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Kh Domains on Brain Polyribosomes: FMRP and Nova in Translational Regulation

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**KH DOMAINS ON BRAIN POLYRIBOSOMES:
FMRP AND NOVA IN TRANSLATIONAL REGULATION**

A Thesis Presented to the Faculty of
The Rockefeller University
in Partial Fulfillment of the Requirements for
the degree of Doctor of Philosophy

by
Claire Elizabeth Fraser

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KH DOMAINS ON BRAIN POLYRIBOSOMES: FMRP AND NOVA IN TRANSLATIONAL REGULATION

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The Rockefeller University, 2007

The regulation of protein synthesis is an important aspect of the control of gene expression in neurons and is thought to contribute to neurologic diseases such as Fragile X mental retardation syndrome. We demonstrate that several neuronal RNA-binding proteins implicated in human disease are associated with brain polyribosomes, namely the Nova and Hu paraneoplastic antigens and the Fragile X mental retardation protein. We use microarray analysis of polyribosomal mRNAs in knockout mouse models of these diseases to identify target mRNAs and analyze the translational profiles of mice lacking Nova-1 or FMRP. The KH2 and RGG box RNA-binding domains of FMRP bind specific RNA motifs that form kissing complex and G-quartet structures, respectively. We find that the association of FMRP with polyribosomes in both mouse brain and human neuroblastoma cells is abrogated by competition with kissing complex RNA, but not by high-affinity G-quartet RNA. In addition, the polyribosome associations of FMRP-interacting proteins FXR1 and FXR2, are specifically abrogated by competition with this kissing complex RNA. FXR1 and FXR2 also bind kissing complex RNA via KH2, and they are competed off polyribosomes by kissing complex RNA even in the absence of FMRP. Kissing complex RNA does not disrupt heterodimerization between FMRP and FXR1 or FXR2. We conclude that the mental retardation associated with the I304N mutation, and likely the Fragile-X syndrome more generally, may relate to a crucial role for RNAs harbouring the kissing complex motif as targets for FMRP translational regulation.

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CHAPTER I: INTRODUCTION

Translational control in neurons

Translation of messenger RNAs into functional proteins is the final step in the gene expression pathway. As such, it represents a vital mechanism for control of protein levels in the cell. Regulation of mRNA translation is relatively fast, lacking requirements for RNA transcription, processing, and nuclear export. Instead, a previously established pool of mRNAs can be held in a translationally inactive state, ready to produce encoded proteins when necessary. Furthermore, this mechanism may be restricted locally; translation of a particular pool of mRNAs in a specific subcellular area undergoes regulation unique to that subcellular location, such as an individual dendritic spine. Tight control of translation is particularly necessary for neurons, since neuronal signaling mechanisms rely on a delicate balance of synaptic components for effective neurotransmission and memory formation. Translational control can be achieved by both global and mRNA-specific mechanisms, and by interventions during translation initiation and/or elongation steps. As such, translational regulation represents a real-time strategy for cellular control of gene expression. (Gebauer and Hentze, 2004; Kindler et al., 2005; Moore, 2005; Wang and Tiedge, 2004)

Global control of translation mainly involves modification of initiation factors. A well-characterized example is that of cap-binding protein eIF4E availability. Interaction of eIF4E with the scaffold protein eIF4G is required for cap-mediated recruitment of the 43S ribosomal complex to an mRNA during initiation. eIF4E-binding proteins (4E-BPs) compete with eIF4G for interaction with eIF4E, preventing recruitment of the 43S complex and resulting in translational inhibition. However, activation of the insulin,

PI3K/Akt/mTOR and MAPK signaling pathways can phosphorylate 4E-BPs to enable interaction of eIF4E with eIF4G and initiation of translation (Gingras et al., 1999; Kelleher et al., 2004a; Kelleher et al., 2004b; Pause et al., 1994; Raught et al., 2000; Ruggero and Sonenberg, 2005; Treisman, 1996). A second example of global translational repression occurs during cell death. The apoptotic protein caspase-3 cleaves initiation factor eIF4G and poly(A)-binding protein (PABP) resulting in inhibition of initiation. (Bushell et al., 2000; Marissen et al., 2004) A location-specific, global mechanism of translational repression can be found in the small, untranslated BC1 and BC200 RNAs, which are specifically targeted to dendrites in neurons. BC1 inhibits translation of a reporter in *Xenopus* oocytes by interacting with both translation initiation factor eIF4A and PABP (Wang et al., 2005).

Blockage of translational initiation factor recruitment is also a goal of some mRNA-specific regulatory mechanisms. Sequence and structural elements in an mRNA such as the presence of a hairpin, internal ribosome entry site (IRES), or binding site for an RNA-binding protein can influence translational state. The iron regulatory proteins IRP1 and IRP2 bind an iron-responsive element (IRE) in the 5'UTRs of the ferritin heavy- and light-chain mRNAs. Protein binding to this stem-loop sequence hinders 43S complex recruitment by the cap-binding complex, thereby preventing translation initiation. (Gray and Hentze, 1994; Muckenthaler et al., 1998) Other regulatory proteins can act as message-specific 4E-BPs. The cytoplasmic element-binding protein (CEBP) binds a CPE sequence in the 3'UTR of target mRNAs. It inhibits translation initiation by forming a complex with Maskin, a protein that binds to eIF4E and displaces eIF4G. On phosphorylation by Aurora kinase, CEBP promotes polyadenylation of the mRNA and

dissociation of Maskin from eIF4E. (Mendez and Richter, 2001) During development of the *Drosophila melanogaster* embryo, Bicoid inhibits the translation of *caudal* mRNA by directly binding to both eIF4E and an element in the 3'UTR (Niessing et al., 2002). In response to interferon- γ , ribosomal protein L13a is phosphorylated and released from the 60S ribosomal subunit; it then binds to a sequence in the 3'UTR of *ceruloplasmin* mRNA and subsequently inhibits the initiation of translation (Mazumder et al., 2003). The RNA-binding proteins hnRNP K (heterogenous nuclear ribonucleoprotein K) and hnRNP E1 bind to a CU-rich sequence known as the differentiation-control element (DICE) in the 3'UTR of 15-lipoxygenase (LOX) mRNA. hnRNP K and hnRNP E1 repress LOX translation by preventing 60S ribosomal subunit binding. (Ostareck et al., 2001; Ostareck et al., 1997; Ostareck-Lederer and Ostareck, 2004) Zipcode binding protein 1 (ZBP1) binds to the 3'UTR of β -actin mRNA and is required for its translocation to sites of active actin polymerization (Eom et al., 2003; Farina et al., 2003; Oleynikov and Singer, 2003; Tiruchinapalli et al., 2003). Like hnRNP K, ZBP1 inhibits translation initiation by impairing the transition from a 48S complex to an 80S ribosome. Translation of β -actin mRNA is activated by Src-dependent phosphorylation of ZBP1, which abrogates RNA-binding ability (Huttelmaier et al., 2005). As described above, most translational control mechanisms yet described act on the translational initiation pathway, and result in the inhibition of protein synthesis.

