). However, FMRP contains a nuclear localization sequence and a nuclear export sequence (Figure 1.2) (41), thus some FMRP can shuttle between the cytoplasm and the nucleus (39). The pattern of FMRP expression begins early in development and continues throughout life (38). FMRP has also been shown to homodimerize and interact with several cytoplasmic and nuclear proteins, including cytoplasmic FMRP-interacting protein (CYFIP1), nuclear FMRP-interacting protein (NUFIP), and its own two autosomal paralogs Fragile-X-related proteins 1 and 2 (FXRP1 and FXRP2) (42).

FMRP has also been shown to be a selective RNA-binding protein that mainly functions to negatively regulate protein synthesis in the brain. Sequence analysis first identified the three most common RNA-binding domains in FMRP (41, 43), including two hnRNP (heterogeneous nuclear ribonucleoprotein) K-homology domains (KH1 and KH2) and the arginine-glycine-glycine (RGG) box. The KH motif is one of the most common conserved motifs identified in RNA-binding proteins, and has been discovered in more than 50 proteins. RNA binding is a common function shared among all the KH-containing proteins, with activities ranging from the regulation of alternative splicing to mRNA localization (44). The RGG box is also commonly found in RNA-binding proteins, but is usually in combination with other RNA-binding domains. A fourth domain at the N-terminus of



**Figure 1.2: Domain organization of dFMRP (1 – 681 amino acids).** TD1/TD2, Tudor domain 1/Tudor domain 2; NLS, nuclear localization signal; KH, K-homology domain; NES, nuclear export signal; RGG, motif rich in arginine and glycine.

FMRP that contains two helix-loop-helix-Tudor motifs has been shown to play a role in RNA binding, dimerization, and protein-protein interactions (Figure 1.2) (45). FMRP binds RNA in a sequence-specific manner mediated by these domains. The current working model suggests that FMRP enters the nucleus to bind to its mRNA targets, and then chaperones them out of the nucleus. FMRP acts as a negative regulator of translation and represses translation of this targeted subset of mRNAs. Subsequent transportation of mRNAs to dendrites and axons might occur. Upon receipt of appropriate synaptic stimuli, FMRP derepresses translation, resulting in synthesis of critical synaptic plasticity proteins to occur at a specific time and location.

FMRP has been shown to bind to ~4% of the mRNAs in mammalian brain (41). A key focus for investigators in understanding FMRP function is to identify its RNA targets. Intensive efforts have been made from various

laboratories, however there has been limited success in deriving a consensus set of FMRP mRNA targets, and only a small number of these target mRNAs have been validated by showing direct biochemical interaction. Techniques including coimmunoprecipitation, microarray analysis (46), antibody positioned RNA amplification (APRA) (47), and bioinformatic approaches (46, 48) have identified hundreds of putative mRNAs that associate with FMRP. Nonetheless, these approaches have inherent signal to noise issues (49, 50) and are impeded by difficulties in bioinformatic prediction of complex RNA folding (48, 51). Only 14 of these mRNA targets have been validated through biochemical methods (Table 1.1).

*In vitro* selection experiments have also shown that the KH2 domain of FMRP recognizes “kissing complex” pseudoknot tertiary motifs in RNA (51), whereas the RGG box recognizes a G-quadruplex structure (52), possibly in a methylation-dependent manner (53). Based on these results, it was proposed that FMRP might bind to mRNAs that possess G-quadruplex or pseudoknot forming sequences and repress their translation. A portion of the 14 validated mRNA targets have been shown to form a G-quadruplex-like structures (Table 1), however, the pseudoknot structure has not been observed in endogenous mRNA to this date. In addition, U-rich sequences have also been proposed to be potential RNA-binding motifs (54, 55).

Interestingly, a recent study used high-throughput sequencing of

Table 1.1: 14 validated FMRP target mRNAs.

| mRNA                                       | G-quadruplex-like structure | Method <sup>a</sup>                  | References   |
|--------------------------------------------|-----------------------------|--------------------------------------|--------------|
| <b>App</b>                                 | +                           | Co-IP                                | (58)         |
| <b>Arc</b>                                 | –                           | Co-IP                                | (59, 60)     |
| <b>CamKII<math>\alpha</math></b>           | –                           | Co-IP                                | (61-63)      |
| <b>eEF1A</b>                               | –                           | Co-IP, <i>in vitro</i>               | (64)         |
| <b>Fmr1</b>                                | +                           | <i>In vitro</i>                      | (52, 65)     |
| <b>GABA<sub>A</sub><math>\delta</math></b> | –                           | APRA, <i>in vitro</i>                | (47)         |
| <b>GluR<sub>1/2</sub></b>                  | –                           | Co-IP                                | (61)         |
| <b>hASH1</b>                               | –                           | <i>In vitro</i>                      | (55)         |
| <b>Map1b</b>                               | +                           | Co-IP, <i>in vitro</i> , biophysical | (46, 48, 66) |
| <b>Psd95</b>                               | +                           | Co-IP, <i>in vitro</i> , CLIP        | (61, 67)     |
| <b>Rgs5</b>                                | –                           | APRA, <i>in vitro</i>                | (47, 68)     |
| <b>Sapap3/4</b>                            | –                           | Co-IP                                | (68, 69)     |
| <b>Sema3F</b>                              | +                           | Co-IP, biophysical                   | (48, 70)     |
| <b>Sod1</b>                                | –                           | <i>In vitro</i>                      | (71)         |

<sup>a</sup> Numerous *in vitro* methods including filter binding, electrophoretic mobility shift assay, affinity capture, and UV-cross-linking assays have been used to validate FMRP-mRNA interactions (56). *In vivo* methods used include coimmunoprecipitation (co-IP), cross-linking immunoprecipitation (CLIP), antibody-positioned RNA amplification (APRA), etc....

RNAs isolated through cross-linking immunoprecipitation (HITS-CLIP) to identify neuronal FMRP mRNA targets in the mouse brain (57). The 842 putative mRNA targets identified were significantly enriched in proteins involved in neuronal and synaptic transmission. Interestingly, 28 of the FMRP target genes identified were candidate genes for autism. About 24% of the newly identified FMRP target transcripts showed overlap with targets identified in a previous study (46). Surprisingly, the new study showed FMRP binding largely within the coding regions of many mRNAs instead of the 5' or 3' untranslated regions (57), which is controversial with the previous hypothesis of FMRP recognizing specific consensus motifs such as the G-quadruplex and pseudoknot structures. None of the FMRP binding sites identified in this study can be folded into a G-quadruplex or pseudoknot structure.

Additionally, a more recent study reported the discovery of RNA sequences known as RNA-recognition elements that are targeted by the two independent KH domains of FMRP. It was shown that the KH1 domain of FMRP binds to mRNAs having the sequence WGGA and the KH2 domain of FMRP binds to mRNAs having the sequence ACUK (in which K = G or U and W = A or U) (72). However, the sequences ACUK or WGGA occur frequently in a random mRNA sequence, about once in every 128 nucleotides. This suggests that the ACUK and WGGA sequences, by themselves, cannot provide the specificity for translational control by FMRP.

Interestingly, in this study it was also shown that FMRP's mRNA binding sites were also predominantly located in the coding sequence, which cannot form G-quadruplex and pseudoknot structures. This accentuates the lack of consensus of FMRP's mRNA targets and the molecular mechanism of how FMRP regulates translation.

#### **1.4 FMRP is a Negative Regulator of Translation**

The majority of FMRP has been shown to cosediment with polyribosomes in both neuronal and nonneuronal cells in subcellular fractionation experiments (73-75). FMRP's association with polyribosomes, together with the knowledge that both FMRP and mRNA are expressed in dendrites and dendritic spines, suggest that FMRP acts as a regulator of local protein synthesis at the synapse. In the presence of puromycin, which disrupts actively translating polyribosomes and inhibits protein synthesis, this association is disrupted. This suggests that FMRP is interacting with actively translating polyribosomes (75). FMRP's interaction with actively translating polyribosomes was abolished in a FXS patient with a distinct I304N missense mutation (73). It is reasonable to speculate that this association is essential to FMRP's normal function. However, the mechanism of how the I304N mutation can interfere with polyribosome association and result in FXS is still unclear. Interestingly, the presence of FMRP mRNA-protein (mRNP)

complexes in p-bodies and stress granules (76), which are assumed to sequester mRNAs whose translation is being suppressed, indicates FMRP may repress translation throughout many phases of translational regulation. Previously the association of FMRP with polyribosomes and/or mRNPs was debated (77); however, now it is acknowledged that FMRP may be implicated in dynamic translational regulation of its mRNA targets. This topic is further described in the introduction of chapter 3.

Several independent *in vitro* and *in vivo* studies demonstrate FMRP predominantly inhibiting translation of most of its target mRNAs. Biochemical assays indicated that *Drosophila* dFMRP inhibits translation of the Map1B ortholog Futsch (16). Purified recombinant FMRP also represses translation of various mRNAs in a dosage-dependent manner when added to rabbit reticulolysate and *Xenopus laevis* oocytes. FMRP was shown to bind to these mRNAs, yet other proteins with similar RNA-binding properties did not repress translation. This suggests that FMRP inhibits translation not merely due to its RNA-binding activity (78). Interestingly, in another study, the ability of FMRP to repress translation was abolished when FMRP-binding sequences were removed from target mRNA (79), suggesting that FMRP binding might be necessary for translation regulation. Direct measurement of the rate of protein synthesis in cortical synaptoneurosomes or hippocampal slices *in vitro* showed a significant increase in the *Fmr1* KO mouse (61, 80). *In vivo* assays in KO mouse also exhibited a global increase in brain protein

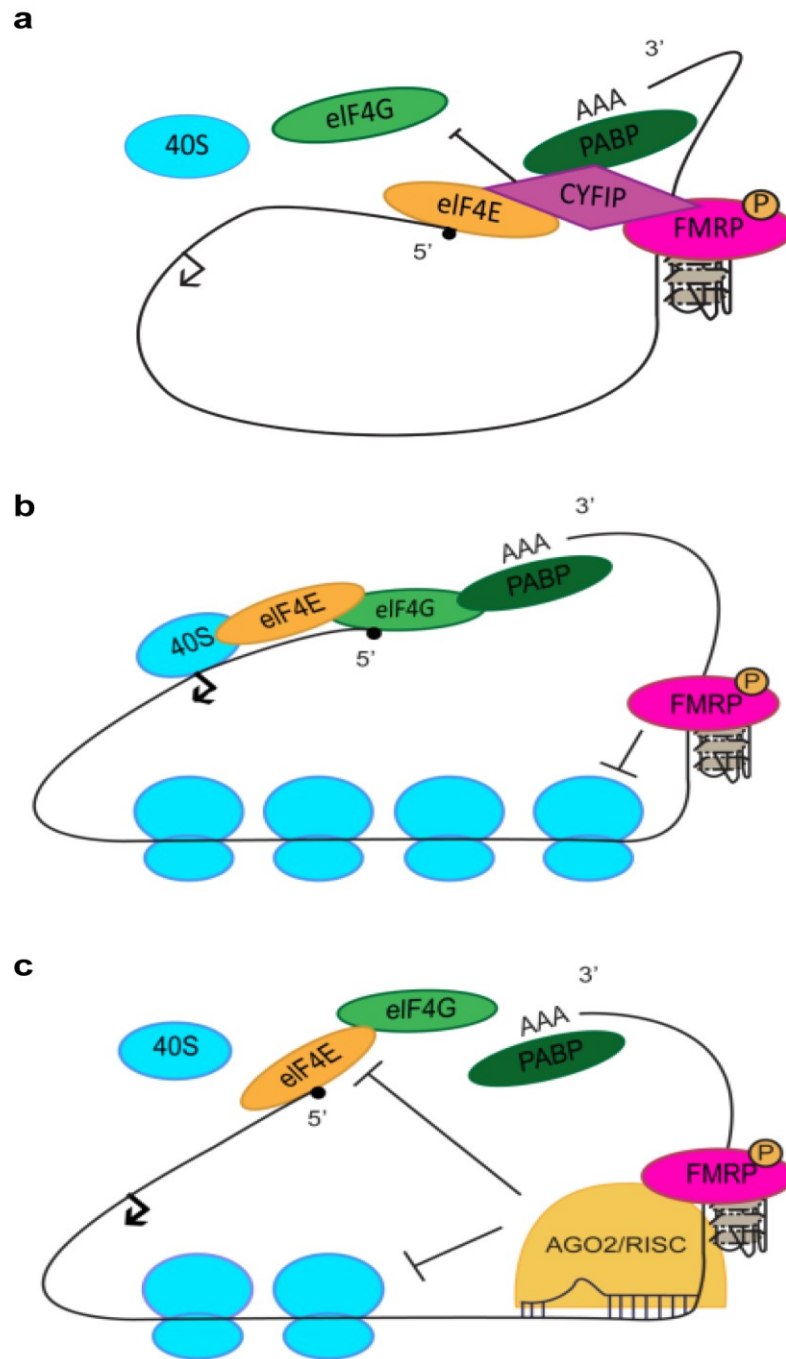
synthesis (81) and demonstrated that FMRP target proteins Map1B, Arc/Arg3.1, and CamKII $\alpha$  are overexpressed (62, 82). The intriguing fact that elevated protein synthesis can be observed in the intact animal *in vivo* has raised the possibility that measurements of protein synthesis could serve as a biomarker of disease (83, 84), and studies testing this hypothesis in human patients with FXS are currently underway.

Unexpectedly, there are a few special cases where FMRP has a novel function as translational activator. Superoxide Dismutase 1 (Sod1) mRNA has been identified to specifically bind to the C-terminal RGG region of FMRP via a novel RNA motif, SoSLIP (Sod1 mRNA Stem Loops Interacting with FMRP). SoSLIP behaves as a translational activator whose action is potentiated by the interaction with FMRP (71) and this results in increased translation of human Achaete-Scute Homologue 1 (hASH1) through an unknown mechanism (55). Furthermore, human nitric oxide synthase 1 (NOS1) translation was also activated by FMRP via interactions with coding region binding motifs (85). These discoveries indicate that FMRP may also activate translation of some transcripts.

### **1.5 Mechanisms of Translational Regulation by FMRP**

It is now well established that FMRP functions to negatively regulate protein synthesis, however, the exact molecular mechanism by which

inhibition is achieved remains controversial and unclear. Some of the main models proposed are: (a) FMRP represses the initiation of translation. In 2008, a known protein binding partner of FMRP, CYFIP1, was found to be a 4E-binding protein (4E-BP) (86). 4E-BPs interfere with the formation of the translation initiation eIF4A-eIF4G-eIF4E (eIF4F) complex. Essential cap-dependent translation in eukaryotes requires the translation initiation eIF4A-eIF4G-eIF4E (eIF4F) complex to associate with the 5' N<sup>7</sup>-methyl guanosine cap of the mRNA template for translation initiation. These discoveries have led to a model in which FMRP recruits CYFIP1 to target mRNAs, subsequently associates with eIF4E, and blocks recruitment of eIF4G (Figure 1.3 a). Evidence supporting this model showed that levels of FMRP targets MAP1B, CamKII $\alpha$ , and APP proteins are elevated in mice that express half of the normal CYFIP1 levels (86). (b) FMRP represses translation by stalling ribosomes during the elongation phase of translation (Figure 1.3 b) (75, 87, 88). This hypothesis is strongly reinforced by a recent study in which FMRP mRNA targets were identified following ultraviolet cross-linking (57). The majority (66%) of mRNA binding was found within the coding region of the 842 transcripts cross-linked to FMRP in mouse brain polyribosomes. A portion of FMRP codsedimented with polyribosomes, even after ribosome runoff experiments in which cells were treated with puromycin or sodium azide (which is a nonspecific inhibitor of translation initiation that does not affect elongation), indicating that FMRP was associated with stalled



**Figure 1.3: Models for how FMRP represses translation.** (a) FMRP prevents translation initiation by recruiting CYFIP1 to block the formation of the eIF4A-eIF4G-eIF4E (eIF4F) complex. (b) FMRP causes ribosomes to stall during the elongation phase of translation. (c) FMRP recruits the RNA-induced silencing complex (RISC) to repress translation (56).

ribosomes (75, 87). Ribosome stalling has not been thoroughly characterized, but is believed to be effected by subcellular localization, folding dynamics of the nascent protein, RNA secondary structure, and transfer RNA (tRNA) availability (89). These results support a model whereby FMRP dynamically represses translation in a complex consisting of target mRNAs and stalled ribosomes. (c) FMRP inhibits translation of specific mRNAs through the RNA interference (RNAi) pathway (Figure 1.3 c). Mouse embryonic fibroblasts (MEFs) from *Fmr1* KO mice showed impaired RNAi compared with wild-type MEFs (90). Another study discovered FMRP selectively associates with several microRNAs (miRNAs) in the mouse brain (91). Furthermore, both *Drosophila* and mammalian FMRP interact with Argonaute 2 and Dicer (90, 92, 93), important components of the RNA-induced silencing complex (RISC), and several specific miRNAs (94) that function together to silence target mRNA, either by translational repression or direct cleavage of transcripts. However, FMRP lacks a canonical miRNA-binding domain, so it currently seems that miRNAs are associated with FMRP via protein-protein interactions with other members of the RISC complex, rather than direct binding to miRNAs. (d) Post-translational modifications of FMRP have also been suggested to regulate translational inhibition. A region modulated by phosphorylation is located between the FMRP central portion and the RGG box, specifically located between amino acids 483 and 521 and conserved across different species. The primary

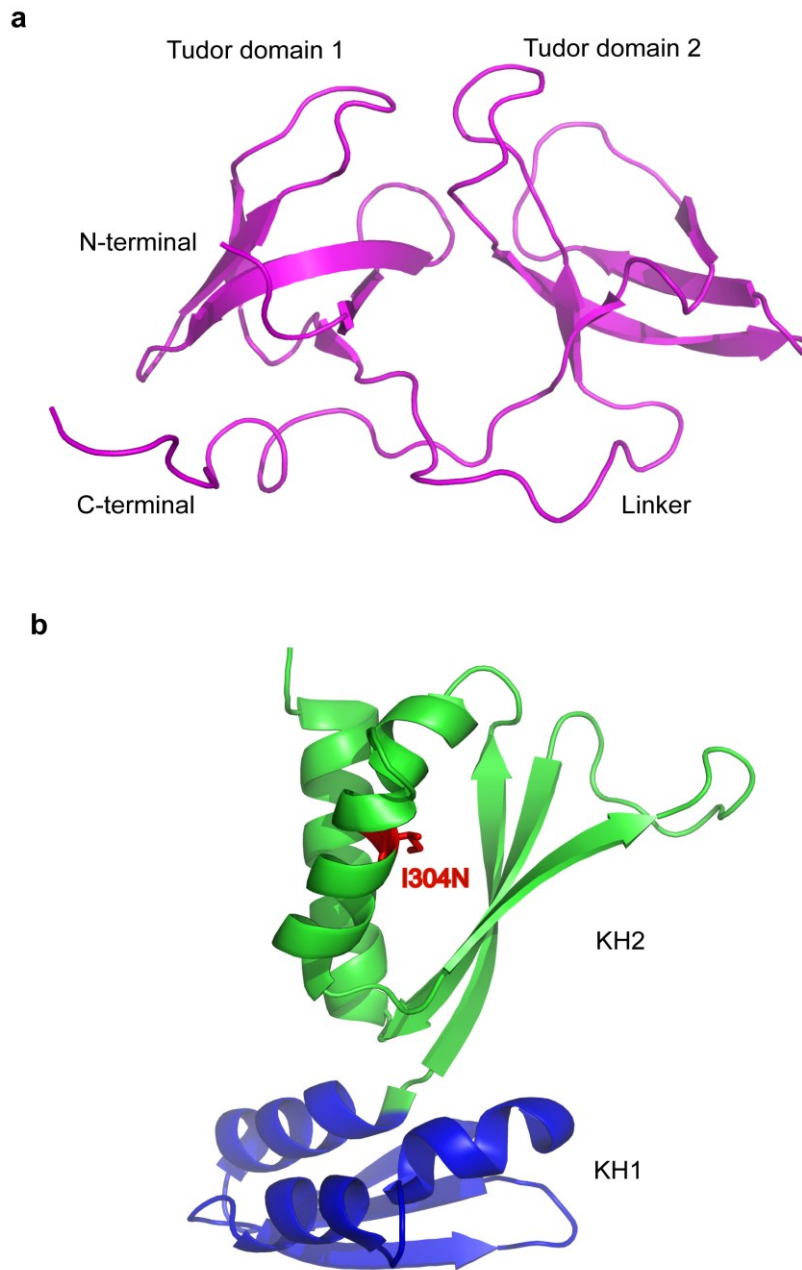
phosphorylated residue is a serine residue that is conserved from *Drosophila* to humans (human Ser500, murine Ser499, *Drosophila* Ser406) (87). Although phosphorylation of FMRP does not affect its ability to bind RNA, it does affect its association with polyribosomes and has been proposed to stall ribosomal translocation (87, 95, 96), whereas unphosphorylated FMRP allows translation to proceed. Phosphorylation of FMRP was also reported to inhibit its association with Dicer, while increasing its affinity for precursor miRNAs (pre-miRNA) complexes (97). These studies propose that miRNAs function as a critical component to modulate FMRP translational regulation and that phosphorylation of FMRP acts as a switch in this pathway. Thus, one way neural activity may gate translation is by regulating FMRP phosphorylation. FMRP can also be methylated on arginine residues and reduce FMRP binding to stem loop G-quartet structures (98).

Nevertheless, the exact mechanism of how FMRP controls translation is still not entirely clear and how these proposed mechanisms are temporally and spatially coordinated with each other still requires further determination. These models of FMRP-regulated translation repression may not necessarily be mutually exclusive. Different mechanisms could occur separately in different locations or at different times in the life of an mRNA molecule, for example, before transport to the synapse or after synaptic stimulation. All these studies demonstrate and underscore that much remains to be learned about the role of FMRP in translational regulation.

## 1.6 An Insight into the FMRP Structure

Understanding the structural properties of FMRP would highly benefit the process to further elucidate FMRP's function. Nevertheless, the structure of the entire FMRP has not been solved so far, and only structures of isolated domains of FMRP and its homologs are known from NMR and X-ray crystallographic studies (13, 45, 99). This is primarily due to aggregation characteristics and instability of full-length FMRP (100), so purification of large quantities of the protein amenable for structural studies has not been very successful.