Translational control can also be achieved through regulation of elongation rather than initiation. NMDA receptor activation in rat brain synaptoneurosomes induces phosphorylation of elongation factor eEF2, a process that reduces the rate of peptide chain elongation. This promotes a general decrease in protein translation, but an increase

in translation of abundant but poorly initiated mRNAs such as that encoding α CamK II. (Scheetz et al., 1997; Scheetz et al., 2000) Translational regulation of the *Drosophila oskar* (*osk*) and *nanos* mRNAs seem to involve inhibition of both initiation and elongation. Initiation is inhibited by binding of Bruno to the 3'UTR of *osk*. Bruno recruits a 4E-BP, Cup, which then prevents binding of eIF4E to eIF4G (Nakamura et al., 2004) Cup is also involved in translational repression of *nanos* by interaction with Smaug bound to the *nanos* 3'UTR (Nelson et al., 2004). However, both *osk* and *nanos* mRNAs are associated with polyribosomes even when *osk* and *nanos* proteins do not accumulate, suggesting that there may be more than one means of repressing the translation of these mRNAs (Baat et al., 2004; Clark et al., 2000). microRNAs (miRNAs) inhibit translation by partial base-pairing to target mRNAs, usually in the 3'UTR (Hutvagner and Zamore, 2002). The mechanism of this repression is largely unknown, but may involve inhibition of elongation, as translational repression of *lin-14* mRNA by *lin-4* miRNA does not alter its association with polyribosomes (Olsen and Ambros, 1999).

Local control of translation is an important aspect of the regulation of gene expression. Although originally studied in developmental processes in *Xenopus* and *Drosophila*, mRNA targeting and localization has become a key aspect of the current theory of activity-dependent synaptic modification. (Schuman et al., 2006; Wang and Tiedge, 2004) In essence, for an mRNA to have a selective impact on a specific synapse, its transport must be correctly targeted to the translational area of that synapse, and either transport or translation of this mRNA must be regulated in a manner that can be altered by activity at that synapse. It follows that locally translated messages should encode

proteins that are in use at the synapse, modify other proteins at the synapse, or serve as messengers to send signals back to other parts of the neuron. Many studies have shown that protein synthesis is required for long-lasting forms of behavioral and synaptic plasticity (Bailey et al., 1996; Mayford et al., 1996; Nguyen and Kandel, 1996). Evidence for this hypothesis includes localization of polyribosomes and associated membranous cisterns to sub-synaptic sites in dendrites (Gardioli et al., 1999; Steward, 1983; Steward and Fass, 1983; Steward and Levy, 1982), assessments of the mRNA complement of dendritic processes (Eberwine, 1996; Eberwine et al., 2002; Eberwine et al., 2001; Miyashiro et al., 1994), protein synthesis in isolated synaptosomal preparations (Chicurel et al., 1993; Rao and Steward, 1991), translation of an mRNA construct transfected into an isolated dendrite (Crino and Eberwine, 1996), studies of polyadenylation-induced translation of the α CaMKII mRNA in synaptosome preparations after NMDA receptor activation (Huang et al., 2002), and the observation that polyribosomes redistribute to dendritic spines from dendritic shafts after induction of long-term potentiation (LTP) (Ostroff et al., 2002). Investigation of the roles for neuron-specific RNA-binding proteins in translational control may yield valuable clues for both synaptic physiology and human disease.

Neurological disease and RNA-binding proteins: autoimmune-mediated

Paraneoplastic neurologic degenerations (PNDs) are cancer-associated neurodegenerative disorders that are thought to arise when a neoplasm expresses a normally neuron-restricted protein. Autoreactive antibodies and T cells are generated against the protein as an antigen of the tumor cell. If the immune response is present in

the cerebrospinal fluid, the antigen may then be recognized in its normal location – a neuron. The development of an immune response to the paraneoplastic antigen may benefit the patient in suppressing tumor growth, but may ultimately lead to loss of function of this protein in the neurons where it normally resides. Such loss of function may result in neuronal death and/or severe neurologic impairment. (Albert et al., 2000; Albert et al., 1998; Albert and Darnell, 2004; Buckanovich et al., 1996; Darnell, 1996; Darnell and Posner, 2003; Musunuru and Darnell, 2001)

It is interesting that a number of RNA-binding proteins (RBPs) have been found to be target antigens in a variety of autoimmune diseases. The Nova family of RNA-binding proteins (RBPs) was first discovered as target antigens of the neurodegenerative disorder, paraneoplastic opsoclonus-myoclonus ataxia (POMA). POMA is most often found in association with tumors of the lung, breast and fallopian tubes. Symptoms are characterized by a loss of inhibitory control of motor neurons in the brainstem and spinal cord, leading to rapid irregular eye movements (opsoclonus), muscle spasms (myoclonus), and a failure of muscular coordination (ataxia). (Buckanovich et al., 1993) The Hu family of proteins was identified as during the investigation of another PND, paraneoplastic encephalomyelopathy/sensory neuropathy (Hu syndrome). Hu proteins contain three RNA-recognition motifs (RRMs) and are thought to be involved in RNA stability, export and translation. (Antic and Keene, 1998; Antic et al., 1999; Darnell and Posner, 2006; Levine et al., 1993) A novel RBP, termed GW182, has been identified as a target antigen using serum from a patient with ataxia and sensory polyneuropathy (Eystathiou et al., 2002). GW182 is a cytoplasmic protein that is involved in miRNA mediated mRNA degradation in cytoplasmic processing bodies (Behm-Ansmant et al.,

2006; Jakymiw et al., 2005; Liu et al., 2005). hnRNP A1, another RBP, is cross-reactive with the viral HTLV-tax protein in individuals with human T-lymphotrophic virus type 1-associated myelopathy/tropical spastic paresis (HAM/TSP) (Levin et al., 2002). These patients develop antibodies to neurons and have symptoms of progressive corticospinal tract damage (weakness, spasticity and pathological reflexes). These autoantibodies inhibit neuronal firing, and are targeted to epitopes within hnRNP A1 that include an RGG type RNA-binding domain, suggesting that this domain is important for normal function of hnRNP A1 (Lee et al., 2006). While human autoimmune disorders have provided the means to identify many of these RBPs, and clues to their physiological functions in neurons, model organisms are required for further understanding of the pathophysiology of these diseases.

Neurological disease and RNA binding proteins: loss-of-function mouse models

Several RBPs have been knocked out in the mouse to create model systems for the study of human diseases. The Nova family consists of two highly conserved genes, Nova-1 and Nova-2. Expression of Nova-1 (commonly targeted in POMA, originally termed the Ri antigen) is expressed in the ventral spinal cord, brainstem, and deep nuclei of the cerebellum, as well as in discrete supratentorial regions such as the cingulum and amygdala. Expression of Nova-2, an antigen targeted in POMA patients with dementia, is largely complementary to Nova-1, and is most prominently expressed in regions such as the cerebral and cerebellar cortices and dorsal spinal cord (Yang et al., 1998). Nova-1 null mice show action tremors and difficulty walking, symptoms similar to those of POMA patients. Apoptotic death of motor neurons is seen in these mice and ultimately

the mice die at about 1-2 weeks of age.(Jensen et al., 2000a) Nova-2 null mice have a lesser degree of ataxia and die at approximately 2-3 weeks of age. (Ule et al., 2003)

In contrast to the severe phenotypes of the Nova-null mice, while there is a transient delay in neurite extension in mutant embryos, HuD-null pups are indistinguishable from their wild type littermates for the first several postnatal weeks. Adult animals, however, develop an abnormal hind-limb clasping reflex and show poor motor ability. (Akamatsu et al., 2005) Thus, the importance of RBPs to neurologic disease is clear.

Fragile X syndrome (FXS) represents another neuropathology in which the mechanism of disease is thought to result from an alteration in translational regulation by an RNA-binding protein. FXS is the most common heritable form of mental retardation in children, with an incidence of about 1 in 4000 males, and 1 in 8000 females. The phenotype of FXS includes mild to severe cognitive deficits, attention-deficit hyperactivity disorder, autistic-like behavior, abnormal facial features such as a prominent jaw and large ears, macroorchidism in postpubescent males, and some connective tissue abnormalities. (Reviewed in Jin and Warren, 2003.) Most cases of Fragile X syndrome are caused by an expansion in the CGG repeat tract located in the 5'UTR of fragile X mental retardation 1 (*FMRI*) on the X chromosome (Fu et al., 1991; Oberle et al., 1991; Verkerk et al., 1991). The expansion from a normal length of 7-54 repeats to a length of over 230 repeats in affected individuals effectively silences the expression of FMRP, since the CGG tract is associated with increased methylation and chromatin deacetylation (Coffee et al., 1999; Hornstra et al., 1993; Oberle et al., 1986; Pieretti et al., 1991; Sutcliffe et al., 1992). However, some fragile X patients have

intragenic deletions or nonsense mutations in *FMRI* instead of a trinucleotide repeat expansion (Hirst et al., 1995; Lugenbeel et al., 1995; Meijer et al., 1994). A single patient has been identified with a missense mutation, I304N, that results in a severe fragile X phenotype (De Boule et al., 1993).

FMRP shuttles between the nucleus and cytoplasm and has been localized to polyribosomes at synapses in neurons (Devys et al., 1993; Eberhart et al., 1996; Feng et al., 1997b; Sittler et al., 1996; Tamanini et al., 1999). The presence of FMRP and its autosomal paralogs, FXR1 and FXR2, at the synapse has implications for its role in the cognitive phenotype of fragile X syndrome. In fact, abnormally long dendritic spines have been noted in autopsy sections of brains from fragile X patients, and in brains for *Fmr1* knockout mice (Comery et al., 1997; Greenough et al., 2001; Hinton et al., 1991; Irwin et al., 2000; Nimchinsky et al., 2001; Rudelli et al., 1985). *Fmr1* knockout mice have subtle defects in behavior and learning, as well as showing increased sensitivity to audiogenic epileptic seizures and increased anxiety (Musumeci et al., 2000; Oostra et al., 1994; Peier et al., 2000). Furthermore, metabotropic glutamate receptor (mGluR) activation in *Fmr1* knockout mouse synaptoneurosomes does not produce the increase in protein synthesis that is seen in synaptoneurosomes from wild type mice (Greenough et al., 2001). Finally, group I mGluR-dependent long-term depression (LTD) is increased in hippocampal slices of *Fmr1* knockout mice and is dependent on the transient degradation of FMRP in wild type mice (Hou et al., 2006; Huber et al., 2002). In *Drosophila*, a single ortholog, *dfmr1* (also called *dfxr*), exists for all three mammalian FMRP family members. *Dfmr1*-mutant flies exhibit increased synaptic growth and branching at the neuromuscular junction, impaired coordinated behavior, arrhythmic circadian activity and reduced

courtship (Dockendorff et al., 2002; Inoue et al., 2002; Morales et al., 2002; Zhang et al., 2001). Activity-dependent synaptic modification depends in part on local protein synthesis in the dendrite (Steward and Schuman, 2003). Since FMRP is associated with polyribosomes at synapses, and since the FMRP null phenotype includes cognitive impairment and synaptic abnormalities, FMRP may be involved in the regulation of local translation in neurons.

KH domain proteins in RNA processing and regulation

The Nova-1 and Nova-2 genes encode proteins that contain two tandem hnRNP K-homology (KH) RNA-binding domains followed by a spacer region of variable length and a third KH domain. Nova-1 and Nova-2 are highly homologous, differing in the length of the spacer region. The larger family of KH domain-containing RNA-binding proteins (RBPs) includes hnRNP K, which, like the Nova proteins, contains three KH domains in the same configuration. FMRP, implicated in Fragile-X syndrome, contains two tandem KH domains and a third RNA-binding domain known as an RGG box (De Boulle et al., 1993; Pieretti et al., 1991). Both hnRNP K and FMRP are thought to function in translational control: hnRNP K by binding a pyrimidine-rich element in the 3'UTR and inhibiting translation initiation (Ostareck et al., 2001), and FMRP by associating with mRNAs on actively translating polyribosomes (Corbin et al., 1997; Khandjian et al., 1996; Khandjian et al., 2004; Lagerbauer et al., 2001; Stefani et al., 2004). Additional members of this family function in various aspects of RNA metabolism and regulation, including RNA stability and translational control (hnRNP E1/E2 and hnRNP K) (Kiledjian et al., 1995; Ostareck et al., 1997), subcellular

localization and translational inhibition (ZBP-1) (Farina et al., 2003; Huttelmaier et al., 2005), and splicing (hnRNP K, KSRP and splicing factor 1) (Arning et al., 1996; Expert-Bezancon et al., 2002).

Our laboratory has shown that Nova-1 and Nova-2 influence alternative splice site choice by binding YCAY-rich sites in both intronic and exonic RNA. Nova proteins regulate pre-mRNA splicing for a network of synaptic proteins, suggesting that this regulatory mechanism is critical for synaptic development and maintenance. (Dredge and Darnell, 2003; Dredge et al., 2005; Jensen et al., 2000a; Jensen et al., 2000b; Musunuru and Darnell, 2004; Ule et al., 2003; Ule et al., 2005b) Nova-1 and Nova-2, like FMRP and hnRNP K, are found both in the nucleus and the cytoplasm (Eberhart et al., 1996; Michael et al., 1997). Although the Nova proteins are predominantly nuclear, they are present in significant quantities in the cytoplasm, and Nova-1 has been co-localized with GlyR α 2 mRNA in post-synaptic regions of the dendrite (Racca et al., submitted). Since Nova proteins bind both coding and non-coding RNA sequences, it is reasonable to suggest that the Nova proteins may be multifunctional, perhaps regulating downstream events for messages that have undergone Nova-influenced alternative splicing. For example, after influencing an alternative splicing decision in the nucleus, Nova may regulate the subcellular localization and/or the association with polyribosomes of a target mRNA. Alternately, the cytoplasmic role(s) of the Nova proteins may affect an entirely separate set of mRNAs than those affected by Nova's splicing function. Nova may bind to the 3' UTR of a message in an analogous fashion to hnRNP K to regulate translation of a message during transport and until such a time when translation of that message is activated. Conversely, Nova may promote association with polyribosomes and activation

of translation at the synapse upon receiving a hypothetical “activity-dependent” signal. A third possibility is that Nova may be associated with an complex that inhibits translation in mRNA trafficking, but also remains bound to the 3'UTR of mRNAs associated with actively translating polyribosomes, perhaps serving as a scaffolding protein in this translational complex.