FMRP is composed of three major regions: a N-terminal region containing two Tudor domains, a central region containing the two KH domains, and a divergent C- terminus with motifs rich in arginine and glycine residues. The structure of the first 134 residues of the N-terminal domain of FMRP in solution was solved by NMR, revealing a composite fold comprising three structural motifs, two repeats of a Tudor motif followed by a short alpha helix (Figure 1.4 a). A barrel-like fold made of four-stranded antiparallel  $\beta$  sheets forms each of the Tudor domains. An unstructured fragment links the two Tudor domains. The Tudor domains are able to bind single-strand (ss) nucleic acids. The structure also reveals hydrophobic pockets on the surface of the two Tudor domains, and these hydrophobic pockets can bind methylated amino acids (45). Though structurally similar, the two repeats



**Figure 1.4: Structures for partial fragments of FMRP.** (a) Ribbon representation of the NMR structure of the two Tudor domains on the N-terminus of FMRP (magenta). Protein Data Bank accession code 2bkd. (b) Ribbon representation of the X-ray crystallography structure of the tandem KH domains in FMRP. The KH1 (blue) and KH2 (green) domains are indicated. Also shown in the KH2 domain is the missense mutation I304N (red). Protein Data Bank accession code 2QND.

have different dynamic and functional properties. The second, more flexible Tudor motif can interact both with methylated lysine and with 82-FIP, one of the FMRP nuclear partners. Extensive interactions are observed between these three structural elements, strongly suggesting that all elements are crucial for the stability of the overall N-terminal domain. It is proposed that the N-terminal composite fold determines an allosteric mechanism that regulates the FMRP functions (45).

The three-dimensional structure of the tandem KH domains located in the central region in human FMRP has been solved by X-ray crystallography (Figure 1.4 b) (13). However, the crystallized construct contains a shortened variable loop in the KH2 domain. The KH domains, which are usually present in multiple copies in a protein, are responsible for the interaction with ssDNA, mRNA, and rRNA. The Type I KH1 and KH2 domains both consist of a  $\beta^1\alpha^1\alpha^2\beta^2\beta'\alpha'$  fold, three-dimensionally oriented as a three-stranded  $\beta$ -sheet domain opposed to a three  $\alpha$ -helices domain. The main hydrophobic core of the domain comprises the buried hydrophobic residues between the hydrophobic faces of the  $\beta$ -sheet and the  $\alpha$ -helices. The  $\beta$ -sheet in each KH domain consists of two parallel  $\beta$  strands,  $\beta^1$  and  $\beta^2$ , that sandwich the  $\beta'$  strand in an all anti-parallel arrangement. Residue I304 is part of the hydrophobic core that stabilizes the three-dimensional folding of the KH2 domain, and the missense mutation I304N has been hypothesized to disrupt the structure and destabilize the protein (13). Additionally, since I304 is

buried in the hydrophobic core and is not solvent accessible, it seems that this residue is not directly involved in nucleic acid binding, but rather the missense mutation causes structural rearrangement which in turn affects the nucleic acid binding. Nonetheless, these partial structures of FMRP can only provide limited insight into the function of FMRP and a larger fragment or the entire protein is needed to understand the overall spatial orientation of the different functional domains within FMRP.

### **1.7 FMRP's Role in Neurological Pathways**

The observation that FMRP associates with polyribosomes and is linked to the regulation of protein synthesis, together with the fact that both FMRP and its mRNA targets are localized to neuronal dendrites, strongly suggested that FMRP plays an important role in synaptic function and structure (101, 102). This raised the question of what FMRP's role would be in specific neurological pathways. The most well understood pathway that FMRP interacts with is the group 1 (Gp1) metabotropic glutamate receptor (mGluR)-dependent long-term depression (mGluR-LTD). FMRP also takes part in other pathways and other regions of the brain, but much less is known about FMRP's role in these pathways, though it is presumed FMRP functions as a translational repressor in these instances as well.

mGluRs are G protein-coupled receptors that link to intracellular signaling pathways, including the Gq/PLC pathway (group 1 mGluRs) and Gi/Go pathways (groups 2 and 3 mGluRs). In 1993, a crucial discovery that one result of activating Gp1 mGluRs (comprising mGluR1 and mGluR5) is increased protein synthesis at synapses (103). Two intracellular signaling cascades have been proposed to couple mGluRs and other synaptic receptors to the translational machinery: the mammalian target of rapamycin (mTOR) pathway and the extracellular signal-related kinase (ERK) pathway. Both of these pathways can stimulate cap-dependent translation by increasing eIF4E activity and initiating the assembly of the eIF4F initiation complex. It is now appreciated that Gp1 mGluRs couple to the synaptic translational machinery at many synapses in the brain and that many functional consequences of Gp1 mGluR activation depend on new protein synthesis (104).

mGluR-LTD is a principal form of synaptic plasticity and is a molecular basis of memory and learning. mGluR-LTD depends on the local protein synthesis of postsynaptic, dendritically localized mRNAs in an activity-dependent manner such as in response to appropriate synaptic stimulation. This local protein synthesis leads to  $\alpha$ -amino-3-hydroxyl-4-isoxazole propionic acid receptor (AMPA) internalization, a crucial step in mGluR-LTD. In 1997, FMRP was discovered to be synthesized following application of the mGluR agonist dihydroxyphenylglycine (DHPG) in synaptoneurosomes

(105). Another study also showed that DHPG, acting through mGluR5, could trigger LTD that requires translation of preexisting mRNA (106, 107). It was hypothesized that FMRP is required for mGluR-LTD and this was supported by evidence of enhanced/exaggerated mGluR-LTD in the hippocampus of *Fmr1* KO mice that does not require new protein synthesis (108, 109). The exaggerated LTD pointed in the direction that FMRP serves as an important brake on mGluR-stimulated protein synthesis and in the absence of FMRP, there is poorly regulated synaptic protein synthesis.

The mGluR theory of FXS proposes that many psychiatric and neurological symptoms of FXS stem from the exaggerated downstream consequences of mGluR1/5 activation (110) caused by the absence of FMRP. This theory predicts that FMRP acts as a negative regulator of its mRNA targets in the normal basal state, but upon mGluR activation, FMRP repression is released and results in the burst of local protein synthesis essential for AMPAR internalization and LTD (110). When FMRP is absent, as in FXS, even in the absence of mGluR activation there is runaway of synaptic protein synthesis, which leads to exaggerated LTD and overactive AMPAR internalization. In agreement with this theory, the levels of several FMRP mRNA targets associated with actively translating polyribosomes are enhanced in response to DHPG in wild-type mouse synaptoneurosomes, but not in *Fmr1* KO synaptoneurosomes (61). Furthermore, mice heterozygous for the *Grm5* gene (which encodes mGluR5) were crossed with *Fmr1* KO

mice, resulting in 50% reduction of mGluR5 protein levels which led to correction of many of the FXS-related phenotypes (80). A significant decrease was seen in induction of audiogenic seizures, a model for the increased susceptibility of epilepsy observed in FXS patients. Moreover, the enhancement of protein synthesis in the hippocampus and the increase in dendritic spines seen in visual cortex of *Fmr1* KO mice were all corrected. Exaggeration of mGluR-LTD and the inability to further increase protein synthesis in an activity-dependent manner are the likely explanations behind the intellectual deficiencies seen in FXS.

Based on the mGluR theory, a prediction that mGluR antagonists might restore normal synaptic protein synthesis and correct some of the FXS phenotypes arose. This hypothesis raised the possibility that a targeted intervention treatment strategy could provide a novel therapeutic approach in curing FXS. The mGluR5 antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP), a potent negative allosteric modulator of mGluR5 that crosses the blood-brain barrier, was shown to rescue behavioral and cognitive deficits in mouse, *Drosophila*, and zebrafish models of FXS (19, 111-113). MPEP was shown to reverse abnormalities in behavioral and structural measures of courtship-related learning and memory in *dFMR1* mutant flies (111). Another group of investigators developed *Fmr1* knockdown zebrafish embryos in medium containing MPEP, and observed reduced disruptions in neurite morphology in the hindbrain and spinal cord of the embryos, as well in

craniofacial development (19). Furthermore, administration of MPEP in *Fmr1* KO mice restored proper AMPAR internalization, significantly corrected the probability of audiogenic seizures, and rescued the abnormal dendritic spine morphology exhibited in *Fmr1* KO neurons. This has lead to potential possibilities for therapeutic intervention in FXS and clinical trials of drugs that target the mGluR pathway.

In light of these exciting discoveries, a number of clinical trials are currently underway and compounds targeting the mGluR signaling pathway are being tested in treating FXS. The first Gp1 mGluR inhibitor tested in clinical trials was fenobam, a compound that was originally developed as a nonbenzodiazepine anxiolytic with an unknown molecular target and was subsequently demonstrated to be a selective mGluR5 antagonist (114). Other potential drugs tested include acamprosate (115), AFQ056, STX107 (see <http://www.clinicaltrials.gov>), and minocycline (116). Unfortunately, none of these compounds are specifically approved for the treatment of FXS to date, and issues such as the developmental time window available for therapeutic intervention are still unresolved concerns. However, promising preliminary results have been obtained and ongoing research will surely lead to more thorough understanding of FXS.

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## **Chapter 2: Inhibition of Protein Synthesis by FMRP**

## Introduction

Loss of function of FMRP is the cause of the leading inherited mental retardation disease, FXS (1, 2). Symptoms of FXS include intellectual disability and behavioral problems. Since FMRP's discovery about twenty years ago, researchers have strived in diverse areas to connect the dots of what the underlying mechanism is and whether therapeutic interventions could provide dependable cures (3-6). Significant progress has been made in our understanding of FMRP's normal function (7) and how its absence leads to symptoms exhibited in FXS patients (8). Most research has been focused on FMRP's function as a translational repressor (9, 10), and extensive studies have been done to attempt to reveal FMRP's mechanism of translational regulation. However, there are conflicting data regarding FMRP's mechanism of translation repression. It may directly interact with its target mRNAs by recognition of specific structural motifs (11-15), or it may rely on adaptor molecules such as brain cytoplasmic 1 (BC1) (16). Recent evidence has also underlined the concept of FMRP acting through the miRNA pathway (17, 18), but the possibility remains that it may inhibit translation initiation or elongation through more direct means. Many lines of inquiry remain open, and validation of several previous studies needs to be done. All things considered, the underlying mechanism of translational regulation is still unclear.

Nonetheless, it is well established that FMRP is a selective RNA-binding protein (19-21) and various labs have utilized different techniques (22-25) to uncover the specific mRNA targets of FMRP. However, there has been little to no consensus between the results obtained, and only a few out of the hundreds of putative mRNAs have been validated with direct biochemical interaction (26). *In vitro* selection experiments identified a G-quadruplex structure (11, 13, 15) and a pseudoknot structure (14) as potential RNA ligands for the RGG and KH2 domains, respectively. A G-quadruplex consists of two to four G quartets or tetrads stacked on top of each other; each G-quartet is made of four guanines in a planar conformation that interact via cyclic Hoogsteen-type hydrogen bonds (27). Since G quadruplexes are hard to predict bioinformatically, biochemical assays confirmed that G-quadruplexes mediate the interaction of FMRP with *Fmr1*, MAP1b, and Sema3F mRNAs (12, 28, 29). The pseudoknot structure was formed by a selection of randomized RNA, and this structure has not been observed in endogenous mRNA.

More recently, Darnell and co-workers used high throughput sequencing of RNAs isolated through cross-linking immunoprecipitation (HITS-CLIP) to identify neuronal mRNAs regulated by FMRP in the mouse brain (30). The 842 unique mRNA targets identified were significantly enriched for proteins involved in neuronal and synaptic transmission. Strikingly, 28 of the FMRP target genes identified in this study were

candidate genes for autism. About 24% of the newly determined FMRP target transcripts overlap with targets identified in a previous study (22). Although previous studies suggested that FMRP inhibits translation by binding either G-quadruplex or pseudoknot secondary structural motifs in the mRNA, none of the FMRP binding sites identified in this study can be folded into a G-quadruplex or pseudoknot structure. Furthermore, results show that 66% of the target FMRP binding sites were within the coding region of the mRNAs (30). It is not clear how FMRP can bind to the coding region of an mRNA that is loaded with polyribosomes and stall translation. Thus, understanding the mechanism of translational control by FMRP at the molecular and cellular level is crucial for developing effective therapeutics to treat FXS.

In chapter 2, we established a sensitive *in vitro* translation system to dissect the mechanism of translational control by FMRP and analyze the role of mRNA structural motifs. Mutational analysis of FMRP was also done for identification of structural domains in FMRP that are essential for translational regulation. In our studies, we are using the *Drosophila* model system. *Drosophila* contains only one *FMR1* gene, whose expression product shares 56% amino acid similarity with mammalian FMRP (31, 32). Importantly, *FMR1* knockout flies exhibit phenotypes that are consistent with the synaptic defects found in human FXS patients and *FMR1* knockout mice (31, 33-38). Specifically, *FMR1* knockout flies exhibit increased growth and

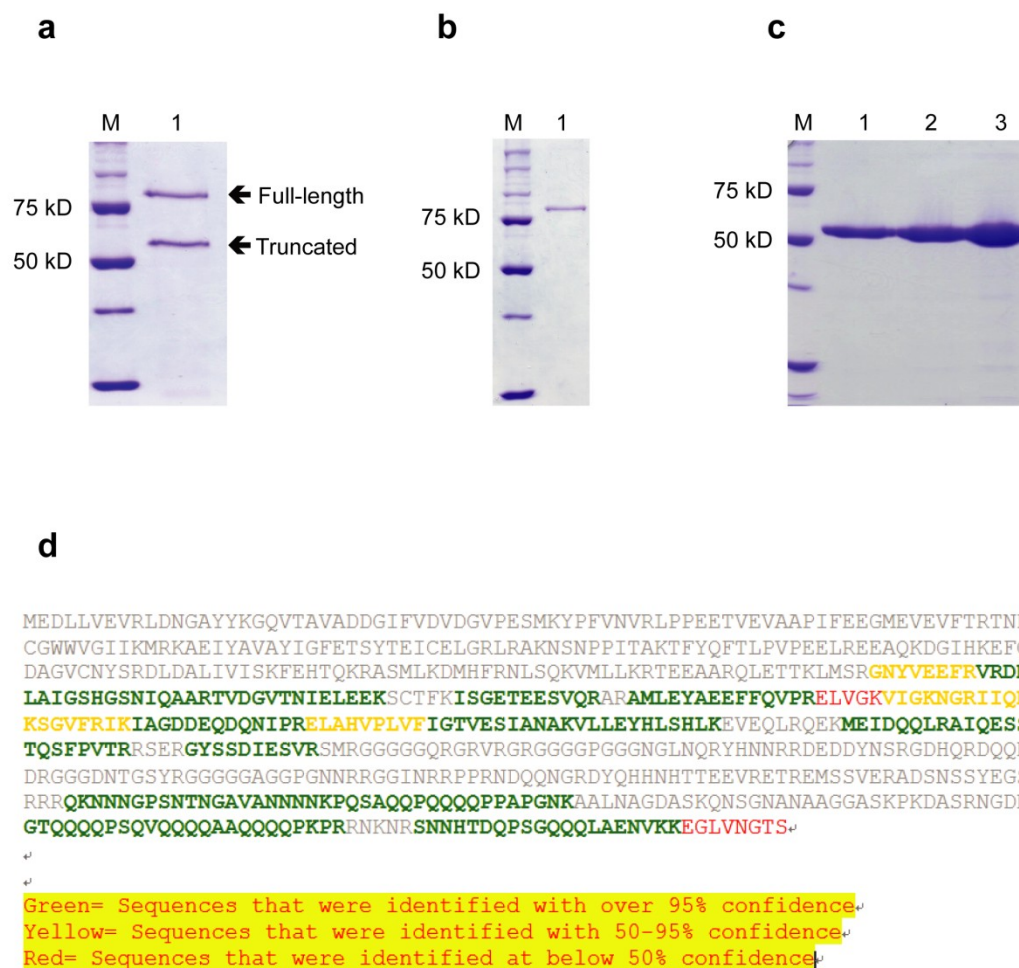
branching of dendritic processes (31). The availability of sophisticated genetic, cellular, and molecular tools make *Drosophila* a simple and attractive model system to study the function of FMRP.

## Results and Discussion

### 2.1 Purification of dFMRP

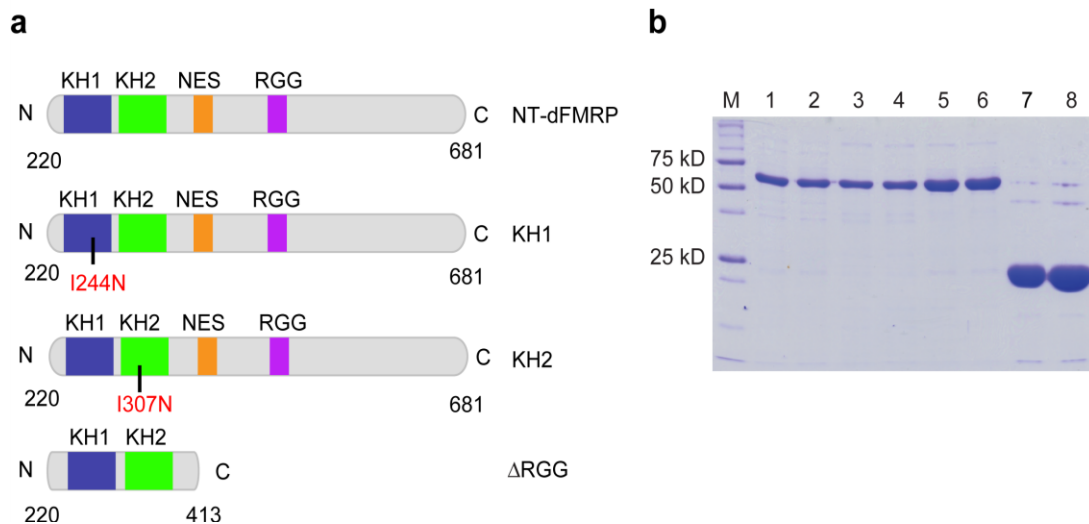
The *Drosophila FMR1* gene was obtained from Professor Gideon Dreyfuss (University of Pennsylvania) (39). dFMRP was overexpressed and purified from *E. coli* using the IMPACT-CN purification system (New England Biolabs). Although we were able to purify the full-length dFMRP, a truncated version would co-purify along with it (Figure 2.1 a). Separation of the two dFMRP versions was successfully done by adding a polyhistidine-tag and passing through subsequent affinity chromatography columns; however, the full-length dFMRP was not very soluble and could not be concentrated (Figure 2.1 b). Previous studies also showed difficulties in production of full-length protein and constructs containing more than one to two domains due to FMRP's tendency to aggregate and/or degrade (19, 39-41).

Analysis of the co-purified truncated dFMRP with mass spectrometry showed the truncation was occurring around residue 220. The truncated dFMRP was much more soluble, so we designed a construct mimicking the



**Figure 2.1: Purification of dFMRP.** (a) Co-purification of a full-length and a truncated version of dFMRP resolved on a 10% SDS-PAGE gel. Lanes: M, molecular weight ladder; 1, full-length and truncated dFMRP (b) Purified full-length dFMRP. Lanes: M, molecular weight ladder; 1, full-length dFMRP. (c) NT-dFMRP is stable and can be concentrated to 1.5 mg/ml. Lanes: M, molecular weight ladder; 1, NT-dFMRP concentration of 0.5 mg/ml; 2, NT-dFMRP concentration of 1 mg/ml; 3, NT-dFMRP concentration of 1.5 mg/ml. (d) The full-length dFMRP sequence. NT-dFMRP construct design was based on mass spectrometry analysis of trypsin digested truncated dFMRP showing truncation at glycine 220.

truncated dFMRP by deleting residues 1 to 219 from the N-terminus. This produced a protein, N-terminal truncated dFMRP (NT-dFMRP), which was more soluble and did not aggregate like the full-length dFMRP (Figure 2.1 c-d). Since NT-dFMRP retains all three RNA binding domains (Figure 2.2 a), it should be capable of binding to its RNA target ligands. Additionally, NT-dFMRP should recapitulate FMRP's functionality more precisely than previous studies working with constructs of one or two domains, so we decided to use NT-dFMRP throughout the majority of our studies



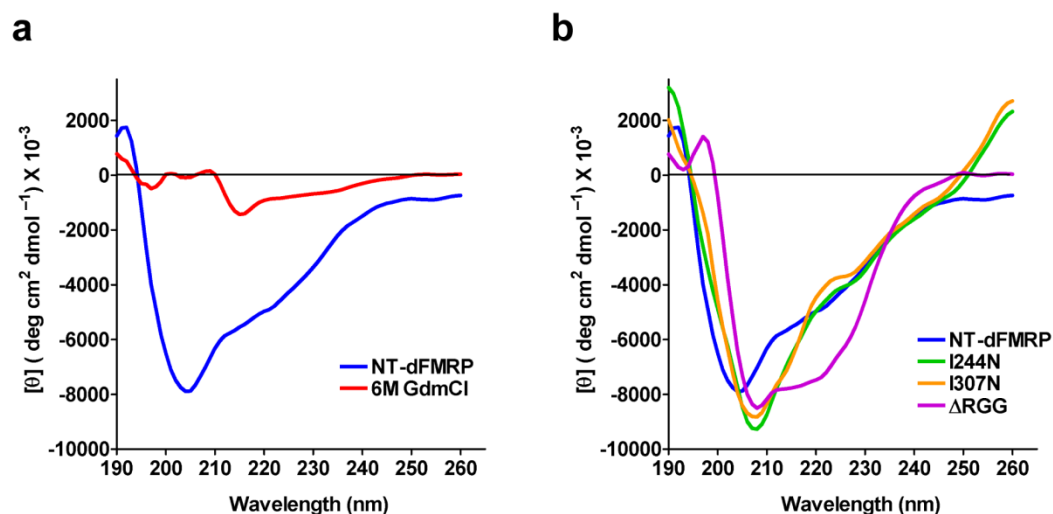
**Figure 2.2: NT-dFMRP and mutants.** (a) Domain organization of NT-dFMRP (220-681 amino acids) and mutants. KH, K-homology domain; NES, nuclear export signal; RGG, motif rich in arginine and glycine. Point mutations are indicated for the KH1 mutant (I244N) and KH2 mutant (I307N). (b) NT-dFMRP and mutants resolved on a 15% SDS-PAGE. M, molecular weight ladder; 1-2, NT-dFMRP; 3-4, KH1 mutant (I244N); 5-6, KH2 mutant (I307N); 7-8, ΔRGG mutant (deleted residues 414-681).