While there is little evidence that Nova proteins are involved in translational control, a related protein, FMRP, is thought to have a primary role in post-synaptic regulation of translation. *FMRI* is a 38kb long gene composed of 17 exons, which are subject to alternative splicing and produce 20 predicted isoforms (4-5 protein bands are detected in various tissues) (Devys et al., 1993; Eichler et al., 1993; Khandjian et al., 1995; Sittler et al., 1996; Verheij et al., 1993; Verheij et al., 1995). FMRP has three conserved RNA-binding domains, two KH domains and one RGG box, and has been shown to exhibit a high affinity for polyG and polyU homoribopolymers *in vitro* (Adinolfi et al., 2003; Ashley et al., 1993; Denman and Sung, 2002; Dolzhanskaya et al., 2003; Mazroui et al., 2003; Siomi et al., 1993). In addition to the RNA-binding domains, FMRP also contains an N-terminal protein-protein interaction domain (NDF), a second protein-protein interaction domain (PPId), a phosphorylation domain (Phd), a nuclear localization signal (NLS) and a nuclear export signal (NES) (Adinolfi et al., 2003; Ceman et al., 2003; Eberhart et al., 1996; Fridell et al., 1996; Siomi et al., 2002; Sittler et al., 1996). FMRP has two autosomal paralogs, FXR1P and FXR2P, which share >60% amino acid identity (Siomi et al., 1995; Zhang et al., 1995). FMRP is normally expressed in most tissues at variable levels, although the highest expression is observed in brain and

testis (Abitbol et al., 1993; Devys et al., 1993; Hergersberg et al., 1995; Hinds et al., 1993).

Several laboratories have made efforts to identify the mRNA targets of FMRP. FMRP has been shown to negatively regulate the translation of several mRNAs, both in an *in vitro* rabbit reticulocyte lysate system and *in vivo* in cell culture (Laggerbauer et al., 2001; Li et al., 2001b; Mazroui et al., 2002; Schaeffer et al., 2001). Target RNA structural motifs have been identified for the RGG box domain (G-quartet RNA) and the second KH domain (kissing complex RNA) (Darnell et al., 2005a; Darnell et al., 2001; Darnell et al., 2005b). FMRP has also been shown to bind its own mRNA (Ashley et al., 1993; Brown et al., 1998; Ceman et al., 1999; Schaeffer et al., 2001). *Fmr1* mRNA contains a G quartet FMRP binding site in the coding region which, when placed 50nt downstream of the 5' cap structure of a luciferase reporter, confers translational repression in the presence of FMRP. Of note, all attempts to reproduce a 3'UTR G quartet site have failed to confer translational repression (Schaeffer et al., 2001; JC Darnell, unpublished data). Other target mRNAs for which there is some evidence of translational regulation by FMRP include elongation factor 1A (EF-1A) (Sung et al., 2003), MAP1B (Brown et al., 2001; Darnell et al., 2001; Zalfa et al., 2003), and PSD-95 (Todd et al., 2003). Transcriptome-wide screening for FMRP target mRNAs has identified mRNAs that preferentially co-IP with FMRP in the presence of EDTA (Brown et al., 2001). Some of these mRNAs also display an altered polyribosomal profile in cells from Fragile X patients. However, this study may have been biased toward identification of RGG box-binding mRNAs, since the co-immunoprecipitation buffer contained 30mM EDTA, which is known to disrupt ribosomes and KH2 binding to RNA (Darnell et al.,

2005a). In addition, mRNA targets differentially associated with polyribosomes in the presence or absence of FMRP were identified from lymphoblastoid cells, not brain. Other potential FMRP targets have been identified by antibody-positioned RNA amplification (Miyashiro et al., 2003) and *in vitro* RNA selection from transcribed sequences in brain (Chen et al., 2003). However, little data at the protein level exist to confirm translational regulation of these mRNAs by FMRP.

Methodologies to elucidate a role for FMRP and Nova proteins in translational control

In order to address potential functions for Nova-1, Nova-2 and/or FMRP in the regulation of translation in neurons, we have devised several approaches. First, we have developed a method to purify and analyze polyribosomes from mouse brain and spinal cord lysates. Subjecting these lysates to various treatments can provide more knowledge on whether these proteins are associated with polyribosomes or with other large complexes. Second, mRNA isolated from purified polyribosomes of wild type and knockout mice can be interrogated by microarrays in order to screen for steady state changes in mRNA translation that are associated with these proteins. Finally, we can use known RNA ligands to these KH domain-containing proteins to investigate whether binding to a particular RNA target has an impact on other protein-protein or protein-RNA interactions. These experiments provide a foundation to address important aspects of translational control in neurons, and the relationship to human neurological disease.

CHAPTER II: MATERIALS AND METHODS

Polyribosome analysis

Polyribosomes from cortex or spinal cord were analyzed as follows. CD1 or FVB mice at various ages were killed by decapitation after anesthesia with chloroform. The brain or spinal cord was removed from the skull and placed in ice-cold buffer (10mM HEPES-KOH, pH 7.4, 150mM KCl, and 5 mM MgCl₂) containing 100 µg/ml of cycloheximide. From this point on, the material was kept ice-cold or at 4°C throughout the preparation. From brain, the cerebral cortex was dissected free of cerebellum, brainstem, mesencephalon, diencephalon, basal ganglia, and much of the underlying white matter. For spinal cord, meninges were carefully removed. Tissue was homogenized in 1 ml of homogenization buffer per cortex or per 4 spinal cords (except as noted) (homogenization buffer: 10 mM HEPES-KOH, pH 7.4, 150 mM KCl, 5 mM MgCl₂, and 0.5 mM DTT) containing protease and RNase inhibitors (complete EDTA-free from Roche and Rnasin from Promega). Tissues were homogenized with 12 strokes at 900 rpm in a motor-driven glass-Teflon homogenizer. The homogenized material was spun at 2000 x g, 10 min, and NP-40 was added to the supernatant (S1) to a final concentration of 0.3% or 1% v/v. After 5 min of incubation on ice, the material was spun at 20,000 x g for 10 min, and the supernatant (S2) was loaded onto 20-50% w/w linear density gradient of sucrose in 10 mM HEPES-KOH, pH 7.4, 150 mM KCl, 5 mM MgCl₂, or 30 mM EDTA where indicated. In the indicated samples, EDTA was added to S2 to a final 30 mM concentration before loading on the gradient. Material obtained from one cortex or one-half cortex was loaded onto each gradient. The gradients were centrifuged at 40,000 x g for 2 hr at 4°C in a Beckman Instruments SW 41 rotor.