To test which RNA binding domains of NT-dFMRP are essential for translational repression, we designed constructs with a missense mutation in the KH1 (I244N) or KH2 (I307N) domains or with the RGG domain deleted (residues 414 to 681) (Figure 2.2 a). The KH domain mutations made are the equivalent of the I304N in human FMRP, which is a point mutation seen in an individual with a severe phenotype of FXS (42). Purification of NT-dFMRP and its mutants were all done using the IMPACT-CN system, which yields extremely pure protein (~95%) and high concentrations as indicated by the clean protein bands seen on the SDS-PAGE gel (Figure 2.2 b).

To validate that the proteins were not completely unfolded, circular dichroism (CD) spectroscopy (43) in the far-UV (190-250 nm) spectral region was measured for NT-dFMRP in the presence or absence of 6 M guanidinium hydrochloride (GdmCl), a strong denaturing agent. At these wavelengths, the chromophore is the peptide bond, and the signal arises when it is located in a regular, folded environment. Addition of strong denaturants is expected to result in loss of CD signal, corresponding to unfolded protein. Overlaid spectra results display extreme differences, indicating NT-dFMRP is not completely unfolded in the absence of 6 M GdmCl; whereas, in the presence of 6 M GdmCl, loss of CD signal and classic random coil characteristics at 215 nm are displayed indicating unfolded protein properties (Figure 2.3 a).

To confirm that the mutations and deletions we made would not completely unfold the proteins, far-UV CD spectrums were also measured for the mutant proteins. As expected, the  $\Delta$ RGG mutant is not entirely unfolded and displays significant structural discrepancies to NT-dFMRP due to the deletion of over 200 residues (Figure 2.3 b). Interestingly, KH1 and KH2 mutants are also not completely unfolded and show similar structural characteristics, but diverge from NT-dFMRP. This strongly suggests these missense mutations are destabilizing the protein and the KH domains may be crucial for maintaining precise translational control. There is special interest in residue I304, which is located in the KH2 domain of hFMRP, because a patient with a particularly severe case of Fragile X syndrome produces normal levels of a mutant form (I304N) of FMRP (42). Three main hypotheses regarding the possible effect of the I304N mutation on protein function include: (1) the I304N mutation causes complete unfolding of the KH2 domain (44); (2) the mutation has no effect on protein structure, but it impairs binding of target RNAs by disrupting a hydrophobic platform on FMRP involved in RNA contact (45); (3) the mutation causes an alteration in the association of FMRP with other proteins *in vivo* (46). Extrapolation of data obtained with different KH domains to the KH domains of hFMRP leads to controversy surrounding the effect of the I304N mutation (45, 47, 48). More recently, Regan and co-workers solved the structure of the tandem KH domains in hFMRP (49), precisely specifying the position of the I304 residue.

All of the atoms of I304 are inaccessible to solvent, indicating I304 cannot participate in direct contacts with ligands and its mutation to asparagine would disrupt the hydrophobic core and destabilize the protein. Our findings correspond to Regan's hypothesis of the point mutations in the KH domains perturbing the structure and destabilizing the protein.

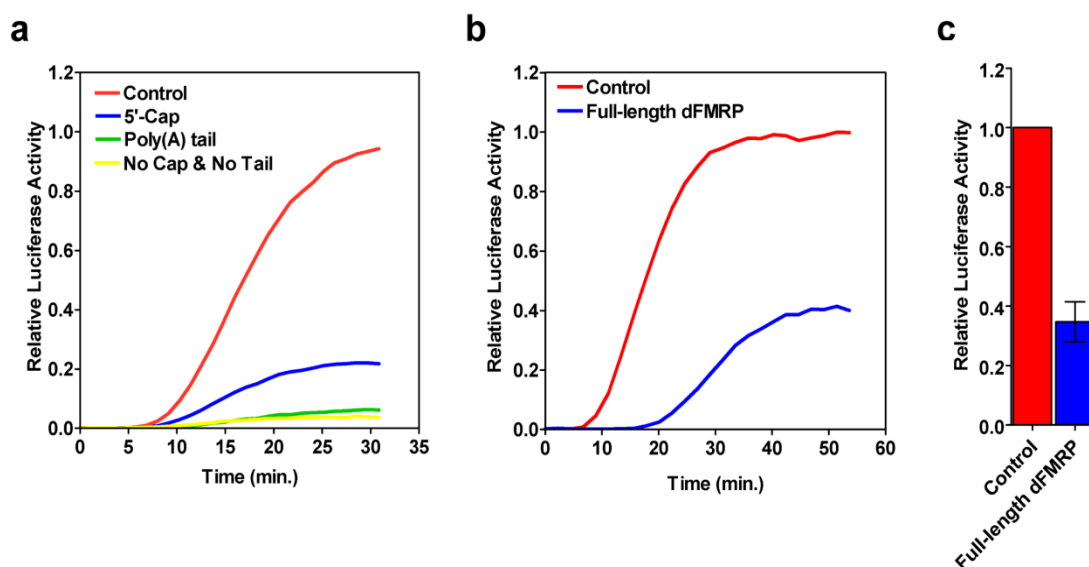


**Figure 2.3: Far UV CD spectra of NT-dFMRP and mutants.** (a) CD spectra of NT-dFMRP in the presence or absence of 6 M GdmCl. Blue, NT-dFMRP in the absence of GdmCl; red, NT-dFMRP in the presence of GdmCl. (b) CD spectra of NT-dFMRP and mutants show structural discrepancies. Blue, NT-dFMRP; green, KH1 mutant; orange, KH2 mutant; purple,  $\Delta$ RGG mutant.

## 2.2 Inhibition of translation by FMRP

In order to test the activity and functionality of NT-dFMRP, we established an *in vitro* translation system (IVTS) using *Drosophila* embryo

extracts (50, 51). *Renilla* luciferase mRNA with a N<sup>7</sup>-methyl guanosine cap at the 5'-end and a poly(A) tail at the 3'-end was used as the reporter for protein synthesis. The time course of luciferase synthesis was monitored by bioluminescence (52). Titrations of various concentrations of Mg(OAc)<sub>2</sub>, spermidine, and KOAc were examined, and it was found that 0.6 mM Mg(OAc)<sub>2</sub>, 0.1 mM spermidine, and 80 mM KOAc achieved optimal bioluminescence signal.



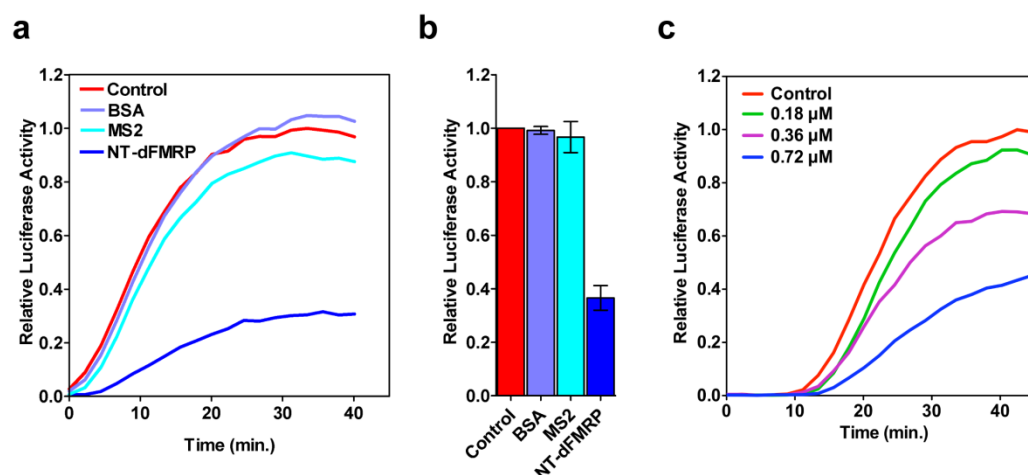
**Figure 2.4: Inhibition of translation by dFMRP.** (a) *In vitro* translation is dependent on 5' cap and poly(A) tail in the mRNA. The graph shows the increase in luminescence over time due to the synthesis of luciferase. Red trace, control mRNA with 5' cap and poly(A) tail; blue trace, mRNA with 5' cap; green trace, mRNA with poly(A) tail; yellow trace, mRNA without 5' cap and poly(A) tail. (b) Full-length dFMRP inhibits translation. Red trace, control translation without dFMRP; blue trace, translation with 0.6  $\mu$ M of full-length dFMRP. (c) Bar graph showing the relative luciferase activity at 30 min. time point for control translation without full-length dFMRP (red bar) and translation with 0.6  $\mu$ M full-length dFMRP (blue bar). Bar graph data was normalized with respect to the control translation without dFMRP. Standard deviations from 3 independent experiments are shown.

By comparing the relative luciferase activity of the control *Renilla* luciferase mRNA which contains a 5' cap structure and a 3' poly(A) tail with *Renilla* luciferase mRNA constructs lacking the 5' cap and/or 3' poly(A) tail, the IVTS more efficiently translates mRNAs having a 5' cap structure and a 3' poly(A) tail (Figure 2.4 a). This demonstrates that this established IVTS follows the canonical pathway of protein synthesis.

We then used the IVTS to analyze the function of NT-dFMRP. To confirm NT-dFMRP recapitulates the functionality of full-length dFMRP, we added close to endogenous levels (10) (0.6  $\mu$ M) of full-length dFMRP into the IVTS, and observed a 60% repression of translation compared to the control reaction (Figure 2.4 b-c). This corresponds to previous studies of FMRP acting as an inhibitor of translation (9, 10). The addition of 0.6  $\mu$ M of NT-dFMRP to the IVTS also inhibited the synthesis of luciferase to a similar extent (~60% repression). In contrast, the addition of 1.2  $\mu$ M bovine serum albumin (BSA) and a RNA binding protein, MS2 coat protein, to the IVTS did not inhibit the synthesis of luciferase (Figure 2.5 a-b). Various amounts of NT-dFMRP were titrated into the IVTS, and results indicated that with increasing concentrations of NT-dFMRP added, enhanced repression of translation was observed (Figure 2.5 c). This strongly suggests that inhibition of translation may correlate with the concentration of FMRP.

NT-dFMRP also inhibited the translation of luciferase mRNAs that do not have a 5' cap or a poly(A) tail, indicating that translation repression is cap

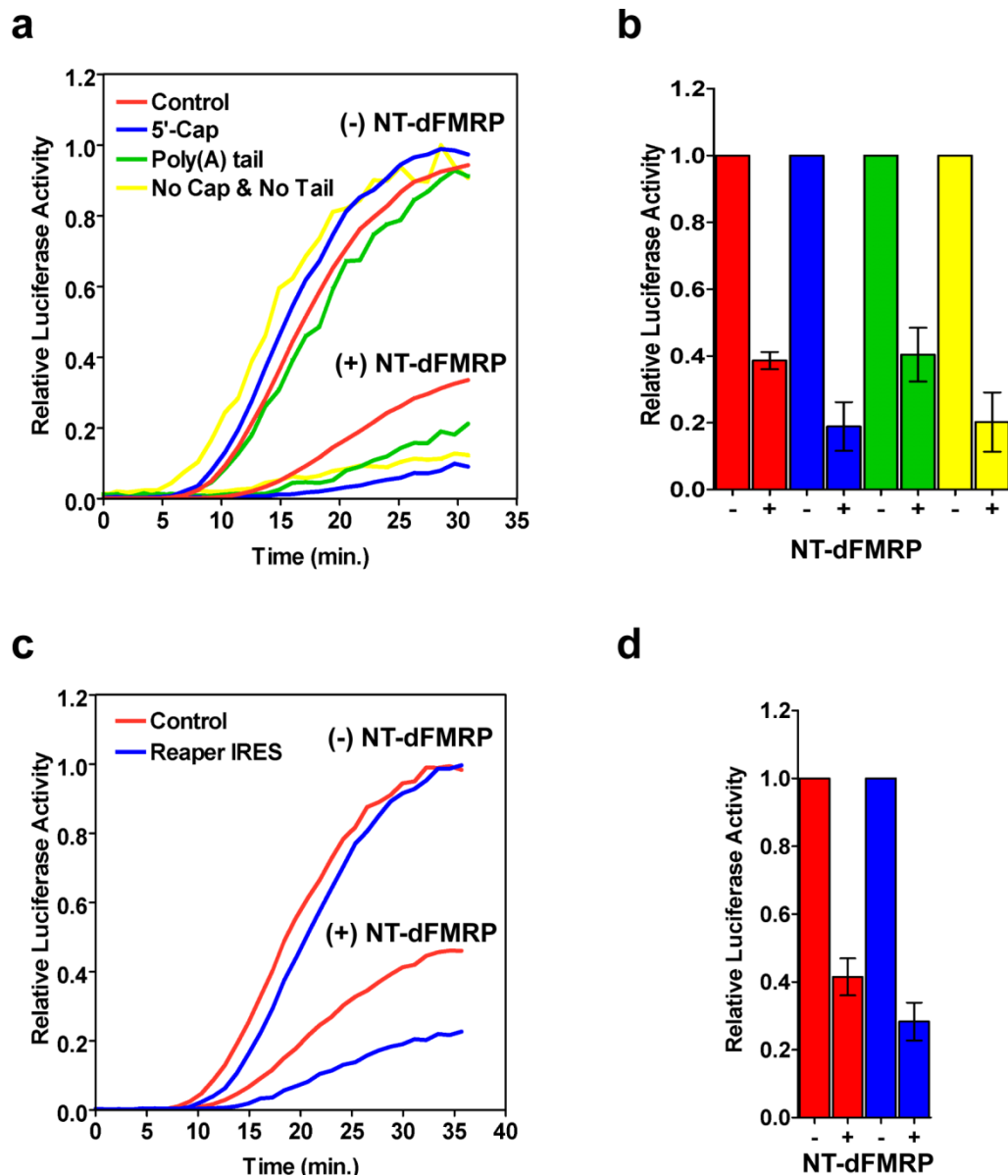
and poly(A) tail independent (Figure 2.6 a-b). To confirm that the repression of translation by NT-dFMRP is cap independent, we synthesized uncapped luciferase mRNA with an internal ribosome entry sequence (IRES) from the *Reaper* mRNA at the 5' end (53). IRES regions are known to attract eukaryotic ribosomal translation initiation complex and thus promote translation initiation independently of the presence of the commonly utilized 5'-terminal N<sup>7</sup>-methyl guanosine cap structure.



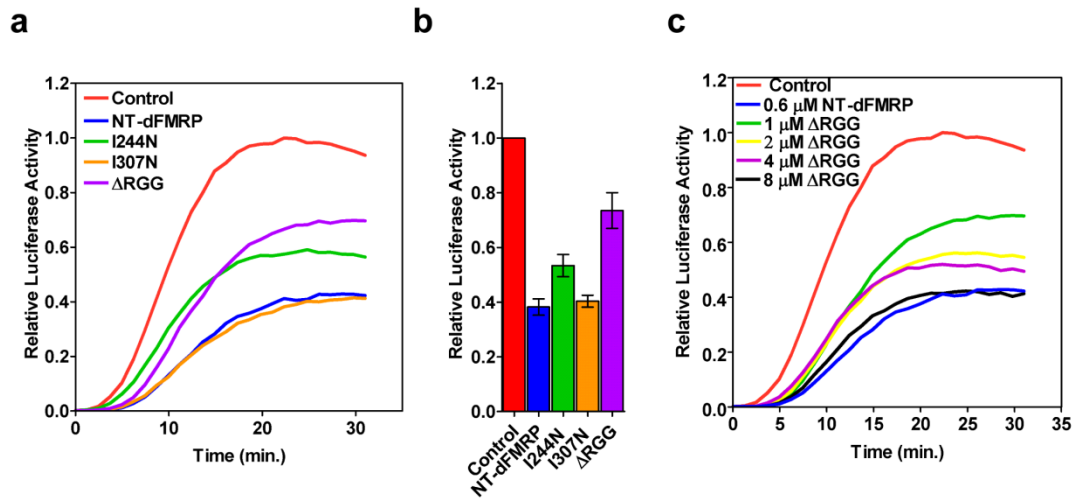
**Figure 2.5: Inhibition of translation by NT-dFMRP is concentration-dependent.** (a) NT-dFMRP inhibits translation of luciferase mRNA. Red trace, control translation without NT-dFMRP; light blue trace, translation with 1.2 μM BSA; cyan trace, translation with 1.2 μM MS2; blue trace, translation with 0.6 μM NT-dFMRP. (b) Bar graph showing relative luciferase activity. Red bar, control translation without NT-dFMRP; light blue bar, translation with 1.2 μM of BSA; cyan bar, translation with 1.2 μM of MS2; blue trace, translation with 0.6 μM NT-dFMRP. Standard deviations from 3 independent experiments are shown. (c) Concentration-dependent inhibition of NT-dFMRP. The indicated amounts of NT-dFMRP were added to the IVTS. Red trace, control translation without NT-dFMRP added.

IRES-dependent translation of luciferase mRNA was as efficient as the 5' capped mRNA. NT-dFMRP inhibited the translation of luciferase mRNA having the IRES element to a similar extent, confirming that the 5' cap is not essential for repression (Figure 2.6 c-d). These results strongly suggest that FMRP inhibits the translation elongation cycle without affecting the initiation step of protein synthesis. One of the current models also hypothesizes FMRP represses translation by stalling the elongation of actively translating ribosomes (30, 46, 54). Our results support and agree with this hypothesis.

To dissect the importance of the RNA binding domains of NT-dFMRP, the three mutants: KH1 mutant (I244N), KH2 mutant (I307N), and  $\Delta$ RGG mutant (deleted residues 414 to 681) were added to the IVTS. The KH2 mutant displayed similar repression effects as NT-dFMRP. Interestingly, the KH1 mutant and the  $\Delta$ RGG mutant exhibited less inhibition compared to NT-dFMRP in the IVTS assay (Figure 2.7 a-b). Titrating increasing concentrations of  $\Delta$ RGG mutant protein showed that it took significantly more to reach the same extent of repression as 0.6  $\mu$ M NT-dFMRP (Figure 2.7 c). Thus, the translation repression by NT-dFMRP partly requires the KH1 and RGG RNA binding domains. Interestingly, in a previous study it was shown that overexpressing the KH1 mutant (I244N) produced a much weaker rough eye phenotype compared to the KH2 mutant (I307N) in the *Drosophila* model system. This was due to the KH1 mutant being much less active than the



**Figure 2.6: Inhibition of translation by NT-dFMRP is 5'-cap and poly(A) tail independent.** (-) NT-dFMRP and (+) NT-dFMRP indicate IVTS performed in the absence and presence of 0.6  $\mu$ M NT-dFMRP, respectively. Bar graphs show standard deviations from 3 independent experiments. (a) Red trace, control mRNA with 5' cap and poly(A) tail; blue trace, mRNA with 5' cap; green trace, mRNA with poly(A) tail; yellow trace, mRNA without 5' cap and poly (A) tail. The data was normalized with respect to the highest signal for each mRNA in the absence of NT-dFMRP. (b) Bar graph of (a) at 30 min. time point. (c) Inhibition of IRES-dependent translation by NT-dFMRP. Red trace, control mRNA with 5' cap and poly(A) tail; blue trace, mRNA with IRES at the 5' end and poly(A) tail at 3' end. (d) Bar graph of (c).



**Figure 2.7: Inhibition of translation by NT-dFMRP depends on the KH1 and RGG domains.** (a) Translation in the presence of the indicated mutant NT-dFMRP proteins. The data were normalized with respect to the control IVTS without NT-dFMRP. Red trace, control translation without NT-dFMRP. (b) Bar graph of (a). Standard deviations from 3 independent experiments are shown. (c) Higher amounts of ΔRGG mutant protein is needed to obtain the same level of repression as NT-dFMRP.

KH2 mutant (32). This corresponds very well with our results showing that the KH1 domain is more crucial for normal FMRP function. However, we cannot rule out the possible importance of the KH2 domain at this stage.

### 2.3 Role of mRNA structures, sequences, and binding for translational repression by FMRP

Since the luciferase mRNA used in our experiments does not have a G-quadruplex or a pseudoknot forming sequence, we wondered what was

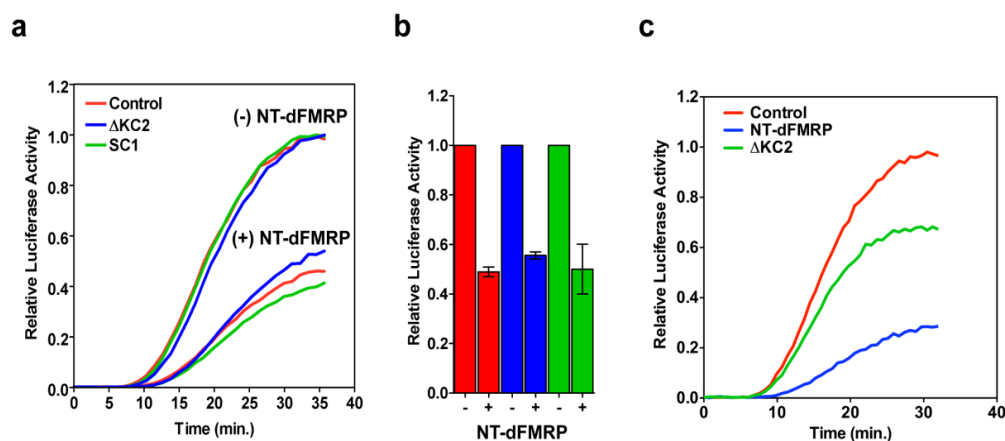
the basis for translational repression by NT-dFMRP. One possibility is that NT-dFMRP is a general translational repressor and inhibits translation independent of the presence of G-quadruplex or pseudoknot structures in the mRNA. Alternatively, NT-dFMRP may inhibit the translation of mRNAs having G-quadruplex or pseudoknot structures to a greater extent than mRNAs that do not have the G-quadruplex or pseudoknot structures.

To test these two possibilities, we inserted SC1 G-quadruplex (11) and  $\Delta$ KC2 pseudoknot (14) forming sequences at the 3' untranslated region of the luciferase mRNA. Interestingly, NT-dFMRP inhibited the translation of the control mRNA and mRNAs with the SC1 G-quadruplex or the  $\Delta$ KC2 pseudoknot forming sequences to a similar extent (Fig. 2.8 a-b). This suggests that translation repression by FMRP is independent of G-quadruplex and pseudoknot forming sequences in the mRNA. However, adding the  $\Delta$ KC2 pseudoknot-forming sequence in trans to the IVTS partially relieved the translation repression by NT-dFMRP (Fig. 2.8 c). This demonstrates that the inhibition of translation by NT-dFMRP is reversible and leads to speculation that the  $\Delta$ KC2 sequence blocks NT-dFMRP from binding to its authentic target required for translation repression.

To test whether at low concentrations of FMRP, authentic target mRNAs will show a greater level of translational repression compared to non-target mRNAs, we did titrations of low concentrations of NT-dFMRP. However, no differential effects on the translation of the control mRNA versus

mRNAs having the G-quadruplex and the pseudoknot forming sequences were seen.

Interestingly, a recent study reported the discovery of RNA sequences known as RNA-recognition elements that are targeted by the two independent RNA-binding regions of FMRP. It was shown that the KH1 domain of FMRP binds to mRNAs having the sequence WGGA and the KH2 domain of FMRP binds to mRNAs having the sequence ACUK (in which K = G or U and W = A or U) (55). However, the sequences ACUK or WGGA



**Figure 2.8: Inhibition of translation by NT-dFMRP is independent of G-quadruplex and pseudoknot forming sequences in the target mRNAs.** (a) (-) NT-dFMRP and (+) NT-dFMRP indicate IVTS performed in the absence and presence of 0.6  $\mu$ M NT-dFMRP, respectively. Red trace, control mRNA; blue trace, mRNA with  $\Delta$ KC2 pseudoknot forming sequence; green trace, mRNA with SC1 G-quadruplex sequence. The data was normalized with respect to the highest signal for each mRNA in the absence of NT-dFMRP. (b) Bar graph of (a). Standard deviations from 3 independent experiments are shown. (c) Inhibition of translation is relieved by adding  $\Delta$ KC2 sequence in trans. Red trace, control translation without NT-dFMRP; blue trace, translation with 0.6  $\mu$ M NT-dFMRP; green trace, translation with 0.6  $\mu$ M NT-dFMRP and 1.5  $\mu$ M of  $\Delta$ KC2 RNA.

occur frequently in a random mRNA sequence, about once in every 128 nucleotides. In other words, an mRNA of 1,000 nucleotides, coding for an average sized ~30 kD protein, will have about 7 ACUK and 7 WGGA sequences. For example, the human insulin mRNA (GenBank ID: X70508), which is one of the smallest transcripts (450 nucleotides), has 2 ACUK and 8 WGGA sequences. To our knowledge, translation of insulin mRNA is not regulated by FMRP. Additionally, the *Renilla* luciferase mRNA used in our *in vitro* translation assays has 7 ACUK and 6 WGGA sequences (Figure 2.9). This suggests that the ACUK and WGGA sequences, by themselves, cannot provide the specificity for translational control by FMRP.

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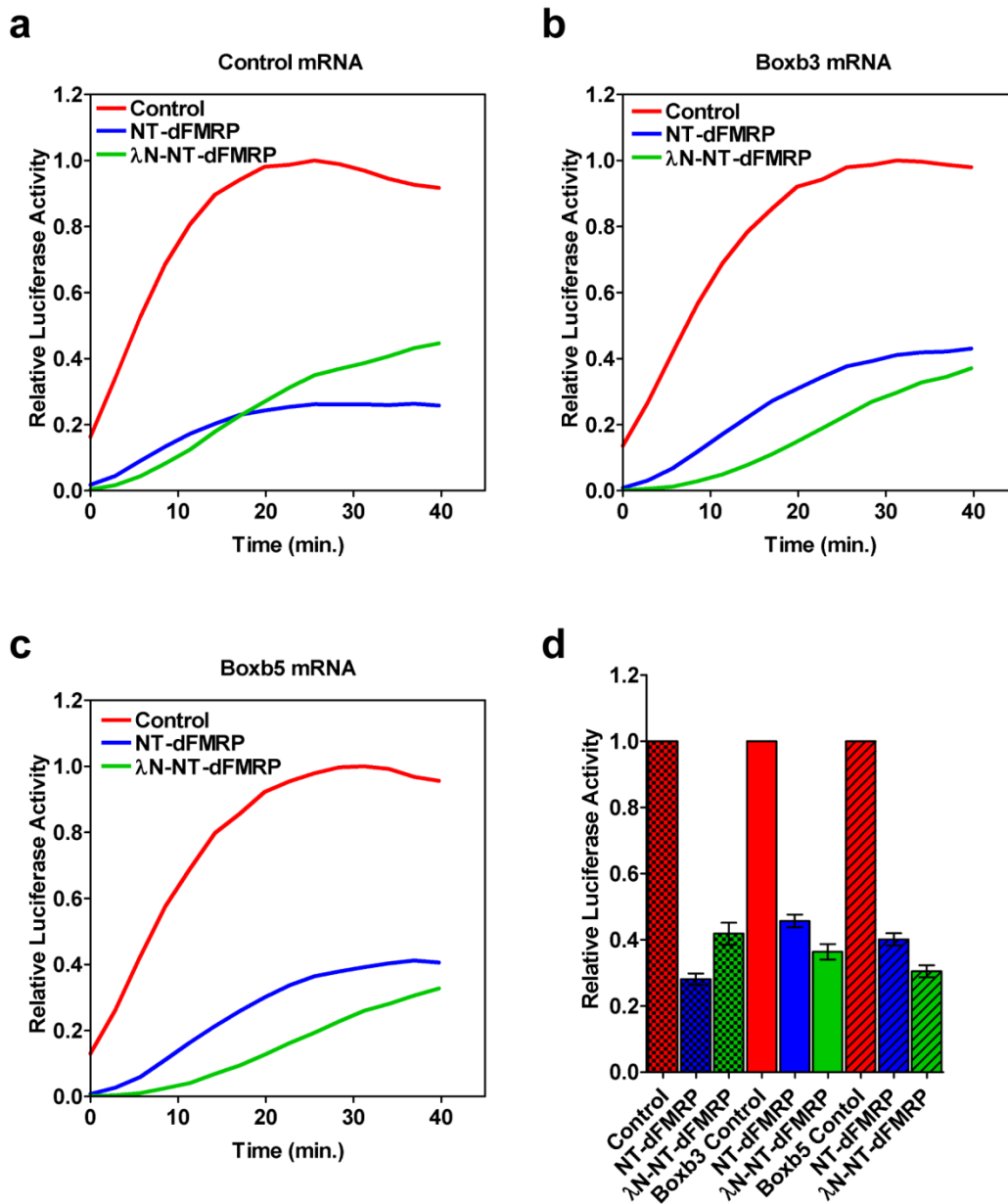
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GACCCAGGAUUCUUUUCCAUGCUAUUGUUGAAGGUGCCAAGAAGUUUCCUAAUACUGAAUUUGUCAAAGUAAAAGG
UCUUCAUUUUUUCGCAAGAAGAUGCACCUAUGAAAUGGGAAAAUAUAUCAAUUCGUUCGUUGAGCGAGUUCUAAAA
AUGAACAAUAA

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**Figure 2.9: The sequence of *Renilla* luciferase mRNA.** The ACUK and WGGA sequences (in which K = G or U and W = A or U) are indicated in green and magenta, respectively.

To verify whether the physical association of FMRP with the mRNA will lead to a greater inhibition of translation, we used the lambda N protein-boxB system to tether NT-dFMRP on the mRNA (56-58). Previous studies have established that a 22 amino acid RNA-binding domain of the lambda N protein ( $\lambda$ N peptide) binds with strong affinity to a specific 19-nt binding site (boxB). Utilization of this tethering technique successfully elucidated the role of proteins in control of mRNA transport, translation, localization, and stability (59).

To test whether mRNA physical interaction with NT-dFMRP was essential for repression of translation, we inserted 3 (Boxb3) or 5 (Boxb5) copies of the boxB sequence at the 3'-UTR of the luciferase reporter mRNA. In addition, the  $\lambda$ N peptide was fused to the N-terminus of NT-dFMRP ( $\lambda$ N-NT-dFMRP). In order to confirm the functionality of  $\lambda$ N-NT-dFMRP, 0.6  $\mu$ M of NT-dFMRP or  $\lambda$ N-NT-dFMRP was added to the control luciferase mRNA. Both proteins displayed similar repression effects on translation, validating the functionality of  $\lambda$ N-NT-dFMRP is not disrupted by addition of the  $\lambda$ N peptide (Figure 2.9 a). NT-dFMRP or  $\lambda$ N-NT-dFMRP was then added to Boxb3 and Boxb5 luciferase mRNAs in the IVTS, and similar inhibition effects were seen in both cases (Figure 2.9 b-d). This indicates that the direct recruitment and association of NT-dFMRP on the target mRNA is not critical for repression of translation.



**Figure 2.10: Physical association of NT-dFMRP with mRNA is not essential for repression of translation.** Red, control translation; blue, translation with 0.6  $\mu$ M NT-dFMRP; green, translation with 0.6  $\mu$ M  $\lambda$ N-NT-dFMRP. (a)  $\lambda$ N-NT-dFMRP has same functionality as NT-dFMRP on control mRNA. (b)  $\lambda$ N-NT-dFMRP and NT-dFMRP inhibit translation similarly on Boxb3 mRNA. (c)  $\lambda$ N-NT-dFMRP and NT-dFMRP inhibit translation similarly on Boxb5 mRNA. (d) Bar graph of (a)-(c) at 30 min. time point. Standard deviations from 3 independent experiments are shown.

Our results show that NT-dFMRP could inhibit the translation of the luciferase mRNA that does not have a G-quadruplex or a pseudoknot forming sequence and incorporation of these sequences in the mRNA does not effect translation repression. Physical interaction of NT-dFMRP with mRNA is also not required for inhibition of translation. This is consistent with previous studies that showed FMRP could repress the translation of a variety of mRNAs (9, 10) and bind to coding regions (30), indicating no specific recognition for secondary structural motifs such as a G-quadruplex or pseudoknots. Nonetheless, there have also been controversial data showing FMRP binding to G-quadruplex and the pseudoknot forming sequences *in vitro* (11, 14). According to our findings, we hypothesize that FMRP may act more as a general translational repressor by binding to the ribosome. Interaction of FMRP with both the mRNA and the ribosome may act synergistically to inhibit translation to a greater extent. Thus, the structural motifs present in target mRNAs could modulate the extent of FMRP-mediated translational inhibition.

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Chapter 2, in full, is currently being prepared for submission for publication of the material. Eileen Chen, Manjuli R. Sharma, Rajendra K. Agrawal, and Simpson Joseph. The dissertation author was the primary investigator and author of this material.

### **Chapter 3: FMRP Binds Directly to the Ribosome**

## Introduction

FXS was the first genetic disorder found to connect RNA regulation to human cognitive function (1). This is the most common form of hereditary autism or autism spectrum disorder associated with a single gene. It results in a range of intellectual disabilities, from mild to severe, and causes patients to exhibit certain physical characteristics (2). In order to develop novel therapies to improve the quality of life for those who suffer from FXS, extensive research has been done to increase our understanding of the molecular basis of FXS.

One particular aspect meticulously studied was the association of FMRP with polyribosomes and/or messenger ribonucleoproteins (mRNPs). Since mRNA translation can be inhibited at the initiation or elongation steps (3, 4), elucidating FMRP's distribution would advance our knowledge on how FMRP represses translation. Years of debate on this subject originated in 1996, when Kandjian and colleagues (5) described, using non-denaturing detergents in extract preparation (0.5% NP-40), the association of FMRP to actively translating polyribosomes. In the same year, Dreyfuss and colleagues (6) published that FMRP is associated to ribonucleoparticles, in particular to the 40S-60S and 80S fractions. However, these findings were obtained using a different protocol that did not include detergent. Interestingly, Warren and colleagues (7, 8) showed that FMRP is equally

distributed between polyribosomes and mRNPs using conditions similar to the ones used by Khandjian and colleagues (0.3% NP-40). Since then, several laboratories have tried to detect the association of FMRP with the polyribosomes and/or mRNPs. Finally, in 2004, Darnell and colleagues (9) definitively showed the presence of FMRP on polyribosomes from the mouse brain and revealed that some previous protocols with addition of the detergent deoxycholate completely disrupts FMRP's interaction with polyribosomes. It is now well established that the majority (90%) of cellular FMRP is associated with polyribosomes (9-11). Whether this association of FMRP with the ribosome is direct or indirect is still unclear. However, the presence of FMRP mRNPs in p bodies and stress granules suggests that FMRP may repress translation throughout many phases of translational regulation.

Structural studies would highly benefit the process to further elucidate FMRP's function. Nevertheless, due to aggregation characteristics and instability of full-length FMRP, purification of large quantities of the protein for structural studies has not been very successful. The structure of the entire FMRP has not been resolved so far, and only atomic structures of small fragments of FMRP and its homologs are known from NMR and X-ray crystallographic studies (12-14). The structure for residues 1-134 of the N-terminal domain, resolved by NMR, reveals the three-dimensional organization of the two Tudor domains, each one formed by a barrel-like fold

made of four-stranded antiparallel  $\beta$  sheet. An unstructured fragment links the two Tudor domains. The structure of the first 134 residues reveals three structural motifs, the two Tudor domain repeats and one  $\alpha$  helix. Extensive interactions are observed between these elements, strongly suggesting that all elements are crucial for the stability of the overall N-terminal domain. The structure also reveals hydrophobic pockets on the surface of the two Tudor domains, and these hydrophobic pockets can bind methylated amino acids (14).

The three-dimensional structure of the tandem KH domains in human FMRP has been solved by X-ray crystallography. However, the crystallized construct contains a shortened variable loop in the KH2 domain. The KH1 and KH2 domains both consist of a  $\beta^1\alpha^1\alpha^2\beta^2\beta'\alpha'$  fold, three-dimensionally oriented as a three-stranded  $\beta$ -sheet domain opposed to a three  $\alpha$ -helices domain. The main hydrophobic core of the domain comprises the buried hydrophobic residues between the hydrophobic faces of the  $\beta$ -sheet and the  $\alpha$ -helices. The  $\beta$ -sheet in each KH domain consists of two parallel, core  $\beta$  strands,  $\beta^1$  and  $\beta^2$ , that sandwich the  $\beta'$  strand in an all anti-parallel arrangement. Residue I304 is part of the hydrophobic core that stabilizes the three-dimensional folding of the KH2 domain, and the missense mutation I304N has been hypothesized to disrupt the structure and destabilize the protein (12). Nonetheless, these partial structures of FMRP can only provide limited insight into the function of FMRP and a structure of a larger fragment

or the entire protein is needed to understand the overall spatial orientation of the different functional domains within FMRP.

Although it is now well established that FMRP functions to negatively regulate protein synthesis, the mechanism by which repression is achieved remains controversial. Mechanisms that have been proposed include: (a) FMRP inhibits the initiation of translation. In cap-dependent translation, which is essential in neurons, initiation requires the eIF4A-eIF4G-eIF4E (eIF4F) complex to associate with the 5' N<sup>7</sup>-methyl guanosine cap of the mRNA template. 4E-binding proteins (4E-BP) interfere with the eIF4G-eIF4E interaction, thereby controlling the formation of the eIF4F complex. In 2008, a known protein binding partner of FMRP, cytoplasmic FMRP-interacting protein (CYFIP1), was found to be a 4E-BP (15). These findings have led to a model in which FMRP recruits CYFIP1 to target mRNAs, subsequently associates with eIF4E, and blocks recruitment of the translation-initiation machinery. The *in vivo* relevance of these interactions, however, has been questioned (9, 16-18). (b) FMRP represses translation by blocking elongation (9, 11, 19). This hypothesis is strongly reinforced by a recent study in which FMRP mRNA targets were identified following ultraviolet cross-linking (20). The majority (66%) of mRNA binding was found within the coding region of the 842 transcripts cross-linked to FMRP in mouse brain polyribosomes. Ribosome runoff experiments in which cells were treated with puromycin or sodium azide (a nonspecific inhibitor of translation initiation

that does not affect elongation) also revealed that a portion of FMRP was associated with stalled ribosomes (9, 11). These results support a model whereby FMRP dynamically represses translation in a complex consisting of target mRNAs and stalled ribosomes. (c) FMRP inhibits translation of specific mRNAs through the RNA interference (RNAi) pathway. Both *Drosophila* and mammalian FMRP interact with Argonaute 2 and Dicer (21-23), important components of the RNA-induced silencing complex (RISC), and several specific miRNAs (24) that function together to silence target mRNA, either by translational repression or direct cleavage of transcripts. However, FMRP lacks a canonical miRNA-binding domain, so it currently seems that this modulation occurs through protein-protein interactions between members of the RISC, rather than direct binding to miRNAs. (d) Post-translational modifications of FMRP have also been suggested to regulate translational inhibition. FMRP can be phosphorylated on a series of serine residues N terminal to the RGG box. Phosphorylation has been proposed to stall ribosomal translocation while maintaining the interaction of FMRP with the mRNA (11, 25, 26), whereas unphosphorylated FMRP allows translation to proceed. Thus, one way neural activity may gate translation is by regulating FMRP phosphorylation. FMRP can also be methylated on arginine residues and reduce FMRP binding to stem loop G-quartet structures (27). Notably, these models of FMRP-regulated translation

repression are not necessarily mutually exclusive, and different mechanisms could apply at different times in the life of an mRNA molecule.

Interestingly, the earlier mindset of FMRP translational regulation was focused on FMRP targeting specific mRNAs with consensus sequences or secondary structural motifs (e.g., G-quadruplex, pseudoknot). However, little to no overlap was shown between the identified mRNA targets of FMRP from several laboratories (8, 28-30). Additionally, more recent studies have shown increasingly controversial data of FMRP binding to coding regions of the mRNAs (20), where there is no possibility of folding into the previously proposed targeted structural motifs of FMRP. Thus, the underlying mechanism of translational repression remains unclear.

In chapter 2, we have already found that mRNA binding and previously proposed structural motifs do not significantly affect translational repression of FMRP. This strongly suggests a mechanism involving a more general suppressor of translation, leading us to the hypothesis of FMRP directly binding to the ribosome. In this chapter, to advance our knowledge of what the true underlying mechanism of FMRP translational regulation is, we have established several reliable methods to test direct and specific binding of NT-dFMRP and its mutants to the ribosome in the absence of mRNA. We have also developed the first fluorescence-based, quantitative assay to monitor the binding of NT-dFMRP and its mutants to the 80S ribosome. Our results indeed show strong and direct binding of NT-dFMRP

to the *Drosophila* 80S ribosome. The binding of NT-dFMRP to the ribosome is also independent of mRNA suggesting that FMRP may function as a general repressor of translation as hypothesized.

Furthermore, to map the binding site of FMRP on the ribosome, we established a chemical crosslinking assay and discovered that NT-dFMRP binds specifically to the large 60S ribosomal subunit protein L5. In addition, mass spectrometry data showed that a peptide fragment from the N-terminus of NT-dFMRP was crosslinked to L5. This correlates well with a previous study, which showed that FMRP could interact with ribosomal proteins L5 and L18 (22), which are part of the central protuberance in the 60S subunit. We next collaborated with Dr. Rajendra Agrawal (Wadsworth Center, Albany, NY) and obtained a cryo-electron microscopy (cryo-EM) map of the 80S ribosome-bound NT-dFMRP. Our preliminary cryo-EM studies show that NT-dFMRP binds at the interface of the large and small ribosomal subunits, suggesting that FMRP can inhibit protein synthesis by blocking the interaction of translation factors with the ribosome or interfere with ribosome assembly. This is a major breakthrough for understanding how FMRP can repress translation via binding to the 80S ribosome. Thus, by combining biochemical, biophysical, and structural approaches, our results are suggesting a new mechanism for FMRP function that could help in identifying novel drug targets to treat FXS.