Fractions of 0.5, 0.75, or 1 ml volume were collected with continuous monitoring at 254 nm using an ISCO UA-6 UV detector. For the RNase experiments, before centrifugation, the S2 fraction was incubated with 1000 U/ml RNase T1 (Ambion) and 44 U/ml RNase A (United States Biochemical) for 10 min at room temperature.

The same procedure was used for the analysis of polyribosomes extracted from cells in culture. A postmitochondrial extract was obtained by lysis of 1×10^7 N2a, IMR32, 293 or 293T cells (American Type Culture Collection) or primary cortical neurons from seven 10-cm-diameter dishes in 1 ml of the following buffer: 20 mM HEPES-KOH, pH 7.4, 10 mM KCl, 5 mM MgCl₂, 0.3% NP-40, 0.5 mM dithiothreitol, 40 U/ml Rnasin (Promega), and complete protease cocktail (Roche). The salt concentration was adjusted to 150 mM with 1 M KCl, and the lysate was spun 12,000 x g, 10 min at 4°C. The resulting supernatant was loaded on sucrose gradients as described above.

The proteins contained in each fraction of the sucrose gradients were TCA-precipitated and analyzed by Western blot.

Western blotting

Anti-FMRP (1C3, Chemicon; 2F5, hybridoma from J. Fallon), anti-S6 ribosomal protein (Cell Signaling Technology), anti-L7 ribosomal protein (GeneTex), anti-P0 ribosomal protein (a kind gift from Dr. Tan, Scripps Institute; Biodesign International), and anti-poly(A) binding protein (PABP) (10E10; Immunoquest), anti-Nova (rabbit polyclonal and human patient sera), anti-Hu (human patient sera), anti-FXR1(830, gift of E. Khandjian and B. Bardoni), anti-FXR2 (1G2, Iowa Hybridoma Bank), anti-CYFIP1,

anti-CYFIP2, anti-NUFIP, anti-83FIP (all gifts of B. Bardoni), anti-hAgo1 and anti-hAgo2 (gifts of T. Tuschl), anti-myoVa (Sigma), anti-puralpha (Abnova), anti-mStaufen (Chemicon), anti-YB1 (Abcam), anti-eIF4E (Santa Cruz), anti-EF1alpha (Novus), anti-Sam68 (Upstate), anti-aPKCzeta (Sigma), and the appropriate HRP-conjugated secondary antibodies (Jackson Immunochemicals) were used for Western blotting. Signal was detected by chemiluminescence. The membranes were exposed on Biomax MR film (Eastman Kodak) and to the digital camera of a Versadoc Imaging System (Bio-Rad).

Pharmacological treatments of cells

Cells in culture were treated with 0.35 mM cycloheximide (Sigma) for 10 min or with 1 mM puromycin (Sigma) for 3 hr before lysis, as described. Cycloheximide was dissolved in methanol, and puromycin was dissolved in water.

In vitro transcription

In vitro transcription of RNA for binding curves was performed with 13 μ L of PCR-generated DNA template, 0.4 μ M NTPs, 1 μ L RNAsin (Promega), 40 μ Ci {alpha}-32P-UTP, 1x transcription buffer (Stratagene), and 1 μ L T7 RNA polymerase (Stratagene). RNA was treated with 3 units of RQ1 DNase for 45 min at 37°C and followed by gel purification on 8% denaturing polyacrylamide gels.

Nitrocellulose filter binding assays

Ten-thousand counts per minute (1-5 fmol) of internally labeled RNA (preheated to 75°C and cooled at room temperature for 5 min) was incubated with the indicated

concentrations of protein in a total volume of 50 μ L in SBB, 10 min at room temperature. Binding solutions were passed through MF-membrane filters (0.45 HA, Millipore) and washed with 4 mL SBB. Filters were air dried and counted in 5-mL ReditSafe scintillant. Data were plotted as percentage of total RNA bound versus log of the protein concentration and K_d 's determined using Kaleidograph software (Synergy Software).

RNA treatment of polyribosome preparations

In vitro transcribed RNAs were prepared as described above, incubated in 100 μ L of 1x SBB for 10 min at 75°C, then incubated for 15 min at room temperature. The supernatant (S1) from the homogenized material was collected and adjusted to 1% NP-40, v/v. Two microliters of rRNasin, and 100 μ L of in vitro transcribed RNA (or yeast tRNA, Roche) in 1x SBB were added to 1.1 mL of S1 and mixed by inversion. After incubation for 15 min at room temperature, S1 lysate was spun at 20,000 x g for 10 min at 4°C and the supernatant (S2) was loaded onto sucrose gradients as described above.

Immunoprecipitation

A post-mitochondrial supernatant (S2) from cerebral cortex was prepared as for polyribosome analysis and pre-cleared with 50 μ L protein A-sepharose (Sigma) for 30min at 4°C. Mixed monoclonal anti-FMRP IP matrix was prepared as follows: 120 μ L Protein A-sepharose was incubated with 12 μ L rabbit anti-mouse Fc γ (2.4mg/ml, Jackson Immunochemicals) for 30 min. at room temperature, then washed three times with 0.1M NaPO₄ buffer (pH 8.0) before incubation with 8 μ L 7G1-1 and 25 μ L 2F5 monoclonal antibodies overnight at 4°C. Final IP matrix was washed three times with 0.1M NaPO₄

buffer before addition to pre-cleared supernatant. Immunoprecipitations were performed at 4°C for 2 hours, after which they were washed twice with brain lysis buffer lacking NP40.

Microarray analysis

Total cytoplasmic or polyribosomal RNA was isolated with TRIzol LS (Invitrogen) extraction followed by RNeasy (Qiagen) purification with DNase treatment on column. Ten µg of RNA was used as input for cRNA generation according to Affymetrix guidelines. Biotin-labeled, fragmented cRNA was hybridized to either MGU74v2 ABC or MG430 2.0 GeneChips (Affymetrix). Data analysis was accomplished as described in Chapter 4 using MicroArray Suite 5.0 (Affymetrix), GeneTraffic (Stratagene), and/or GeneSpring (Agilent).

Lentivirus production

All lentiviral constructs and packaging reagents were obtained from D. Trono (Geneva, Switzerland). Packaging plasmids psPAX2 (15µg) and pMD2.G (6µg) were introduced with 20µg pLVTt into 1×10^7 80% confluent 293T cells (ATCC) by calcium phosphate transfection (Invitrogen). Virus-containing media was collected 48 hours after transfection and filtered through a 0.4µm membrane.

CHAPTER III: NEURONAL RNA-BINDING PROTEINS AND THEIR ASSOCIATION WITH POLYRIBOSOMES IN MOUSE BRAIN

Introduction

Translation of mRNA messages into functional proteins can be regulated in various ways by RNA-binding proteins in cells. Once transcribed and spliced in the nucleus, mRNAs must be exported to the cytoplasm, transported to their subcellular destinations, protected from or encouraged to undergo translation, and degraded under the appropriate circumstances. (Reviewed in (Moore, 2005)) However, these events in the lifetime of an mRNA do not exist in isolation, as supported by recent advances in the fields of transcription, pre-mRNA splicing, mRNA export, nonsense-mediated degradation (NMD) and translation. Instead, they are coupled to the events that precede and follow. Many proteins that bind a specific RNA at a given step in processing remain bound and function in downstream events. It is through this network of regulatory mechanisms that additional layers of complexity are added to our genomic program for tissue-specific cellular function in a temporal and spatial fashion.