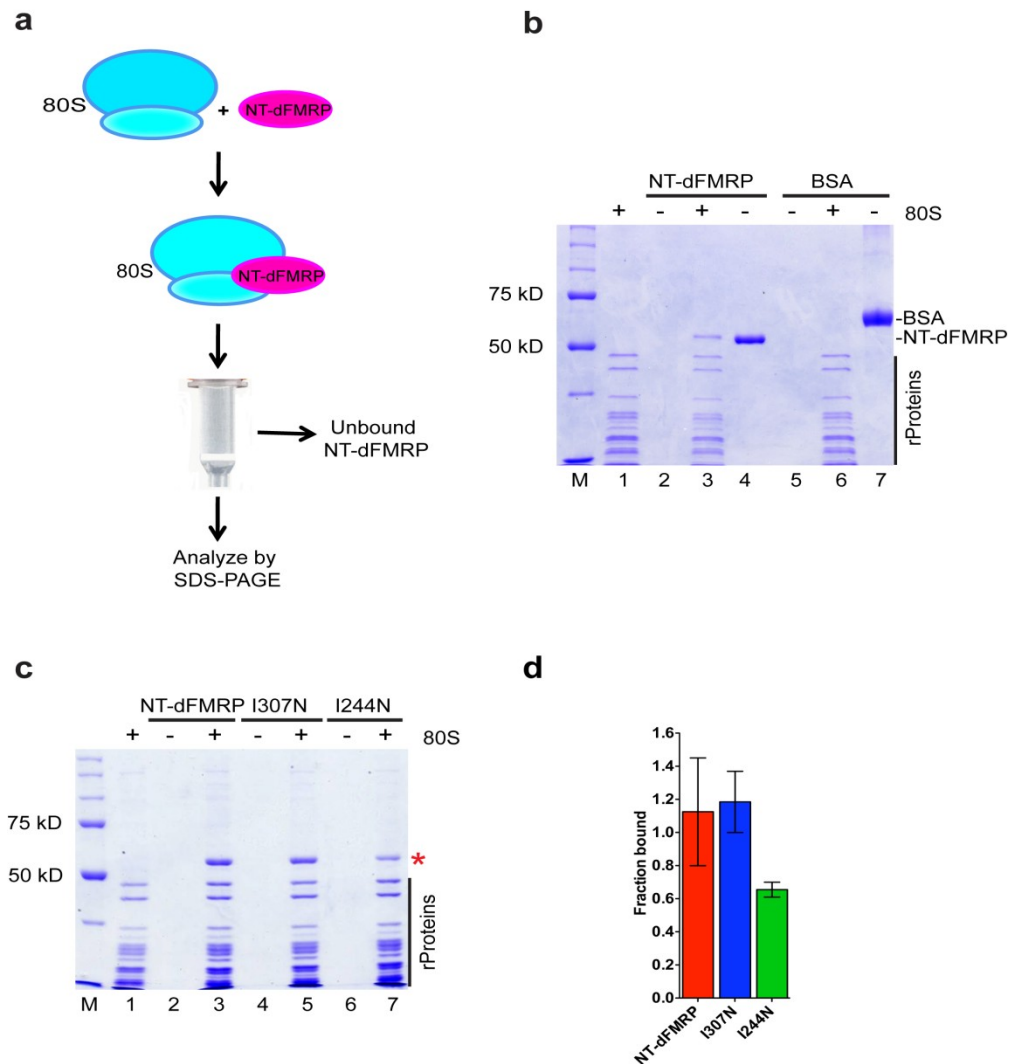
## Results and Discussion

### 3.1 FMRP binds directly to the ribosome

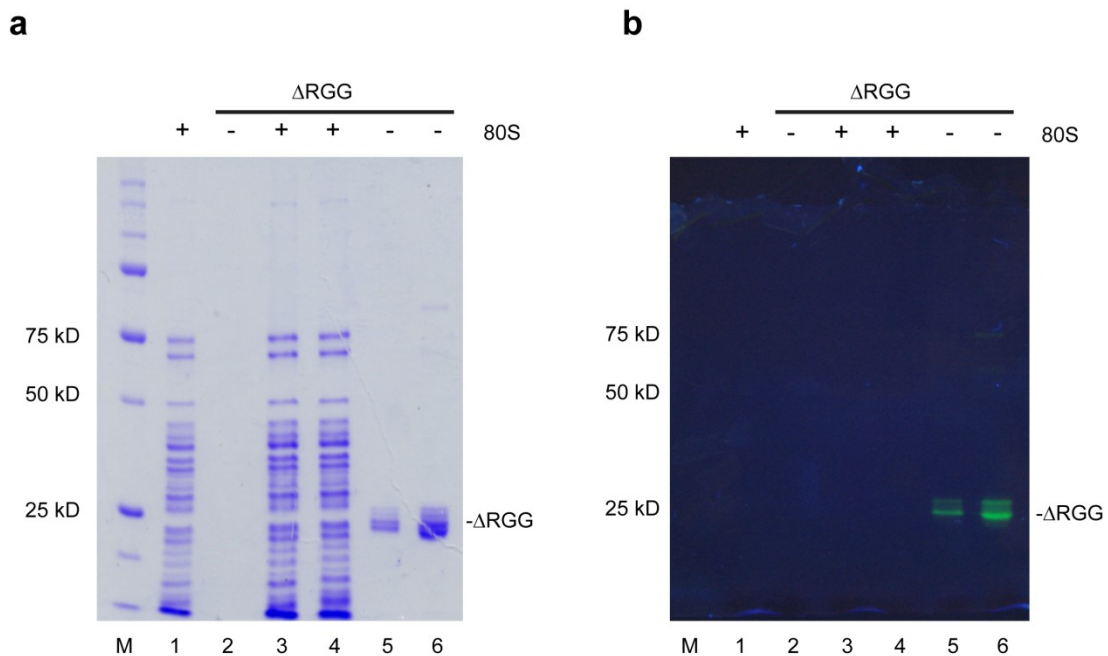
Previous studies have indicated that FMRP can directly associate with the ribosome (5, 9, 22, 31). However, other reports showed that FMRP binds to the ribosome via the mRNA or as an mRNP complex (19, 32-34). It is not clear whether mRNA or other components are required for the association of FMRP with the ribosome. As binding to the reporter mRNA is not critical for inhibiting translation (shown in chapter 2), we hypothesize that FMRP may bind directly to the ribosome to inhibit translation. To test this, ribosomes were purified from *Drosophila* embryos by sucrose gradient centrifugation. To analyze whether NT-dFMRP can bind directly to the ribosome we used a gel filtration chromatography method. NT-dFMRP (2.4  $\mu$ M) was incubated with purified 80S ribosome (0.4  $\mu$ M) and the reaction was applied to a Sephacryl-S300 gel filtration column (Figure 3.1 a). Yeast total tRNA (30  $\mu$ g/ml) was included in the binding reactions to reduce nonspecific binding. The pore size of this matrix is such that the 80S ribosome will be eluted from the column in the void volume, whereas the column will retain unbound NT-dFMRP (51 kD). Analysis of the sample eluted from the column by SDS-PAGE showed that NT-dFMRP elutes along with the 80S ribosome, indicated clearly by the 51 kD band corresponding to NT-dFMRP (Figure 3.1 b, lane 3)

that was not observed in the control reactions (Figure 3.1 b, lanes 1-2). The amount of NT-dFMRP bound to the ribosome appears to be stoichiometric, even though excess amount of NT-dFMRP was present in the binding reaction (Figure 3.1 b, lanes 3-4). In contrast, BSA (4.8  $\mu$ M) used as a control does not bind to the ribosome and was retained by the column (Figure 3.1 b, lanes 5-7). These results demonstrate that NT-dFMRP can directly bind to the ribosome without contacting the mRNA and also correlates with the data from chapter 2, where we discovered NT-dFMRP, but not BSA, represses translation. This strengthens our hypothesis of NT-dFMRP repressing translation by binding directly to the ribosome.

We next tested the binding of NT-dFMRP with mutations in the KH1 (I244N), KH2 (I307N), or RGG ( $\Delta$ RGG) domains. By comparing the relative protein band intensities, it was shown that the KH1 mutant (2.4  $\mu$ M) displayed a 2-fold reduced binding activity to the 80S ribosome (0.4  $\mu$ M), while the KH2 mutant (2.4  $\mu$ M) bound to a similar extent as NT-dFMRP (Figure 3.1 c-d). The  $\Delta$ RGG mutant (2.4  $\mu$ M) did not show any binding to the 80S ribosome (Figure 3.2 a). However, to exclude the possibility of overlapping with ribosomal protein bands migrating similarly on the SDS-PAGE, we fluorescently labeled the  $\Delta$ RGG mutant using 5-iodoacetamidofluorescein (5-IAF) and confirmed that that the  $\Delta$ RGG mutant does not bind to the 80S ribosome under conditions tested (Figure 3.2 b). Our findings demonstrate that the KH1 and RGG domains are crucial for



**Figure 3.1: NT-dFMRP binds directly to the ribosome.** (a) Experimental setup for gel filtration chromatography ribosome binding assay. (b) SDS-PAGE gel showing that NT-dFMRP elutes with the 80S ribosome, whereas BSA remains in the gel filtration column. Lanes: M, molecular weight ladder; 1, 80S only; 2, NT-dFMRP only; 3, 80S + NT-dFMRP; 4, input NT-dFMRP directly loaded on the gel; 5, BSA only; 6, 80S + BSA; 7, input BSA directly loaded on the gel. Positions of NT-dFMRP (51 kD) and BSA (67 kD) are indicated. Vertical black bar indicates ribosomal proteins. (b) KH1 domain of FMRP is important for binding to the ribosome. Lanes: M, molecular weight ladder; 1, 80S only; 2, NT-dFMRP only; 3, 80S + NT-dFMRP; 4, KH2 (I307N) mutant only; 5, 80S + KH2 (I307N) mutant; 6, KH1 (I244N) mutant only; 7, 80S + KH1 (I244N) mutant. [\*] indicates NT-dFMRP and mutants. (c) Bar graph of ribosome binding data. Standard deviations from three independent experiments are shown.



**Figure 3.2: The  $\Delta$ RGG mutant does not bind to 80S ribosome.** (a) Coomassie staining of the SDS-PAGE gel shows no binding of the  $\Delta$ RGG or 5-IAF- $\Delta$ RGG mutant to the 80S ribosome. Lanes: M, molecular weight ladder; 1, 80S only; 2, 5-IAF- $\Delta$ RGG mutant only; 3, 80S +  $\Delta$ RGG mutant; 4, 80S + 5-IAF- $\Delta$ RGG mutant; 5, 5-IAF- $\Delta$ RGG mutant (2.4  $\mu$ M) directly loaded on the gel; 6, 5-IAF- $\Delta$ RGG mutant (4.8  $\mu$ M) directly loaded on the gel. Position of  $\Delta$ RGG mutant (~22 kD) is indicated. (b) Same gel as panel a, showing clearly under ultraviolet light the fluorescently labeled 5-IAF- $\Delta$ RGG mutant does not bind to the 80S ribosome (lane 4). Position of  $\Delta$ RGG mutant (~22 kD) is indicated.

binding to the ribosome. The binding results are also consistent with our *in vitro* functional data (chapter 2), which showed that the KH1 and RGG domains are important for translational repression by NT-dFMRP.

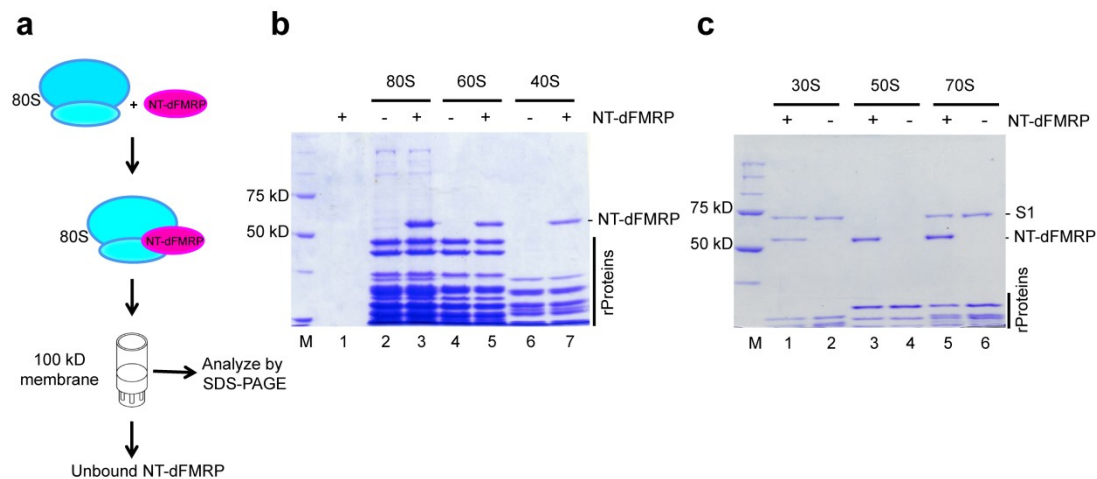
Furthermore, we used a second ultrafiltration method to validate the binding of NT-dFMRP to the ribosome. Similar binding reactions used for the gel filtration chromatography method were set up. NT-dFMRP was

incubated with the 80S ribosome and the reaction was applied to an Amicon filter with a 100 kD cut-off. The filter was then extensively washed with binding buffer (contains 30  $\mu$ g/ml of yeast total tRNA to reduce nonspecific binding) to remove NT-dFMRP that was not bound to the ribosome. The retentate was then analyzed by SDS-PAGE (Figure 3.3 a). NT-dFMRP (51 kD) was present in the retentate, indicating that it binds to the 80S ribosome (Figure 3.3 b, lane 3). As expected, in the absence of the ribosome, NT-dFMRP passed through the Amicon filter (Figure 3.3 b, lane 1).

To further dissect and map the binding site of NT-dFMRP to the ribosome, association of NT-dFMRP to purified 60S and 40S ribosomal subunits were also tested. Interestingly, NT-dFMRP bound to both large and small ribosomal subunits (Figure 3.3 b, lanes 4-7). However, as indicated from relative protein band intensities, NT-dFMRP displayed much less binding affinity toward the 40S small ribosomal subunit (Figure 3.3 b, lane 7). Our results correlate well with previous studies, which have shown that FMRP coimmunoprecipitates with 60S large ribosomal subunits, and that small amounts of FMRP cosediment with 40S ribosomal subunits in linear sucrose gradients. Nevertheless, it was not clear at that time (6) whether this interaction was direct or indirect and the significance was not fully understood. It has been hypothesized that FMRP may associate with the ribosome at a site that is at a junction of the two interacting ribosomal subunits (6), and our studies seem to support that. Moreover, our results

definitively show direct binding of NT-dFMRP to the ribosome in the absence of mRNA.

We also used the ultrafiltration method to test association of NT-dFMRP with the *E. coli* 70S ribosome, 50S large ribosomal subunit, and 30S small ribosomal subunit. Intriguingly, NT-dFMRP exhibited the ability to bind to the 70S ribosome, the 50S, and 30S ribosomal subunits (Figure 3.3 c). Similar to association of the 40S small subunit, relative protein band

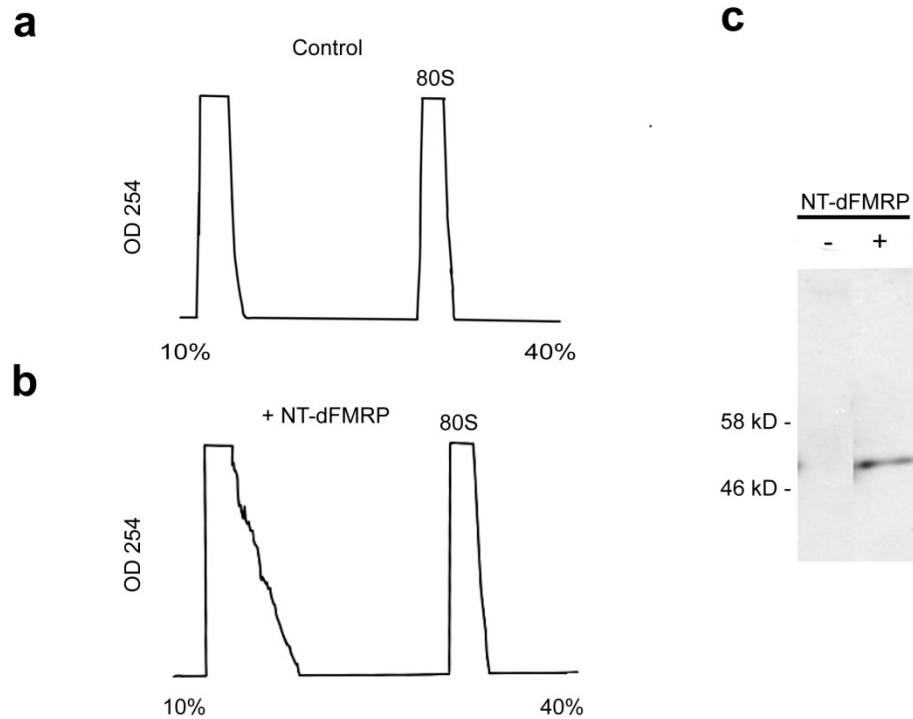


**Figure 3.3: NT-dFMRP binds directly to the eukaryotic and prokaryotic ribosomes.** (a) Experimental setup for ultrafiltration ribosome binding assay. (b) NT-dFMRP binds to the 80S ribosome, 60S, and 40S ribosomal subunits. Lanes: M, molecular weight ladder; 1, NT-dFMRP only; 2, 80S only; 3, 80S + NT-dFMRP; 4, 60S only; 5, 60S + NT-dFMRP; 6, 40S only; 7, 40S + NT-dFMRP. Position of NT-dFMRP is indicated. Vertical black bar indicates ribosomal proteins. (c) NT-dFMRP binds to *E.coli* 70S ribosome, 50S, and 30S ribosomal subunits. Lanes: M, molecular weight ladder; 1, 30S + NT-dFMRP; 2, 30S only; 3, 50S + NT-dFMRP; 4, 50S only; 5, 70S + NT-dFMRP; 6, 70S only. Positions of NT-dFMRP and ribosomal protein S1 are indicated. Vertical black bar indicates ribosomal proteins.

intensities exhibited reduced binding of NT-dFMRP to the 30S small ribosomal subunit (Figure 3.3 c lane 1). This strongly implies that NT-dFMRP may be binding to a conserved region of the eukaryotic and prokaryotic ribosomes. Our preliminary chemical modification and footprinting studies suggest that NT-dFMRP binds near the A-site finger in the *E. coli* 70S ribosome. However, further studies need to be done to strengthen this proposition.

To further authenticate the association of NT-dFMRP with the 80S ribosome, *in vitro* translation reactions (described in Chapter 2) in the absence or presence of excess NT-dFMRP were fractionated through a 10%-40% linear sucrose density gradient (Figure 3.4 a-b). Purified 80S ribosomes were also sedimented through the 10%-40% linear sucrose density gradient to indicate which fraction corresponds to the 80S ribosome. The 80S ribosome fractions were collected and treated with trichloroacetic acid (TCA) to precipitate the proteins, then Western blotting analysis with anti-dFMRP antibody revealed NT-dFMRP in the 80S fraction (Figure 3.4 c). We also detected excess unbound NT-dFMRP in the top fraction, corresponding to specific and stoichiometric binding of NT-dFMRP to the 80S ribosome. These results indicate a strong interaction of NT-dFMRP with the 80S ribosome.

Taken together, our results show that NT-dFMRP is capable of associating directly with the 80S ribosome in the absence of mRNA. The



**Figure 3.4: Sucrose gradient profiles and immunoblots show NT-dFMRP associates strongly with the 80S ribosome.** (a) Control *in vitro* translation reaction sedimented on a 10%-40% linear sucrose density gradient in the absence of excess NT-dFMRP. The 80S fraction is indicated. From left to right are top to bottom (10% to 40%) fractions. (b) *In vitro* translation reaction fractionated on a 10%-40% linear sucrose density gradient in the presence of excess NT-dFMRP. The 80S fraction is indicated. (c) Western blot confirms cosedimentation of NT-dFMRP (51 kD) with the 80S ribosome in the 80S fraction collected from panel b.

direct association of NT-dFMRP with the 80S ribosome may be important for translation repression. Moreover, the binding site of NT-dFMRP seems to be within the inter-subunit space of the ribosome. This region of binding could interfere with ribosome assembly, block elongation, or give rise to structural changes of the ribosome that may influence steps after translation initiation. We speculate that this results in inhibition of protein synthesis.

### **3.2 Development of FRET-based quantitative assay for measuring the affinity of FMRP for the ribosome**

To determine the binding affinity of NT-dFMRP to the 80S ribosome, we strived to develop a fluorescence-based assay. Our goal was to obtain a binding curve to calculate the equilibrium dissociation constant ( $K_D$ ) using the fluorescence signal difference of unbound NT-dFMRP and NT-dFMRP bound to the ribosome. We initially labeled NT-dFMRP (10 nM) with 5-IAF or N-(1-Pyrene)maleimide and titrated with increasing concentrations of the unlabeled 80S ribosome (0-100 nM). However, the fluorescence signal was unstable due to aggregation in the binding reaction. The addition of 0.015% Nikkol, a nonionic detergent, was found to stabilize the fluorescence signal and solve this matter. Nevertheless, problematic light scattering issues arose with the addition of increasing amounts of 80S ribosome. Due to this, the fluorescence signal change could not be accurately calculated. To

resolve this setback, we had to establish an assay where we could titrate increasing amounts of NT-dFMRP instead of the 80S ribosome and maintain optimal fluorescence signal change.

Förster Resonance Energy Transfer (FRET) is widely applied in measuring structural changes within macromolecules, the proximity of molecules, as a signal of biochemical events, and as a sensor of local conditions (35-37). In FRET, one fluorophore (donor) spontaneously transfers its energy by a nonradiative dipole-dipole interaction to another molecule (acceptor) after being excited by an initial photon of light (usually supplied by a laser or a filtered arc lamp). The probability of energy transfer in this way is related to both the spectral characteristics of the fluorophores involved and the distance between them, making the technique useful for proximity and structural measurements.

To determine the binding affinity of NT-dFMRP for the ribosome we developed a FRET-based assay using the fluorescent dyes Cy3 (donor) and Cy5 (acceptor) (Figure 3.5 a). Cy3 and Cy5 dyes form an efficient FRET donor-acceptor pair and energy transfer can only occur within distances of 1-100 Å (38). We attached the fluorescent dye Cy5 to the single cysteine at position 263 in dFMRP using the manufacturer's procedure (GE Healthcare). To label the purified *Drosophila* ribosome, we used an amine reactive Cy3 dye that reacts with all the exposed amino groups in the ribosomal proteins. Various concentrations of Cy5-NT-dFMRP were incubated with a constant

amount of Cy3-ribosome to form the ribosome•NT-dFMRP complex. In parallel, we performed control experiments with similar concentrations of Cy3-ribosome only and Cy5-NT-dFMRP only. The sample containing both Cy3-ribosome and Cy5-NT-dFMRP clearly showed a decrease in Cy3 fluorescence emission (near 575 nm) and an increase in the Cy5 fluorescence emission (near 665 nm) compared to the two controls. This shows that NT-dFMRP binds to the 80S ribosome resulting in FRET (Figure 3.5 b-c).

The increase in FRET efficiency because of NT-dFMRP binding to the ribosome was used to measure the  $K_D$ . The FRET efficiency is the proportion of light absorbed by the donor that is transferred in a nonradiative manner to the acceptor. FRET efficiency (%) was calculated by the equation  $I_D/(I_D+I_A)*100$  (39).  $I_D$  stands for the intensity of donor at 575 nm and  $I_A$  stands for the intensity of acceptor at 665 nm. FRET efficiency was then fitted to a quadratic equation using Graphpad prism to obtain the  $K_d$  values.

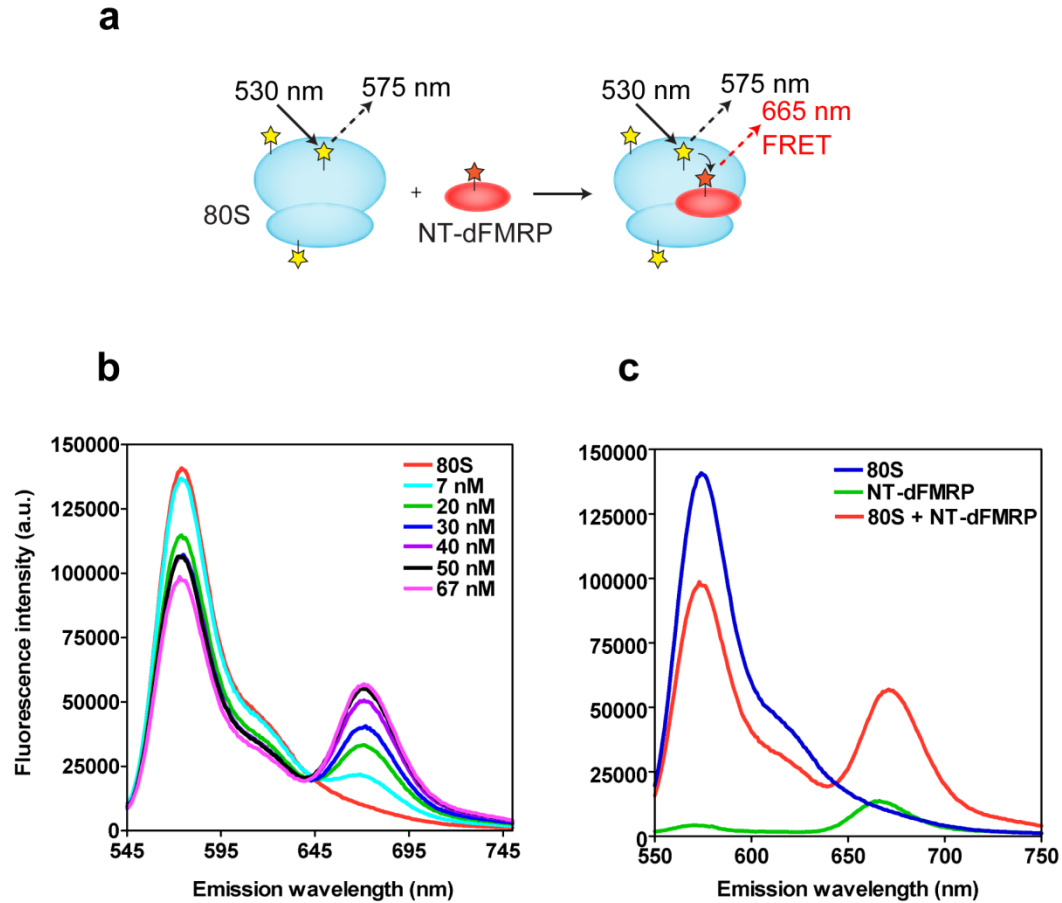
Cy3-ribosome (2 nM) was titrated with Cy5-NT-dFMRP (0-125 nM) with excitation at 530 nm. Emission maxima for the donor Cy3-ribosome and the acceptor Cy5-NT-dFMRP were determined at 575 nm and 665 nm, respectively. Under the same conditions, buffer was also titrated with Cy5-NT-dFMRP and each buffer emission spectrum was subtracted from the corresponding fluorescence emission spectrum. The FRET efficiency data

was then used to calculate the  $K_D$ . The  $K_D$  for NT-dFMRP is  $20 \pm 3$  nM showing that it binds with high affinity to the ribosome (Figure 3.6 a).

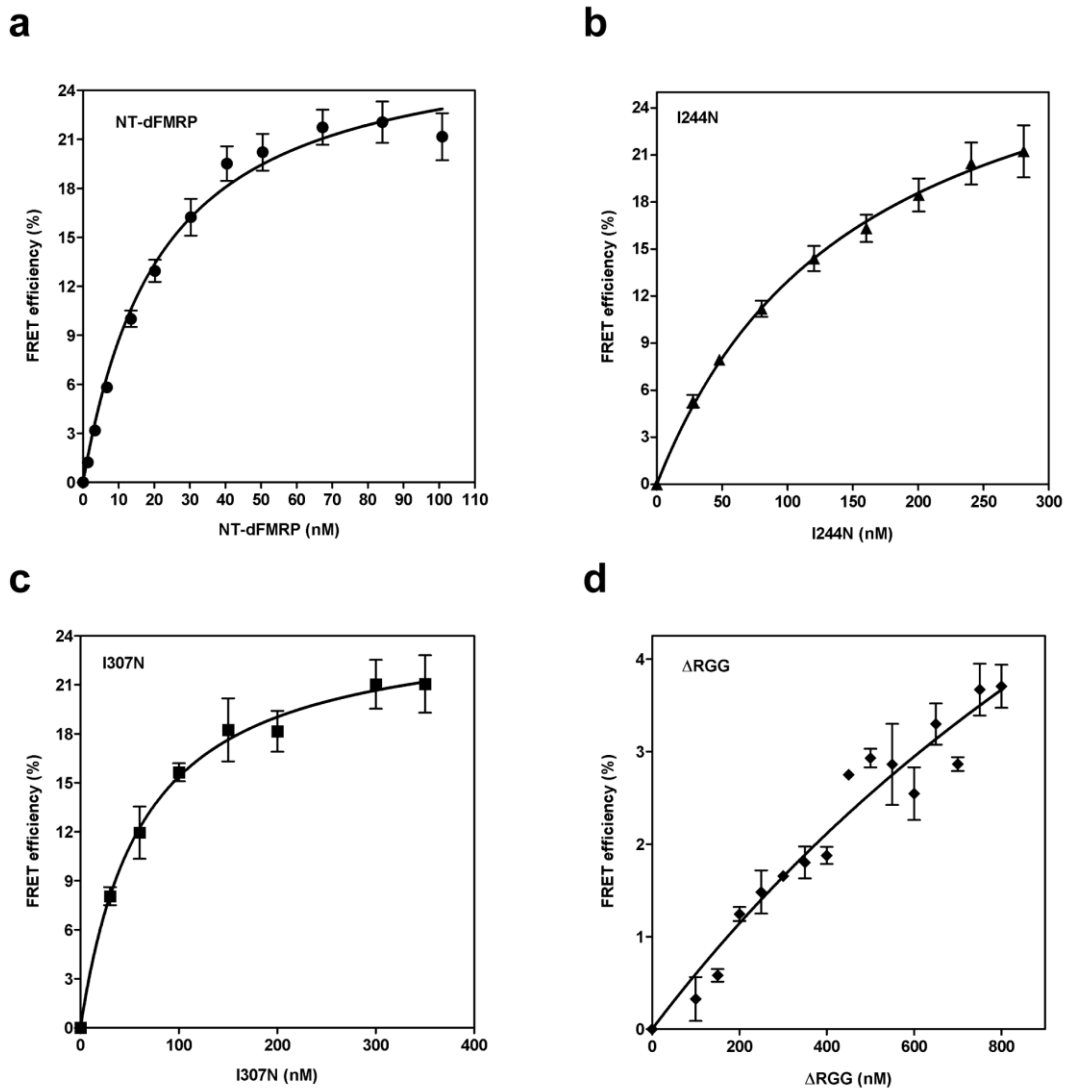
We also analyzed the KH1, KH2, and the  $\Delta$ RGG mutants using this quantitative FRET-based binding assay. Our results showed that the  $K_D$  for the KH1 and KH2 mutants are  $130 \pm 5$  nM and  $61 \pm 2$  nM, respectively (Figure 3.6 b-c). In contrast, the  $\Delta$ RGG mutant bound poorly to the ribosome with a  $K_D$  that is  $> 2$   $\mu$ M (Figure 3.6 d). These results correlate very well with the *in vitro* translation assay, which showed that the  $\Delta$ RGG mutant was defective and the KH1 mutant was slightly defective in repressing translation compared to the KH2 mutant and the wild type NT-dFMRP. Previous studies with the individual KH domains showed that the I244N mutation in the KH1 domain was shown to unfold the protein (40), whereas the I307N mutation in the KH2 domain was shown to adopt a native-like fold (41). This may explain why the KH1 (I244N) mutant binds with lower affinity to the ribosome compared to the KH2 (I307N) mutant. These findings confirm that NT-dFMRP can bind directly to the ribosome to inhibit translation.

Therefore, we have established a sensitive FRET-based assay for analyzing the binding of FMRP to the ribosome. Most studies in the field utilize techniques such as coimmunoprecipitation, linear sucrose gradients, etc., to determine association of FMRP to other components. However, it is difficult to interpret direct or indirect interactions using these methods. To our knowledge, our FRET-based assay is the first fluorescence-based,

quantitative assay in the field, which should greatly assist in understanding the mechanism of translational regulation.



**Figure 3.5: FRET-based assay for determining the binding affinity of NT-dFMRP for the ribosome.** (a) Schematic representation of the FRET assay. Ribosome (cyan) is labeled with Cy3 (yellow stars). NT-dFMRP (pink) is labeled with Cy5 (red star). The binding of NT-dFMRP to the ribosome results in FRET (emission at 665 nm). (b) Emission spectrum for the titration of indicated amounts of Cy5-NT-dFMRP with a constant amount of 80S ribosome (2 nM) showing changes in fluorescence. Red trace is the control with 80S (2 nM) only. (c) Emission spectrum displaying the changes in fluorescence intensity due to NT-dFMRP binding to the ribosome. Blue trace, 80S ribosome (2 nM) only control reaction; green trace, NT-dFMRP (100 nM) only control reaction; red trace, 80S ribosome (2 nM) + NT-dFMRP (100 nM) complex.



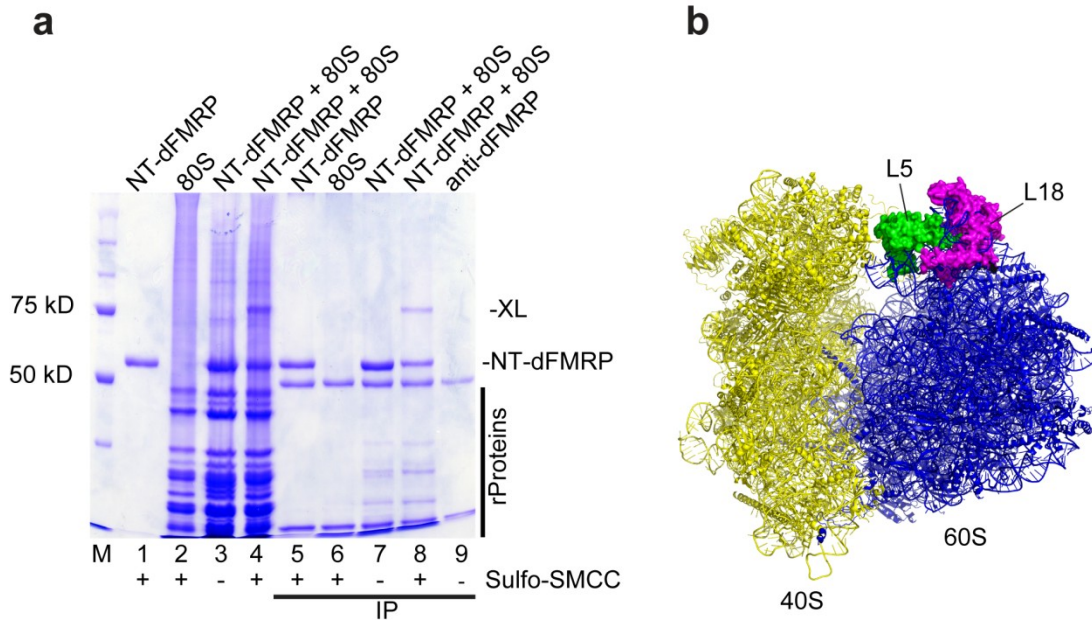
**Figure 3.6: Binding of NT-dFMRP and its mutants to the 80S ribosome.** (a) Binding curve for NT-dFMRP. (b) Binding curve for KH1 (I244N) mutant. (c) Binding curve for KH2 (I307N) mutant. (d) Binding curve for the  $\Delta$  RGG mutant. The y- axis shows the % FRET efficiency and the error bars show standard deviation from three independent experiments.

### **3.3 Mapping the binding site of FMRP on the ribosome by chemical crosslinking**

After discovering that NT-dFMRP can bind directly to the ribosome and that this interaction correlates with repressing translation, revealing the binding site on the ribosome would provide crucial insight for understanding the mechanism of FXS. To map the binding site of NT-dFMRP on the ribosome, we used the chemical crosslinking reagent sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC). Sulfo-SMCC is a water-soluble, amine-to-sulfhydryl crosslinker that contains NHS-ester and maleimide reactive groups at opposite ends of a medium-length cyclohexane spacer arm (8.3 Å). NT-dFMRP bound to ribosome was treated with Sulfo-SMCC and the reaction was analyzed by SDS-PAGE. We observed the appearance of a new ~80 kD band and the amount of the NT-dFMRP band was also decreased (Figure 3.7 a, lane 4 and labeled XL). The ~80 kD band was not present in the control reactions. Purification of the crosslinked sample with the anti-dFMRP 5B6 monoclonal antibody (Developmental Studies Hybridoma Bank) and mass spectrometry analysis indicated that the ~80 kD band consists of FMRP and the large 60S ribosomal subunit protein L5 (previously called L11 in yeast and human ribosomes, but see (42) for more unified nomenclature for the ribosomal proteins).

We next tested a cysteine-free NT-dFMRP mutant by changing the single cysteine at position 263 to a serine. Crosslinking with the 80S ribosome using Sulfo-SMCC showed identical results as the wild type NT-dFMRP. This indicates that the contribution of NT-dFMRP for the crosslink is an amine instead of sulfhydryl group. Furthermore, thorough analysis of the mass spectrometry data showed that a peptide fragment from the N-terminal end of NT-dFMRP was crosslinked to L5, which provides important constraints for the arrangement of NT-dFMRP on the ribosome.

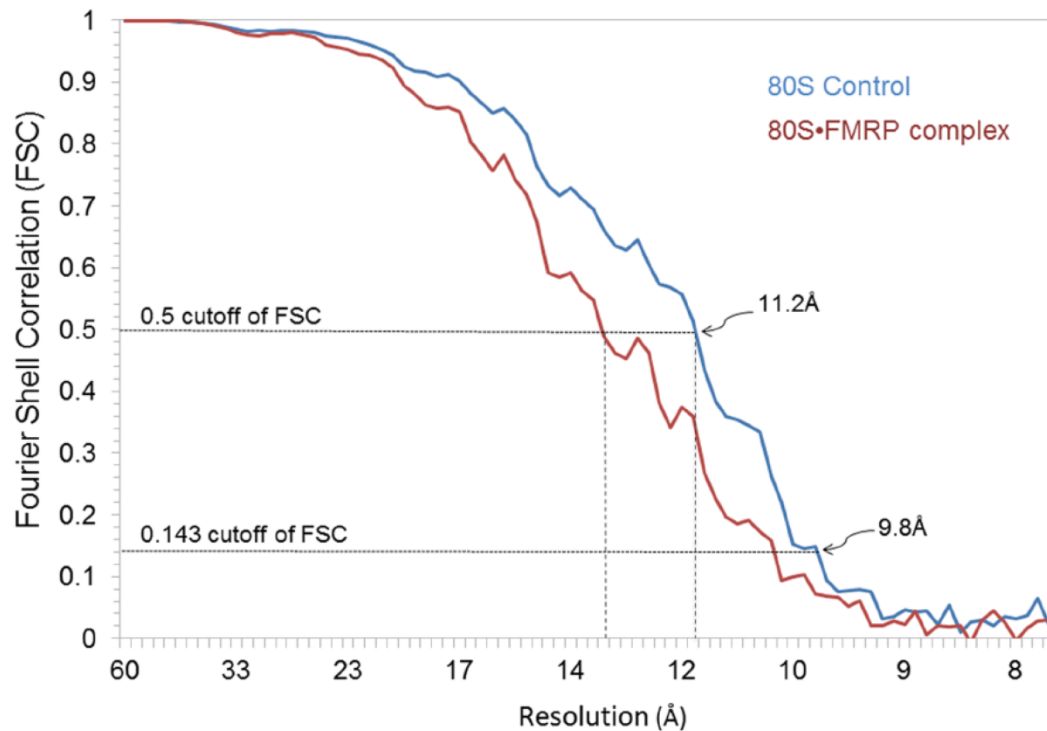
Our results agree with a previous study, which showed that FMRP could interact with ribosomal proteins L5 and L18 (22). However, their results indicate that the last 150 residues in the C-terminal of FMRP is the region important for binding to ribosomal proteins L5 and L18. This region has been known to exhibit nonspecific binding characteristics, and it is also unclear whether this interaction is direct or indirect. In contrast, our crosslinking results are done in the presence of yeast total tRNA to reduce nonspecific binding and we show definitive evidence of direct binding to the ribosomal protein L5. The ribosomal protein L5 is part of the central protuberance in the 60S subunit (43, 44) (Figure 3.7 b). This region of the ribosome is close to the binding site of several translation factors and tRNAs. It is possible that the binding of FMRP to the ribosome blocks the binding of translation factors, which may explain the inhibition of translation.



**Figure 3.7: NT-dFMRP binds near ribosomal protein L5.** (a) SDS-PAGE gel showing crosslinking of NT-dFMRP to the 80S ribosome. Lanes: M, molecular weight ladder; 1, NT-dFMRP only; 2, 80S only; 3, 80S + NT-dFMRP; 4, 80S + NT-dFMRP. Sulfo-SMCC was added to samples in lanes 1, 2, and 4. Lanes 5-8 correspond to samples in lane 1-4 after immunoprecipitation (IP) with anti-dFMRP antibody and purification. Lane 9 shows the heavy and light chains of the anti-dFMRP antibody. Black bar, ribosomal proteins; XL, position of the crosslinked proteins. (b) Crystal structure of the eukaryotic 80S ribosome. The large 60S ribosomal subunit (dark blue) and small 40S ribosomal subunit (yellow) are indicated. NT-dFMRP crosslinked to ribosomal protein L5 (green). Also shown is L18 (magenta).

### 3.4 Determine the structure of the ribosome-FMRP complex by cryo-electron microscopy

In collaboration with the Agrawal laboratory, (Wadsworth Center, Albany, NY) we obtained a cryo-electron microscopy (cryo-EM) map (45-48) of the *Drosophila* 80S ribosome•NT-dFMRP complex to determine the three-dimensional (3D) binding position of NT-dFMRP on the ribosome (49-52). The resolution at 0.5 cutoff of Fourier shell correlation (FSC) for the control 80S ribosome and ribosome•NT-dFMRP complex maps were 11.2 Å (or 9.8 Å, using 0.143 cutoff of FSC (51)) and 12.8 Å (or 10.4 Å, using 0.143 cutoff of FSC), respectively (Figure 3.8). Subtraction of the 3D map of the control *Drosophila* 80S ribosome from that of the 80S ribosome•NT-dFMRP complex shows a complex and elongated mass of density within the ribosomal inter-subunit space that spans from central protuberance (CP) to  $\alpha$ -sarcin/ricin stem-loop (SRL) region of the 60S subunit (Figure 3.9). One end of the elongated difference mass interacts with the central protuberance and A-site finger (ASF) of the large subunit, while its other end is situated between the protein S12 region of the small (40S) subunit and SRL region of the 60S subunit. However, the portion of difference density that interacts with the CP is much stronger than the portion that is situated between the S12 and SRL. Based on our cross-linking data, suggesting that the N-terminus of the NT-dFMRP construct interacts with the CP protein L5, we assign that portion of

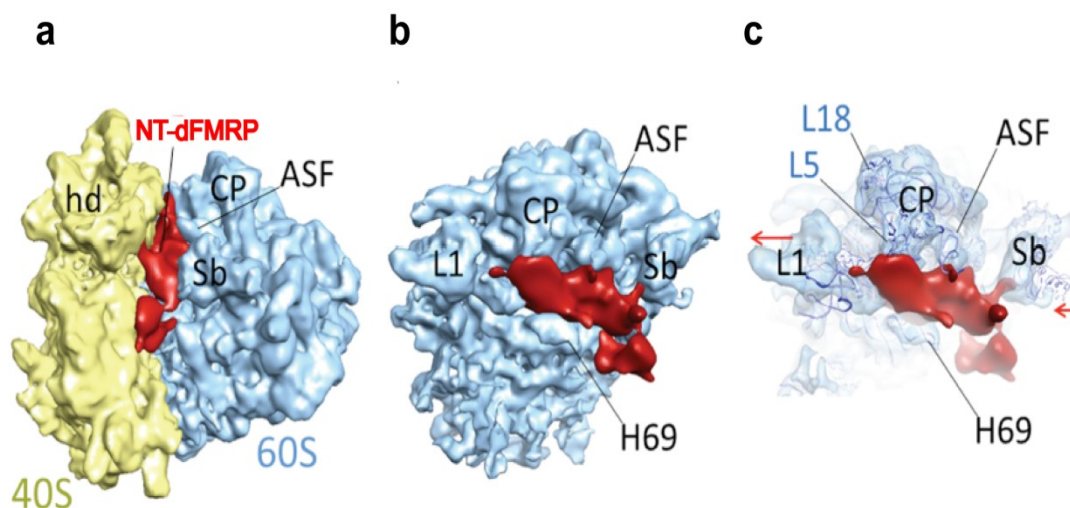


**Figure 3.8: Resolution curves for the cryo-EM maps of the control *Drosophila* 80S ribosome and the 80 ribosome•NT-dFMRP complex.** The resolution at 0.5 cutoff of Fourier shell correlation (FSC) for the control 80S ribosome and ribosome•NT-dFMRP complex maps were 11.2 Å (or 9.8 Å, using 0.143 cutoff of FSC (51)) and 12.8 Å (or 10.4 Å, using 0.143 cutoff of FSC), respectively.