Local control of translation is an important aspect of the regulation of gene expression, especially in neurons, which can extend processes and create synapses enormous distances from the cell body. Although originally studied in developmental processes in *Xenopus* and *Drosophila*, mRNA targeting, localization, and translation have become a key aspects of the current theory of activity-dependent synaptic modification. (Richter and Lorenz, 2002; Steward and Schuman, 2003; Sutton and Schuman, 2005) In essence, for an mRNA to have a selective impact on a specific synapse, its transport must be correctly targeted to the translational area of that synapse. It follows that locally

translated messages should encode proteins that are in use at that synapse, modify other proteins at the synapse, or serve as messengers to send signals back to other parts of the neuron. Finally, transport and/or translation of this mRNA must be regulated in a manner that can be altered by activity at that synapse.

Many studies have shown that protein synthesis is required for long-lasting forms of behavioral and synaptic plasticity. (Govindarajan et al., 2006; Kang and Schuman, 1996; Kelleher et al., 2004a; Mayford et al., 1996; Nguyen and Kandel, 1996) Evidence for this hypothesis to date includes: localization of polyribosomes and associated membranous cisternae to sub-synaptic sites in dendrites, assessments of the mRNA complement of dendritic processes, protein synthesis in isolated synaptosomal preparations, translation of an mRNA construct transfected into an isolated dendrite, and studies of polyadenylation-induced translation of the α CaMKII mRNA in synaptosome preparations after NMDA receptor activation. (Crino and Eberwine, 1996; Eberwine et al., 2001; Gardiol et al., 2001; Huang et al., 2002; Miyashiro et al., 1994; Steward and Levy, 1982)

Analysis of the subcellular distribution of a given RNA-binding protein by gradient centrifugation may offer clues to its function *in vivo*. In particular, if the protein in question cosediments with polyribosomes, it may be involved in the regulation of translation of its target mRNAs. Three target antigens for paraneoplastic neurologic disease (PND), Hu (family members HuB, HuC and HuD), Nova (family members Nova1 and Nova2), and Trim9 are neuronal RNA-binding proteins that may function in this manner. Fragile-X mental retardation protein (FMRP) is a related RNA-binding protein that is also implicated in neurologic disease. We show that the while only 10-

25% of cytoplasmic Nova proteins are bound to polyribosomes, the majority of FMRP and the neuronal Hu proteins are associated with actively translating polyribosomes in brain and neuronal cells in culture.

Results

Polyribosomal protein analysis by gradient centrifugation

Velocity sedimentation of cytoplasmic components by centrifugation through a sucrose gradient allows subsequent fractionation and simultaneous measurement of absorbance at 254nm. This method was first used in 1961 to work out the basic mechanisms of protein synthesis, describing complexes that included multiple ribosomes and messenger RNAs, and thereby generating a polyribosomal profile for a given cell population (Becker et al., 1963; Gierer, 1963; Marks et al., 1962; Penman et al., 1963; Rich et al., 1963; Risebrough et al., 1962; Scherrer et al., 1963; Slayter et al., 1963; Spyrides and Lipmann, 1962; Staehelin et al., 1963a; Staehelin et al., 1963b; Warner et al., 1963a; Warner and Knopf, 2002; Warner et al., 1963b; Wettstein et al., 1963). The UV absorbance tracing of post-mitochondrial supernatant sedimented through a continuous 20-50% (w/w) sucrose gradient displays clearly distinguishable peaks for the 40S and 60S subunits, 80S ribosomal monomer, and subsequent increases in the number of ribosomes associated with mRNA. Protein analysis of polyribosome gradients by Western blotting is one tool to understand whether a given RNA-binding protein is associated with ribosome-loaded mRNAs; such an association may indicate a role for that protein in the regulation of mRNA translation. For example, FMRP co-sediments with polyribosomes from tissue culture cells (Corbin et al., 1997; Khandjian et al., 1996).

There are data to show that FMRP can negatively regulate the translation of several mRNAs, both in an *in vitro* rabbit reticulocyte lysate system and *in vivo* in cell culture (Laggerbauer et al., 2001; Li et al., 2001b; Mazroui et al., 2002; Schaeffer et al., 2001)

In our studies of neuronal RNA-binding proteins, we have developed a method to fractionate polyribosomes from mouse cerebral cortex in order to ascertain the polyribosomal distributions of these proteins. Several RNA-binding PND antigens demonstrate partial polyribosomal association *in vivo*. There are several commonly used assays to demonstrate true polyribosome association and distinguish it from co-migration on a sucrose gradient. Treatment with EDTA, translational initiation inhibitors that allow run-off of translating ribosomes, or RNase result in disruption of the polyribosomal profile and a change in the sedimentation rate of ribosomal proteins. If a protein is indeed associated with polyribosomes, its sedimentation rate should mirror that of ribosomal proteins under these conditions. Since EDTA chelates the magnesium ions necessary for 80S ribosome formation and stabilization, an EDTA-treated lysate will produce a profile showing two large peaks for ribosomal subunits (often off-chart and merged into a single peak) but no peaks corresponding to 80S ribosomes or polyribosomal complexes. A polyribosome-associated protein may be bound to actively translating or stalled complexes. To distinguish between these types of polyribosomes, sodium fluoride (NaF) and sodium azide (NaN₃) can be used to inhibit ribosome re-initiation in a cell lysate, allowing previously bound ribosomes to run-off the mRNA and complete translation. The resultant UV profiles are shown in Figure 3.1A. Protein from each fraction of each gradient was precipitated with trichloroacetic acid and analyzed by western blotting. The S6 ribosomal protein shows the position of the small (40S)