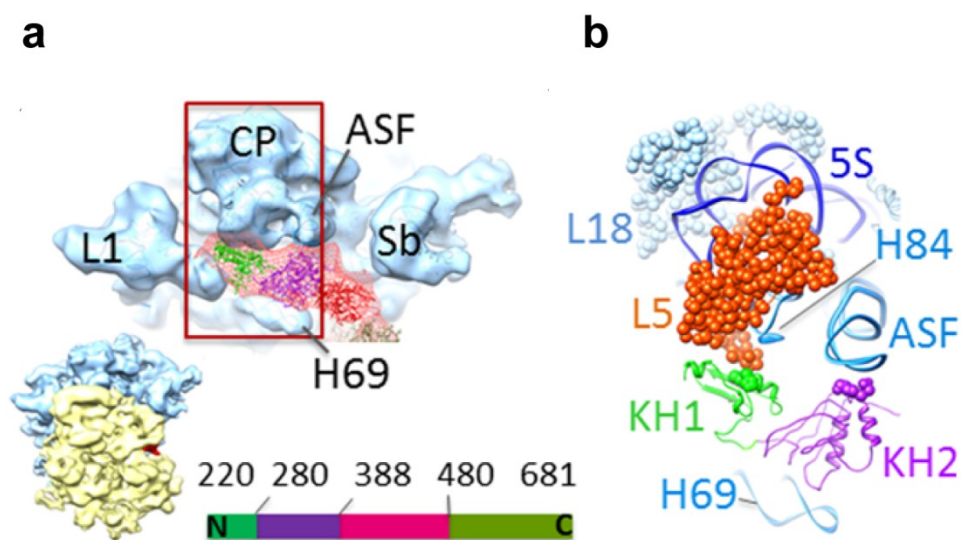
difference map to the N-terminus, and tentatively assign the portion between S12 and SRL to the C-terminus domain of FMRP. Both our crosslinking and cryo-EM results agree with a previous study, which showed that FMRP could interact with ribosomal proteins L5 and L18 (22), which are part of the CP (43). Indeed, we observe a direct interaction of NT-dFMRP with ribosomal protein L5. The previous interaction reported with protein L18 could involve the N-terminus of the full-length FMRP that was absent in our construct.

Atomic structures of small fragments of FMRP and its homologs are known from nuclear magnetic resonance (NMR) and X-ray crystallographic studies (12-14). An I-TASSER (53) homology model generated for our NT-dFMRP construct suggests that NT-dFMRP should be an elongated structure with four structural domains, each linked through long linker regions. Each domain was fitted independently as a rigid body into the cryo-EM density corresponding to NT-dFMRP. Based on our crosslinking data, we first assigned the N-terminus domain (amino acid residues 1-60 of the construct, containing the KH1 motif) to the portion of density closest to the CP of the 60S ribosome. We next fitted the immediately next structural domain (amino acid residues 61-168, containing the KH2 motif), followed by the third structural domain (amino acid residues 169-260, containing first three RGG motifs), and then finally the fourth structural domain (amino acid residues 261-466, containing the remaining two RGG motifs). These independent fittings tentatively place the KH1 motif close to the ribosomal protein L5 and the 28S rRNA helix 84 within the CP of the ribosome, and KH2 motif close to the 28S rRNA helix 38, also known as the A-site finger (Figure 3.10).

The overall features and placements of all four domains match and explain most of the cryo-EM density corresponding to NT-dFMRP. However, the cryo-EM density corresponding to structural domains 3 and 4 are relatively weak, suggesting that the RGG motif containing domains and the C-terminus of FMRP is relatively flexible on the ribosome. In addition, we



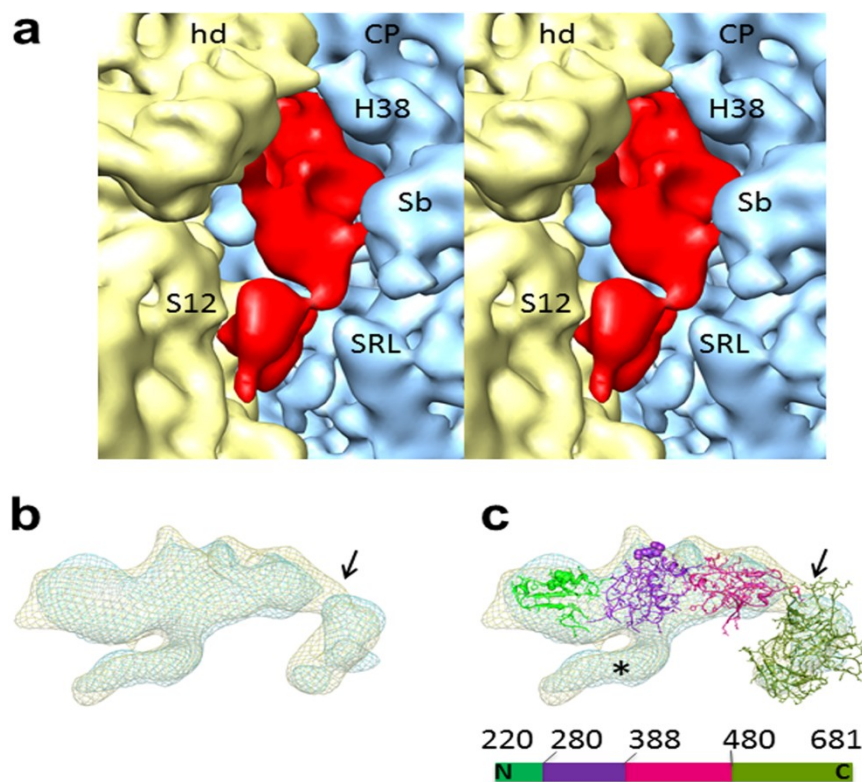
**Figure 3.9: Cryo-electron microscopy structure of the *Drosophila* 80S ribosome•NT-dFMRP complex.** (a) Localization of NT-dFMRP on the *Drosophila* 80S ribosome. The difference map (red) is superimposed on to the cryo-EM map of the control 80S ribosome, with 40S (yellow) and 60S (blue) subunits identified. (b) Same as panel a, but the 40S subunit has been removed and NT-dFMRP density is shown from the inter-subunit face of the 60S ribosomal subunit. (c) Atomic structure of the yeast 60S ribosomal subunit (42) has been docked as a single rigid body, aligning the CP region, into the cryo-EM map. A cutting plane has been applied from the far end to remove the portion of the 60S subunit cryo-EM map for visual clarity. Red arrows depict the movements in L1 and Sb regions with respect to the crystallographic structure. Landmarks: hd, 40 subunit head; L1, protein L1 protuberance; CP, central protuberance; Sb, L7/L12 stalk base; ASF, A-site finger; H69, helix 69 of the 28S rRNA (see Figure 3.11 for a stereo representation of the NT-dFMRP binding region).



**Figure 3.10: Cryo-electron microscopy structure showing overall binding position of NT-dFMRP.** (a) A portion of the 60S subunit map is shown from its interface side to reveal the overall binding position of NT-dFMRP. The fitted I-TASSER model of the NT-dFMRP is displayed onto the 60S subunit with fitted X-ray coordinates of the yeast 60S ribosome. Four structural domains of the NT-dFMRP are identified according to the color in the bar-diagram shown at the bottom. Thumbnail at lower left depicts the overall orientation of the ribosome in panels a and b. (b) The boxed area in panel a is enlarged to reveal putative interactions of KH1 and KH2 domains of NT-dFMRP with the 60S subunit components. The cryo-EM density has been removed for clarity. Landmarks of the 60S subunit: L1, L1 protein protuberance; CP, central protuberance; Sb, L7/L12 stalk base; 5S, 5S rRNA; H69 and H84, 28S rRNA helices 69 and 84, respectively; ASF, A-site finger, or 28S rRNA helix 38; SRL, α-sarcin-ricin stem-loop; and 60S ribosomal proteins L5 and L18 are shown as space-filled models.

observe another weak mass of unexplained density. This density is situated next to the inter-subunit bridge (bridge B2a) forming segment (the helix 69 of the 28S rRNA within the 60S ribosomal subunit) and could result either from a conformational change in the helix 69 region or from an alternative conformation adopted by the FMRP in a small fraction of the ribosome population. Further characterization of this unexplained mass of density may be achieved through more rigorous classification using a much larger cryo-EM data set (Figure 3.11).

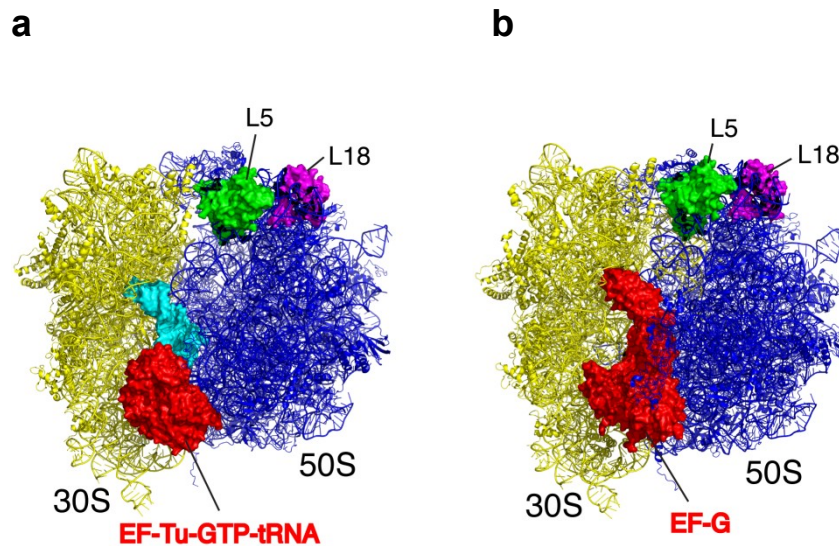
The binding position of NT-dFMRP at the inter-subunit space on the 80S ribosome suggests that FMRP would directly inhibit association of several translation factors and tRNA that are known to bind to the overlapping region on the ribosome (54). This is supported by crystal structures of the 70S prokaryotic ribosome with EF-Tu-GTP-tRNA or EF-G docked in the inter-subunit space that have been resolved (Figure 3.12 a-b). Eukaryotic elongation factor 2 (eEF2) binds between the large and small ribosomal subunits and catalyzes the translocation of the mRNA-tRNA complex in the ribosome. Furthermore, the eukaryotic elongation factor 1A (eEF1A) forms a ternary complex with guanosine triphosphate (GTP) and aminoacyl tRNA and delivers the aminoacyl tRNA to the ribosome during each round of the polypeptide elongation cycle. It is conceivable that the binding of FMRP to the ribosome inhibits the binding of eEF1A•GTP•aminoacyl-tRNA ternary complex. This raises intriguing



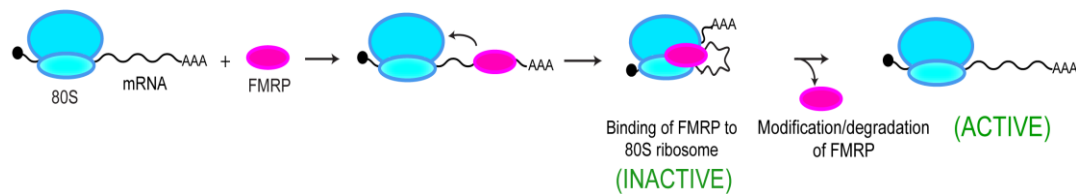
**Figure 3.11: Stereo view of the NT-dFMRP binding region.** (a) A stereo view representation of the binding region of NT-dFMRP (red) on the *Drosophila* 80S ribosome. The orientation of the ribosome is same as in Figure 3.9 a. (b) A comparison of the cryo-EM densities corresponding to NT-dFMRP obtained before and after supervised classification. Superimposed densities of the difference map (light yellow meshwork), obtained by subtracting the cryo-EM map of the 80S control from that of the 80S•NT-dFMRP complex (before classification), and segmented density corresponding to NT-dFMRP (light blue meshwork), as seen within the cryo-EM map computed from classified images. The arrow points to a region of absence of mass in the segmented density that could result due to flexibility of the NT-dFMRP's C-terminal domain (CTD). (c) Fitted positions of four structural domains of the NT-dFMRP homology model into the corresponding cryo-EM density. The space-filled segments within domains 1 (light green) and 2 (magenta) point to KH1 and KH2. As described in the text, the density corresponding to CTD is relatively weak in both NT-dFMRP maps. In addition, we observe another weak mass of unexplained density (marked with an asterisk [\*] in panel c). A bar diagram depicting the four structural domains of the NT-dFMRP homology model in matching colors is shown at the lower right.

possibility that the binding of FMRP to the ribosome may block the binding of translational factors that are important for protein synthesis. This may provide an explanation to the previously reported inhibition of the elongation step of protein synthesis by FMRP (1, 20, 55-57). In conclusion, we hypothesized an innovative model that FMRP inhibits translation by docking on the ribosome and blocking the binding of translation elongation factors (eEF2 and eEF1A) to the ribosome (Figure 3.13). Further studies using our FRET-based assay should validate whether the binding of FMRP blocks the elongation cycle of protein synthesis and broaden our understanding of the underlying mechanism. However, we speculate it is highly possible that the binding of FMRP to the ribosome inhibits protein synthesis by interfering with the binding of tRNAs and translation elongation factors to the ribosome.

The cryo-EM structure not only corresponds well with our biochemical studies showing NT-dFMRP binds directly to the ribosome and more specifically to the large ribosomal protein L5, it also correlates well with several previous studies. Previous studies demonstrate that FMRP associates with the large 60S ribosomal subunit (5, 6) and further identifies interaction with ribosomal proteins L5 and L18 (22). However, most of these studies utilize methods such as ultracentrifugation in sucrose density gradients followed by immunoblot analysis to determine association. Interpretation of direct or indirect association of FMRP could not be elucidated. Our studies take it one step further and show definitive evidence



**Figure 3.12: Crystal structure of the 70S ribosome with EF-Tu-GTP-tRNA or EF-G docked in the inter-subunit space.** (a) Crystal structure showing EF-Tu-GTP-tRNA bound to the 70S ribosome in between the small 30S (yellow) and large 50S (dark blue) ribosomal subunits. EF-Tu is shown in red and tRNA is shown in cyan. Also indicated are ribosomal protein L5 (green) and ribosomal protein L18 (magenta). (b) Crystal structure of EF-G bound to the 70S ribosome in between the 30S (yellow) and 50S (dark blue) ribosomal subunits. EF-G is shown in red. Also indicated are ribosomal protein L5 (green) and ribosomal protein L18 (magenta).



**Figure 3.13: Model proposed for how FMRP can repress translation.** FMRP inhibits translation by docking on the ribosome and blocking the binding of translation elongation factors (eEF2 and eEF1A) to the ribosome.

of FMRP binding directly to the ribosome. Furthermore, we show that the binding of FMRP to the ribosome is independent of mRNA, suggesting that FMRP may function as a general repressor of translation. The cryo-EM structure also reveals the binding site of FMRP, which is at the interface of the large and small ribosomal subunits. This may interfere with the binding of crucial tRNA and translational factors to the ribosome and prevent protein synthesis. All things considered, our results are suggesting a new mechanism for FMRP function that could help in identifying novel drug targets to treat FXS.

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Chapter 3, in full, is currently being prepared for submission for publication of the material. Eileen Chen, Manjuli R. Sharma, Rajendra K. Agrawal, and Simpson Joseph. The dissertation author was the primary investigator and author of this material.

## **Chapter 4: Conclusions and Future Directions**

FXS is the most common cause for inherited mental retardation. There is currently no cure for this devastating disease. Recent genome-wide searches have revealed thousands of mRNA targets of FMRP (1), however, there does not seem to be a common structural motif as previously suggested. Mechanisms have been proposed to explain the underlying reason for the cause of FXS, however, controversial data has been published and lines of inquiry are still open.

From our studies, we have hypothesized a novel mechanism suggesting FMRP acts as a more general suppressor of translation by binding to the ribosome versus the approach of FMRP regulating and recognizing specific mRNA targets with particular structural motifs. This is supported by *in vitro* translation assays where NT-dFMRP represses the translation of a *Renilla* luciferase reporter mRNA that does not have G-quadruplex or a pseudoknot forming sequence, which are the previously proposed FMRP-binding elements (2, 3). In addition, we have shown that NT-dFMRP binds with high affinity ( $K_D = 20 \pm 3$  nM) to the *Drosophila* 80S ribosome near the 60S large ribosomal subunit protein L5 in the absence of mRNA. Mutational analysis also suggested that the KH1 and RGG domains play a more important role in FMRP's function of repressing translation. Finally, a cryo-EM structure indicated definitively that NT-dFMRP is indeed binding specifically to the intersubunit space of the ribosome near the L5 protein. This region of binding overlaps with potential docking areas of

translation elongation factors such as translation elongation factor 2 (eEF2) and translation elongation factor 1A (eEF1A). In conclusion, we hypothesized an innovative model that FMRP inhibits translation by docking on the ribosome and blocking the binding of translation elongation factors (eEF2 and eEF1A) to the ribosome.

One significant future direction that can be pursued to further support our proposed model is to determine whether the binding of FMRP to the ribosome blocks the elongation cycle of protein synthesis, specifically by interfering with the docking of eEF2 and eEF1A. This can be tested by the FRET-based assay we developed. FRET-based binding studies can be performed with fixed concentrations of Cy3-labeled 80S *Drosophila* ribosome and Cy5-labeled NT-dFMRP (at a concentration that is at least 10-fold higher than the  $K_D$ ). To this increasing concentrations of eEF2-GDPPNP will be titrated and the change in FRET efficiency can be determined. This data will be analyzed to determine the  $IC_{50}$  values and the relative affinity of eEF2 for the ribosome. This will reveal whether the binding of FMRP to the ribosome inhibits the binding of eEF2.

Similarly, we can also test whether the binding of FMRP to the ribosome inhibits the docking of eEF1A with the FRET-based assay. Titrating increasing concentrations of eEF1A-GDPNP-aminoacyl-tRNA ternary complex may block the binding of Cy5-labeled NT-dFMRP to the Cy3-labeled 80S ribosome. Determination of the  $IC_{50}$  value and the relative

affinity of the ternary complex for the ribosome can then be done. Furthermore, filter binding experiments can be carried out with radioactively labeled aminoacyl-tRNA to test whether FMRP blocks the binding of eEF1A-GDPNP-aminoacyl-tRNA ternary complex to the ribosome. These studies will show whether FMRP interferes with binding of the elongation factors on the ribosome.

Another interesting future direction is to identify the structural domains in FMRP that are crucial for translational control. Our preliminary results showed that the KH1 and RGG domains are important for translational regulation, as KH1 and RGG mutants exhibit defects in repressing translation and binding to the ribosome compared to the wild-type. However, more extensive and thorough efforts to pinpoint which domain, even which amino acids are crucial for translational control would provide further insight into FMRP's function. A recent study showed that mutating the conserved GXXG sequence found in KH domains to GDDG would block the binding of the KH domains to its RNA substrate (4). Mutations of the GXXG sequence in the KH1 and KH2 domains and mutations of highly conserved amino acids in the KH domains in dFMRP can be used to test the ability of these mutants to repress translation of the luciferase reporter used in our *in vitro* translation assay. The binding of these mutants to the ribosome can also be analyzed using the FRET-based assay. These studies will elucidate the role of the KH domains of FMRP in translational repression.

Furthermore, in a previous study, it was shown that a highly conserved region of dFMRP (5) (residues 359 to 472) is required for binding to the 60S large ribosomal subunit. However, structure/function analysis of this region has not been conducted. To further strengthen our hypothesis of FMRP binding to the ribosome being essential for translational control, we can target this area for mutational analysis and test whether translational repression can still occur using the *in vitro* translation assay. In addition, the ability of these mutants to bind to the ribosome can be tested with the FRET assay. This will reveal if there is a correlation between translational repression and the ability of FMRP to bind to the ribosome.

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## **Chapter 5: Materials and Methods**

## 5.1 Expression and purification of full-length dFMRP

The *Drosophila FMR1* gene (GenBank accession no. AF305881) was obtained from Prof. Gideon Dreyfuss (University of Pennsylvania). Full-length dFMRP was subcloned into PTYB1 (New England Biolabs) to produce a fusion protein with a C-terminal intein and chitin binding domain. To separate the two co-purified versions of dFMRP, a polyhistidine-tag was added to the N-terminus of full-length dFMRP. For production of the His-dFMRP-intein fusion protein, the plasmid PTYB1 was introduced into Rosetta (DE3) bacteria. Separation was achieved by purifying first through a chitin column (IMPACT-CN system, New England Biolabs) followed by a Nickel-NTA agarose column (Qiagen).

4 liters of Luria Broth (LB) with ampicillin and chloramphenicol supplemented was inoculated with 40 ml of cells from saturated overnight cultures. Cultures were incubated at 37 °C shaking until OD<sub>600</sub> reached 0.5-0.8. Protein expression was induced with 0.8 mM IPTG (isopropyl  $\beta$ -D-thiogalactoside) for 16 hours at 15 °C. Cells were harvested by centrifuging at 5,000 g for 10 min. at 4 °C, then resuspended in 75 ml cold cell lysis buffer A (20 mM Tris-HCl (pH 8), 500 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 10% glycerol, supplemented with Complete Mini EDTA-free protease inhibitor cocktail tablets (Roche)), and lysed by ultrasound sonication. Cell lysate was clarified by centrifugation at 20,000 g for 30 min. at 4 °C twice and

loaded onto a 10 ml chitin column. The column was then washed extensively with 120 ml (12 column volumes) of column buffer A (20 mM Tris-HCl (pH 8), 500 mM NaCl, 1mM EDTA, 10% glycerol). 30 ml (3 column volumes) of cleavage buffer A (20 mM Tris-HCl (pH 8), 500 mM NaCl, 1mM EDTA, 10% glycerol, 60 mM DTT) was then passed through the column to initiate the self-cleavage of the protein from the column. The chitin affinity matrix was then incubated in cleavage buffer A at 4 °C for 48 hours with rotation. The cleaved protein was eluted from the column using 20 ml of column buffer A. Buffer was exchanged (to remove DTT) into 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 500 mM NaCl, 10% glycerol, 10 mM imidazole, 0.1% Triton X-100 (pH 8) by overnight dialysis. Amicon Ultra-15 centrifugal filter devices (Millipore) with 10 kD cutoff were used to concentrate eluted proteins. Protein was then loaded onto Nickel-NTA affinity matrix columns, and the flow through contained only the truncated version of dFMRP. The column was washed extensively with wash buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 500 mM NaCl, 10% glycerol, 20 mM imidazole, 0.1% Triton X-100 (pH 8)). Full-length dFMRP was then eluted from the column using elution buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 500 mM NaCl, 10% glycerol, 500 mM imidazole, 0.1% Triton X-100 (pH 8)). Protein was concentrated, dialyzed overnight into storage buffer A (20 mM Tris-HCl (pH 8), 250 mM NaCl, 1 mM DTT, 10% glycerol) and flash frozen to be stored at -80 °C. Protein purity was assessed by Coomassie-Blue stained SDS-PAGE and concentrations were estimated by Bradford assay.

## 5.2 Expression and purification of NT-dFMRP and mutant proteins

N-terminal truncated dFMRP (NT-dFMRP) spanning residues 220 to 681 was subcloned into pTYB1 (New England Biolabs) to produce a fusion protein with a C-terminal intein and chitin binding domain. I244N (KH1 mutant) and I307N (KH2 mutant) NT-dFMRP constructs were then made by QuikChange Site-Directed Mutagenesis (Stratagene).  $\Delta$ RGG construct spanning residues 220 to 413 was also subcloned into pTYB1.  $\lambda$ N-peptide was subcloned into the N-terminus of NT-dFMRP to produce  $\lambda$ N-NT-dFMRP. All proteins were purified following the IMPACT-CN system manufacturer manual (New England Biolabs). Preparations of cell cultures were done as described above. Cells were harvested at 5,000 g for 10 min. at 4 °C, then resuspended in 75 ml cold cell lysis buffer B (24 mM HEPES (pH 7.5), 500 mM NaCl, 1mM EDTA, 0.1% Triton X-100, 10% glycerol, supplemented with Complete Mini EDTA-free protease inhibitor cocktail tablets (Roche)), and lysed by ultrasound sonication. Crude cell lysate was clarified by centrifugation at 20,000 g for 30 min. at 4 °C twice and applied onto a 10 ml chitin column. The column was then washed extensively with 120 ml (12 column volumes) of column buffer B (24 mM HEPES (pH 7.5), 500 mM NaCl, 1mM EDTA, 10% glycerol). 30 ml (3 column volumes) of cleavage buffer B (24 mM HEPES (pH 7.5), 500 mM NaCl, 1mM EDTA, 10% glycerol, 60 mM DTT) was then passed through the column to initiate the self-cleavage of the

protein from the column. The chitin affinity matrix was then incubated in cleavage buffer B at 4 °C for 48 hours with rotation. The cleaved protein was eluted from the column using 20 ml of column buffer B. Overnight dialysis and Amicon Ultra-15 centrifugal filter devices (Millipore) with 10 kD cutoff were used to exchange the buffer and concentrate the proteins. Proteins were flash frozen and stored in storage buffer B (24 mM HEPES (pH 7.5), 250 mM NaCl, 10% glycerol, 1 mM DTT) at -80 °C. Protein purity was assessed by Coomassie-Blue stained SDS-PAGE and concentrations were estimated by Bradford assay.

### **5.3 Circular dichroism spectroscopy**

CD spectra were recorded at 0.2 mg/ml protein concentration (10 mM sodium phosphate pH 7.5, 90 mM NaCl, 10% glycerol, 0.1 mM DTT) at 25 °C in a 0.1-cm path-length cuvette using AVIV circular dichroism spectrophotometer (AVIV Instruments Inc.) in the absence or presence of 6 M guanidine hydrochloride. The average time at each wavelength was 5 seconds and the spectra were recorded in the range of 190 – 260 nm.

### **5.4 Preparation of *Drosophila* embryo extracts**

Dechlorinated frozen *Drosophila* embryos were resuspended in DEI buffer (10 mM HEPES (pH 7.4), 5 mM DTT, supplemented with Complete Mini EDTA-free protease inhibitor cocktail tablets (Roche)) with a ratio of 1 g embryos per 2 ml DEI buffer. Embryos were homogenized on ice with the Dounce homogenizer, 5 strokes of pestle A (loose-fitting) followed by 5 strokes of pestle B (tight-fitting), and clarified by centrifugation at 13,000 r.p.m. for 20 min. at 4 °C. The clear aqueous interphase (embryo extract) was collected and glycerol was added to a final concentration of 10%. Embryo extracts were then aliquoted, flash frozen in liquid nitrogen, and stored at -80 °C.

### **5.5 *In vitro* translation assay**

*Renilla* luciferase reporter mRNA constructs in the pRL-null vector (Promega) were capped and/or polyadenylated using the T7 mScript™ Standard mRNA Production System (CellScript). *In vitro* transcription reactions produced 40-50 µg of mRNA from ~1 µg of linearized DNA template. DNase I treatment was then done to remove the DNA template from the reaction. Purification of the mRNA product was subsequently done using phenol/chloroform extraction, spin column chromatography, and ethanol precipitation. This step removes residual proteins and unincorporated NTPs from the reaction. Capping and/or polyadenylation of

the mRNA were next done if needed. Heat denaturation of the purified mRNA was done by incubating at 65 °C for 5-10 min., and then the reaction was transferred to ice. In parallel, a capping cocktail was pre-mixed in a separate tube: 10X ScriptCap Capping Buffer, 20 mM GTP, 20 mM SAM, ScriptGuard RNase inhibitor, ScriptCap 2'-O-Methyltransferase (100 U/μl). Just prior to starting the capping reaction, the ScriptCap Capping Enzyme was added to the cocktail and then combined with the uncapped mRNA. Incubation of the capping reaction at 37 °C for 30 min. resulted in capped mRNA. Subsequent addition of 10X A-Plus Tailing buffer, 20 mM ATP, and A-Plus-Poly (A) polymerase (4U/μl) followed by incubation at 37 °C for 30 min. produced ~150 b long poly (A) tails. Purification of the capped and/or polyadenylated mRNA was done as previously described with organic extraction, spin column chromatography, and ethanol precipitation. mRNA was then aliquoted and stored at -80 °C.

SC1, ΔKC2 and boxb (3 or 5 repeats) sequences were subcloned into the 3'-UTR of the luciferase mRNA. Reaper IRES (168 nucleotides) was synthesized by DNAWorks and inserted into the 5'-UTR of luciferase mRNA. *In vitro* translation assays were conducted as described previously (1). *In vitro* translation reactions were typically performed in a final volume of 50 μl containing 60 μM amino acids, 24 mM HEPES (pH 7.5), 0.6 mM Mg(OAc)<sub>2</sub>, 0.1 mM spermidine, 80 mM KOAc, 17 mM creatine phosphate, 80 ng/μl creatine kinase, 38% embryo extract, tested protein, and 15 ng *Renilla*

luciferase mRNA construct. Coelenterazine (3  $\mu$ M final conc.) was added to the reactions to monitor the time course of luciferase synthesis at 25 °C using a 96-well plate reader (Genios, Tecan). Data was normalized with respect to the highest signal for each mRNA in the absence of NT-dFMRP.

## **5.6 Ribosome purification**

Dechlorinated frozen fly embryos were resuspended in buffer 1 (50 mM Tris-HCl (pH 7.5), 10 mM  $MgCl_2$ , 100 mM KCl, 0.5 mM EDTA, 0.5 mM PMSF, 0.1 mM benzamidine, 5 mM 2-mercaptoethanol) with addition of 250 mM sucrose. Resuspended embryos were lysed with the Dounce homogenizer, 5 strokes of pestle A (loose-fitting) followed by 5 strokes of pestle B (tight-fitting), and clarified by centrifugation at 15,000 r.p.m. for 30 min. at 4 °C. Supernatant was then applied to a 30% sucrose cushion in buffer 1 and spun at 39,000 r.p.m. for 17 hours at 4 °C. Crude ribosome pellets were resuspended in buffer 2 (50 mM Tris-HCl (pH 7.5), 10 mM  $MgCl_2$ , 500 mM KCl, 0.5 mM PMSF, 0.1 mM benzamidine, 5 mM 2-mercaptoethanol) and spun at 13,000 r.p.m. for 20 min. at 4 °C. Puromycin was added to the supernatant with a ratio of 1 mg puromycin per 100 mg of ribosomes, incubated 30 min. on ice and 15 min. at 37 °C. Solution was clarified by centrifugation for 20 min. at 13,000 r.p.m.. Supernatant was then loaded on 10%–40% sucrose density gradients made in buffer 3 (50 mM

Tris-HCl (pH 7.5), 10 mM  $\text{MgCl}_2$ , 25 mM KCl, 5 mM 2-mercaptoethanol) in a SW-28 rotor and spun at 20,000 r.p.m. for 17 hours and 30 min. Gradients were fractionated using a gradient fractionator and UA-6 detector (ISCO/BRANDERL). 80S ribosome peaks were pooled and diluted 2-fold with buffer 3 (this helps to dilute the sucrose percentage and improves the recovery of ribosomes) and spun at 35,000 r.p.m. for 17 hours. Purified ribosome pellets were resuspended in 10 mM HEPES (pH 7), 10 mM  $\text{MgCl}_2$ , 50 mM KCl, 5 mM 2-mercaptoethanol and stored at  $-80^\circ\text{C}$ . Ribosome concentration was calculated using 1 A260 unit = 20 pm/ml 80S ribosome.

### **5.7 Ribosome binding assay**

NT-dFMRP (2.4  $\mu\text{M}$ ) or BSA (4.8  $\mu\text{M}$ ) were incubated with purified 80S ribosome (0.4  $\mu\text{M}$ ) in binding buffer (50 mM KOAc, 50 mM TrisOAc (pH 7.7), 10 mM DTT, 5 mM  $\text{Mg}(\text{OAc})_2$ , 30  $\mu\text{g}/\text{mL}$  tRNA) for 10 min. at room temperature. Yeast total tRNA was included in the binding reactions to reduce potential nonspecific binding. The reactions were applied to Illustra MicroSpin Sephacryl-300 columns (GE Healthcare) or Amicon filters with 10 kD cutoff (Millipore) and the eluate or retentate, respectively, was analyzed on 10% SDS-PAGE and stained with Commassie brilliant blue. Identical ribosome binding reactions were also done with the mutant proteins.

## 5.8 Sucrose density gradient analysis

*In vitro* translation reactions as described in 5.4 were assembled and incubated at 25 °C for 35 min., then cooled on ice for 2 min. and clarified by centrifugation at 10,000 g for 15 min. at 4 °C. The supernatant was then loaded on 10-40% sucrose density gradients made in buffer 3 (50 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 25 mM KCl, 5 mM 2-mercaptoethanol) in a SW-41 rotor and spun at 39,000 r.p.m. for 2 hours and 45 min. at 4 °C. Gradients were fractionated using a gradient fractionator and UA-6 detector (ISCO/BRANDER). 0.5 ml fractions were collected and incubation overnight at 4 °C with addition of a final concentration of ~14 % trichloroacetic acid (TCA) was done to precipitate proteins. Reactions were then subsequently centrifuged at 13,000 r.p.m. for 30 min. at 4 °C. 200ul of ice-cold acetone was added twice to wash the protein pellets. Acetone was removed and the pellets were then resuspended in SDS loading buffer.

## 5.9 Western blot

SDS-PAGE gels (10% or 12%) were run for 45 min. and next placed in 1X SDS-running buffer with 10% methanol. In parallel, the nitrocellulose membrane and blotting pads were pre-soaked in the same buffer. To transfer to the nitrocellulose membrane, stacking was done in the order (from

bottom to top): blotting pad, nitrocellulose membrane, SDS-PAGE gel, blotting pad. This was run at 25 V for 1 hour. The membrane was then blocked with addition of dilution/blocking buffer (25 mM Tris-HCl (pH 7.5), 0.14 M NaCl, 5% BSA, 0.01% Tween 20) at room temperature (RT) for 1 hour on a shaker. After discarding the dilution/blocking buffer, incubation with the primary antibody (anti-dFMRP 5B6, 1:250 dilution in dilution/blocking buffer) was done overnight at 4 °C (or 2 hr RT) on a shaker. The membrane was then washed with wash buffer (25 mM Tris-HCl (pH 7.5), 0.14 M NaCl, 0.01% Tween 20) 3-4 times (each time 10 min) at RT. Incubation with the secondary antibody (anti-mouse 1:10,000 in dilution/blocking buffer) was done for 1 hour at RT on a shaker. The membrane was washed 3-4 times with wash buffer at RT. Proteins were then detected using the Western lighting plus-ECL (enhanced chemiluminescence substrate) kit. Film was exposed and developed.

### **5.10 Fluorescent labeling**

NT-dFMRP, KH1, KH2, and  $\Delta$  RGG mutants, which each have one natural cysteine at amino acid 263, were treated with tris(2-carboxyethyl)phosphine (TCEP) for 15 min. at RT to reduce free oxidized cysteines. TCEP-treated NT-dFMRP and mutants were labeled for 2 hours at RT followed by 16 hours at 4 °C in the dark with excess of Cy5 mono

maleimide (GE Healthcare). Unreacted fluorophores were removed by dialysis and gel filtration columns (Bio-Spin 6, Bio-Rad) and buffer was exchanged to 24 mM HEPES (pH 7.5), 250 mM NaCl, 15% glycerol, 2 mM DTT.

The 80S ribosome was diluted to a concentration of 2 mg/ml in 0.1 M sodium carbonate pH 9.1, 10 mM  $MgCl_2$ , 50 mM KCl, 5 mM 2-mercaptoethanol and then labeled with Cy3 mono-reactive dye (GE Healthcare) for 45 min. at RT. Unreacted fluorophores were removed with gel filtration columns (Bio-spin 6, Bio-Rad) and buffer was exchanged to 10 mM HEPES (pH 7), 10 mM  $MgCl_2$ , 50 mM KCl, 5 mM 2-mercaptoethanol.

### 5.11 FRET assay and $K_D$ determination

Cy3-ribosome (2 nM) was titrated with Cy5-NT-dFMRP (0-125 nM) or Cy5-NT-dFMRP mutants (0- 400 nM) in Binding Buffer with excitation at 530 nm. Emission maxima for the donor Cy3-ribosome and the acceptor Cy5-NT-dFMRP or Cy5-NT-dFMRP mutants were displayed at 575 nm and 665 nm, respectively. Under the same conditions, buffer was also titrated with Cy5-NT-dFMRP or Cy5-NT-dFMRP mutants, and each buffer emission spectrum was subtracted from the corresponding fluorescence emission spectrum. FRET efficiency (%) was then calculated by the equation  $I_D/(I_D+I_A)*100$ .  $I_D$  stands for the intensity of donor at 575 nm and  $I_A$  stands for

the intensity of acceptor at 665 nm (2). FRET efficiency was then fitted to a quadratic equation using Graphpad prism to obtain the  $K_D$  values.

## **5.12 Crosslinking and immunoprecipitation**

NT-dFMRP-ribosome complexes (1  $\mu$ M) were formed in 1X PBS (100 mM sodium phosphate, 150 mM NaCl, pH 7.2) using the ribosome binding assay as described above. 30  $\mu$ M of freshly prepared Sulfo-SMCC (sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate) was added to the complexes and incubated for 40 min. at 4 °C, then analyzed on 10% SDS-PAGE. For immunoprecipitation, monoclonal anti-dFMRP 5B6 (Developmental Studies Hybridoma Bank) was added to the crosslinked reactions and incubated for 1 hour at 4 °C. 25  $\mu$ l of Protein G Magnetic Beads (New England Biolabs) were then added and reactions were incubated with agitation for 1 hour at 4 °C. Beads were washed extensively and resuspended in 5X SDS Sample Loading Buffer and analyzed on a 10% SDS-PAGE stained with Coomassie brilliant blue. Crosslinked protein bands were then cut out, digested with trypsin, and analyzed by liquid chromatography (LC) in combination with tandem mass spectroscopy (MS/MS) using electrospray ionization. Peptide identifications were made using paragon algorithm executed in Protein Pilot 2.0 (Life Technologies).

### 5.13 Mass spectrometry in gel digest

The gel slices were cut to 1mm by 1 mm cubes and destained 3 times by first washing with 100  $\mu$ l of 100 mM ammonium bicarbonate for 15 minutes, followed by addition of the same volume of acetonitrile (ACN) for 15 minutes. The supernatant was removed and samples were dried in a speedvac. Samples were then reduced by mixing with 200  $\mu$ l of 100 mM ammonium bicarbonate-10 mM DTT and incubated at 56 °C for 30 minutes. The liquid was removed and 200  $\mu$ l of 100 mM ammonium bicarbonate-55mM *iodoacetamide* was added to gel pieces and incubated at RT in the dark for 20 minutes. After the removal of the supernatant and one wash with 100 mM ammonium bicarbonate for 15 minutes, the same volume of ACN was added to dehydrate the gel pieces. The solution was then removed and samples were dried in a speedvac. For digestion, enough solution of ice-cold trypsin (0.01  $\mu$ g/ $\mu$ l) in 50 mM ammonium bicarbonate was added to cover the gel pieces and set on ice for 30 min. After complete rehydration, the excess trypsin solution was removed, replaced with fresh 50 mM ammonium bicarbonate, and left overnight at 37°C. The peptides were extracted twice by the addition of 50  $\mu$ l of 0.2% formic acid and 5 % ACN and vortex mixing at RT for 30 min. The supernatant was removed and saved. A total of 50  $\mu$ l of 50% ACN-0.2% formic acid was added to the sample, which was vortexed again at RT for 30 min. The supernatant was removed and combined with

the supernatant from the first extraction. The combined extractions were analyzed directly by liquid chromatography (LC) in combination with tandem mass spectroscopy (MS/MS) using electrospray ionization.

#### **5.14 LC-MS/MS analysis**

Trypsin-digested peptides extracted from SDS-PAGE as described above in 5.13 were analyzed by liquid chromatography (LC)-MS/MS with electrospray ionization. All nanospray ionization experiments were performed by using a QSTAR-Elite hybrid mass spectrometer (AB/MDS Sciex) interfaced to a nanoscale reversed-phase high-pressure liquid chromatograph (Tempo) using a 10 cm-180 ID glass capillary packed with 5- $\mu$ m C18 Zorbax<sup>TM</sup> beads (Agilent). The buffer compositions were as follows. Buffer A was composed of 98% H<sub>2</sub>O, 2% ACN, 0.2% formic acid, and 0.005% TFA; buffer B was composed of 100% ACN, 0.2% formic acid, and 0.005% TFA. Peptides were eluted from the C-18 column into the mass spectrometer using a linear gradient of 5–60% Buffer B over 60 min at 400  $\mu$ l/min. LC-MS/MS data were acquired in a data-dependent fashion by selecting the 4 most intense peaks with charge state of 2 to 4 that exceeds 20 counts, with exclusion of former target ions set to "360 seconds" and the mass tolerance for exclusion set to 100 ppm. Time-of-flight MS were acquired at  $m/z$  400 to 1600 Da for 1 s with 12 time bins to sum. MS/MS

data were acquired from  $m/z$  50 to 2,000 Da by using "enhance all" and 24 time bins to sum, dynamic background subtract, automatic collision energy, and automatic MS/MS accumulation with the fragment intensity multiplier set to 6 and maximum accumulation set to 2 s before returning to the survey scan. Peptide identifications were made using paragon algorithm executed in Protein Pilot 2.0 (Life Technologies) (3).

### **5.15 Cryo-electron microscopy, image processing, and three-dimensional reconstruction**

The 80S ribosome-NT-dFMRP complex was prepared under the ribosome binding assay conditions described in 5.6, except that the tRNA was excluded from the reaction mixture. The complex was diluted to 35 nM in the same binding buffer, but containing 25-fold molar excess of NT-dFMRP. Cryo-EM grids were prepared according to standard procedures, using Vitrobot. Data were collected on a Philips FEI Tecnai F20 field emission gun electron microscope with a magnification of 50,760. 102 micrographs for the control 80S and 98 micrographs for the 80S ribosome-NT-dFMRP complex were scanned on a Zeiss flatbed scanner with a step size of 14  $\mu\text{m}$ , corresponding to 2.78 Å on the object scale and were sorted into 28 and 20 defocus groups for the 80S and the 80S•NT-dFMRP complex, respectively. A total of 78,267 images for the control and 82,481 images for

the 80S•NT-dFMRP complex were manually selected. SPIDER was used for all image processing, including 2D image classification, reconstruction, and refinement. A previously determined cryo-EM structure of the yeast 80S ribosome was used as the reference to align images for an initial reconstruction from fewer images, using projection-matching procedure. The 3D volume so obtained was low pass filtered and used subsequently for alignment of a larger data set. The original reconstruction and subsequent refinement yielded a weak mass of density corresponding to NT-dFMRP. To enhance the density of the factor in the 80S ribosome complex, the method of supervised classification was applied (4). 59,313 particle images were used in the supervised classification. 19,520 particle images classified with the reference projection images generated from the original map of the NT-dFMRP-bound 80S ribosome. The 3D map determined from these images showed a significantly enhanced density for NT-dFMRP. The final resolution of the control 80S and 80S• NT-dFMRP complex maps were 11.2 Å (at 0.5 cutoff of FSC; or 9.8 Å, using 0.143 cutoff of FSC (5)), and 12.8 Å (at 0.5 cutoff of FSC; or 10.4 Å, using 0.143 cutoff of FSC), respectively.

Atomic structures of small fragments of FMRP and its homologs are known from NMR and X-ray crystallographic (6-8) studies. The homology model for the full NT-dFMRP construct was obtained using the I-TASSER server (9), which predicted an elongated structure with four distinct structural domains that were connected to each other through long linkers. Docking of

the model, using each domain as an independent rigid body, into the corresponding cryo-EM map density tentatively places the KH1 and KH2 domains of NT-dFMRP interacting with the CP and ASF, respectively, of the 60S subunit. The overall features and placements of all four domains match and explain most of the cryo-EM density corresponding to NT-dFMRP. However, the cryo-EM density corresponding to structural domains 3 and 4 are relatively weak, suggesting that the RGG motif containing domains and the C-terminus of FMRP is relatively flexible on the ribosome (Figure 3.11). All modeling, fitting and visualization were done using Chimera software (10).

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