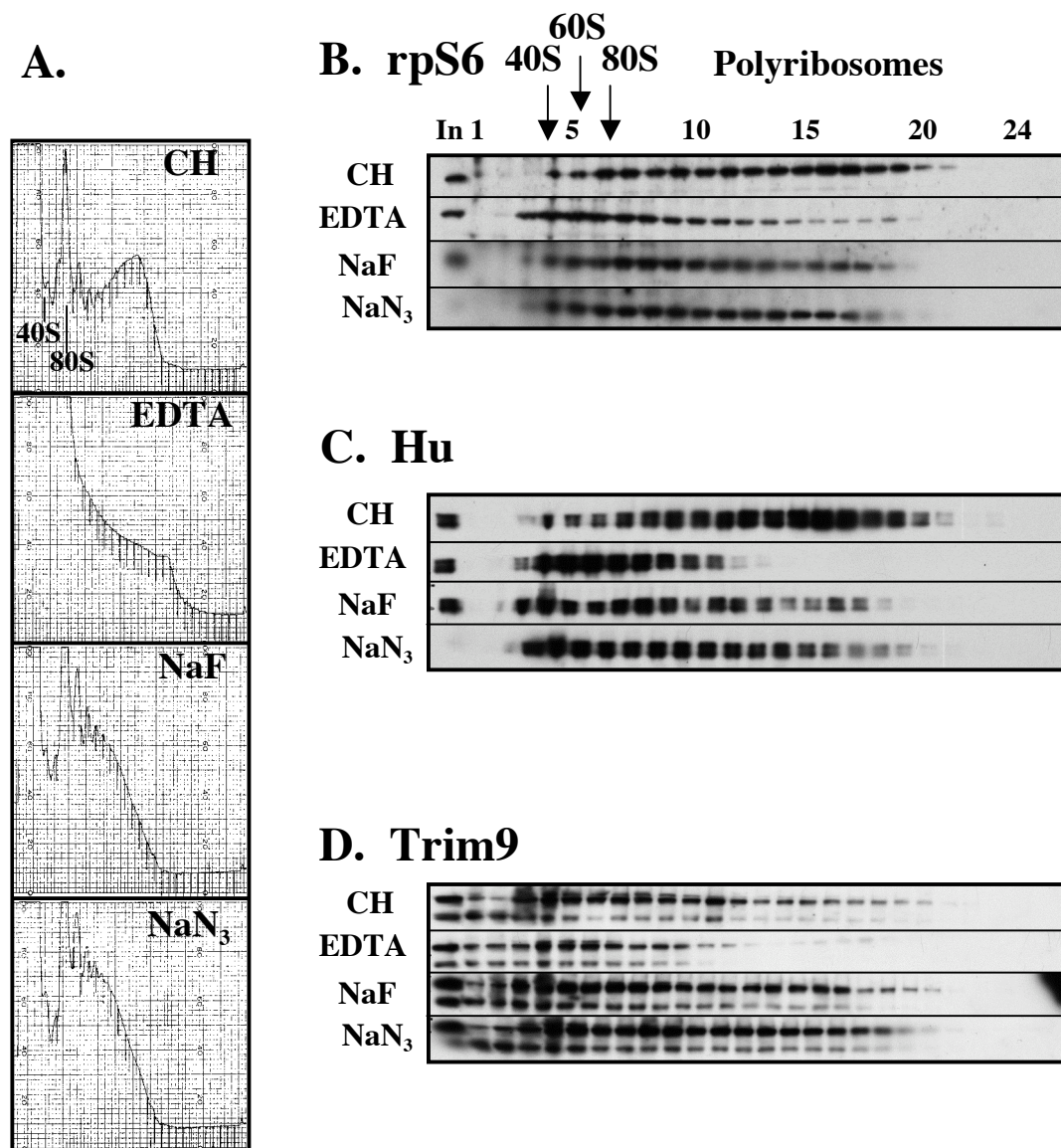


Figure 3.1 Polyribosomal profiles and protein distributions: comparison of treatment with EDTA or translation initiation inhibitors. (A) UV absorbance profiles at 254nm of mouse brain lysate treated as indicated and centrifuged through 20-50% sucrose gradients. Western blots for ribosomal protein S6 (B), the neuronal Hu proteins (C), and Trim9 (D). (CH: 100ug/ml cycloheximide; EDTA: 30mM ethylenediaminetetraacetic acid; NaF: 30mM sodium fluoride; NaN₃: 20mM sodium azide)

ribosomal subunit throughout the gradients (Figure 3.1B). 49% of total S6 protein is found in fractions 12-20, which correspond to polyribosomal complexes with more than five ribosomes. A 25% shift in the distribution of S6 is seen with EDTA treatment. Smaller shifts (16%) are evident in the NaF- and NaN_3 - treated samples. These data confirm that 30mM EDTA disrupts polyribosomes, and that ribosome re-initiation inhibitors do have an impact on the number of ribosomes per message under these conditions. Despite the incomplete translational run-off achieved here, partial sensitivity to NaF and NaN_3 is still informative for our analysis of polyribosomal proteins.

HuB, HuC and HuD

The neuronal ELAV-related RNA-binding proteins HuB, HuC and HuD were visualized using sera from a patient with anti-Hu PND. Although the ubiquitously expressed family member HuR (HuA) has been shown to associate with polyribosomes in cell culture and other tissues (Sheflin et al., 2001), this association had not been demonstrated for the CNS-specific HuB, HuC and HuD proteins. HuB and HuC have been shown to be important for neuronal development in both the CNS and PNS. (Akamatsu et al., 1999) The Hu proteins contain RRM-type RNA-binding domains. HuR has been shown to increase the stability of its target mRNAs, as well as increasing the translation of these messages into protein. (Antic and Keene, 1997; Antic and Keene, 1998; Antic et al., 1999; Gallouzi et al., 2000; Levine et al., 1993) Figure 3.1C shows that 52% of neuronal Hu proteins are associated with polyribosomes containing more than 5 ribosomes (fractions 12-20) in mouse cerebral cortex. When treated with EDTA, Hu proteins shift to the upper region of the gradient, mirroring the dissociation of

ribosomes into subunits shown by ribosomal protein S6. Furthermore, when treated with NaF or NaN₃ in a run-off translation assay, Hu proteins show a 30% shift from their control peak at fractions 12-16 to several peaks between fractions 3 and 10. This evidence supports a role for neuronal Hu proteins in actively translating polyribosomal complexes.

Trim9

Another PND antigen, tripartite motif (RING, B-box and coiled-coil domains) 9, or Trim9, shows partial association with polyribosomes. While the amount of Trim9 peaks in fractions 3-9 in both control and EDTA-treated samples, there is 25% less signal in fractions 12-22 of the EDTA-treated sample (Figure 3.1D). However, Trim9 does not seem to be associated with actively translating polyribosomes, since a shift is not seen in the NaF- and NaN₃-treated samples. It is possible that Trim9 could be associated with stalled polyribosomes or other large complexes that are magnesium dependent. Trim9 has been shown to bind RNA (W. Y. Park, unpublished data). In addition, recent work indicates that it binds microtubules and is localized to the synapse. (Li et al., 2001a; Short and Cox, 2006)

Onconeural ventral antigens Nova1 and Nova2

Nova1 and Nova2 are closely related target antigens of paraneoplastic opsoclonus myoclonus ataxia (POMA). Both Nova proteins contain three KH-type RNA-binding domains, and have been shown to bind YCAY-rich sequences. (Buckanovich et al., 1993; Jensen et al., 2000a) The roles of Nova1 and Nova2 in the regulation of alternative

splicing in neurons have been extensively documented by our laboratory (Dredge and Darnell, 2003; Jensen et al., 2000a; Ule et al., 2003; Ule et al., 2005b). However, while RNA splicing is almost always a nuclear process, approximately 40-60% of Nova is present in the cytoplasm (J. Ule, doctoral thesis). Since RNA-binding proteins are often involved in more than one processing event, it is a reasonable hypothesis that Nova could be involved in the translational regulation of target mRNAs. To test this hypothesis, the polyribosomal distribution of both Nova1 and Nova2 was visualized using high-titer POMA patient sera. Figure 3.2A shows that the majority of Nova is found in the first two (lightest) fractions of the gradient, likely in small protein-only or mRNP complexes. UV absorbance profiles were not obtained for this experiment since fractions were collected through a pinhole at the bottom of the gradient tube. This technique prevents accidental contamination of the heaviest fractions by proteins present in other regions of the gradient. For control purposes, unchelated and EDTA-treated gradients were assayed for a number of proteins, as shown in Figure 3.2A, including non-polyribosomal proteins gamma-tubulin, PTEN and MAPK. In order to better quantify the percentage of cytosolic Nova that is associated with large magnesium-dependent complexes, Western blot chemiluminescence was quantified using the Versadoc Imaging System (Bio-Rad) for control and EDTA-treated polyribosome gradients. Figure 3.2B illustrates that there is a sharp visual difference between the amount of Nova present in fractions 12-19 of control versus EDTA-treated polyribosome profiles, and 11.8% of cytosolic Nova shifts out of these fractions per gradient (one brain). Conversely, 22.4% of total Nova signal is shifted to fractions 4-11 of the EDTA-treated gradient. A repetition of this experiment yields an EDTA-dependent shift of 18.6% of total cytosolic Nova to fractions 4-11 (data not

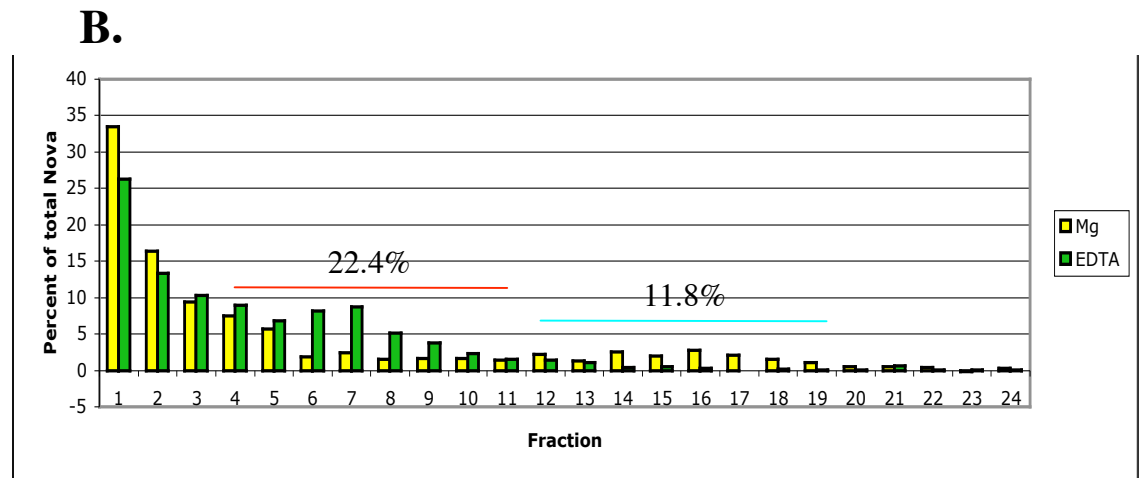
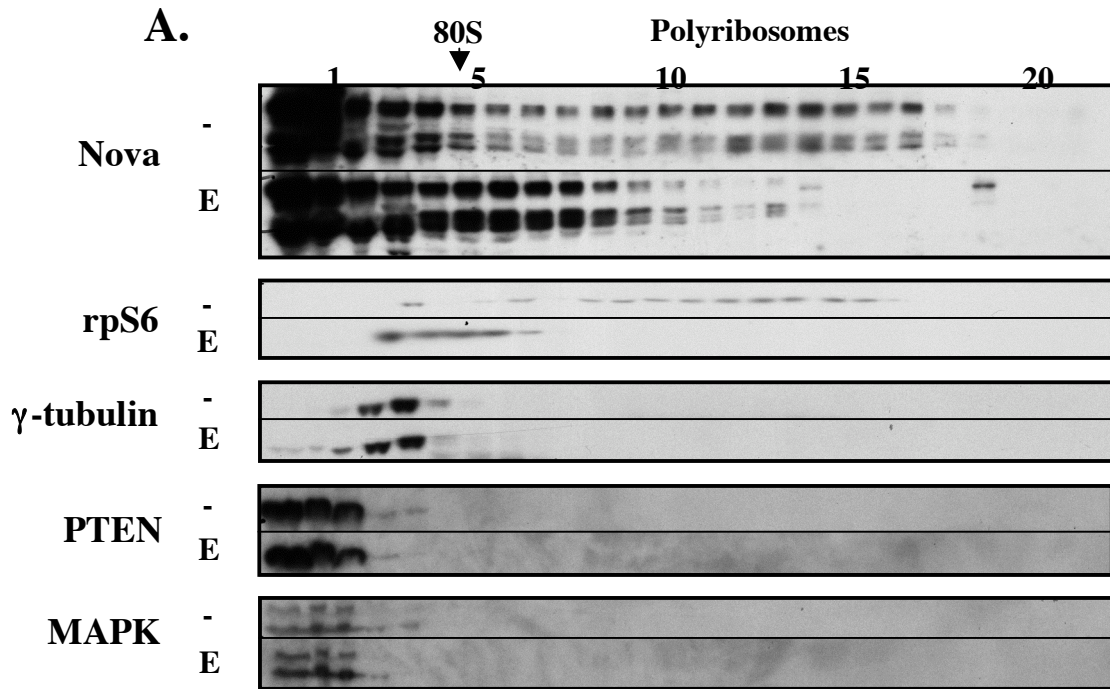


Figure 3.2 10-25% of non-nuclear Nova proteins associate with polyribosomes in mouse cerebral cortex. (A) Western blots of polyribosomal distributions in the absence (-), or presence of EDTA (E) for Nova-1 and Nova-2 proteins (Nova), ribosomal protein S6 (rpS6), gamma-tubulin, PTEN and MAPK. (B) Western blots for Nova in (A) quantitated by fraction as a percentage of total Nova present in the post-mitochondrial supernatant.

shown). Thus, we can estimate that approximately 10-25% of brain cytosolic Nova is associated with large complexes in a magnesium-dependent manner that may in fact contain polyribosomes.

Fragile-X Mental Retardation Protein

Converging lines of evidence support a role for FMRP in translational control. FMRP is found associated with polyribosomes in various non-neuronal cell lines. (Corbin et al., 1997; Eberhart et al., 1996; Feng et al., 1997a; Khandjian et al., 1996) In addition, the abundance of a set of mRNAs in the polyribosomal fraction is altered in human cell lines derived from Fragile X syndrome patients. (Brown et al., 2001) The *Drosophila* ortholog of FMRP, dFXR, has been shown to repress the expression of *futsch* at the translational level. (Zhang et al., 2001) Finally, the ability of FMRP to act as a translational repressor of reporter constructs has been demonstrated in vitro and in transfected cells. (Laggerbauer et al., 2001; Li et al., 2001b; Mazroui et al., 2002) It has been suggested that the loss of proper regulation of translation of FMRP target mRNAs underlies the morphological and functional abnormalities observed at some synapses of *Fmr1*-null mice. (Huber et al., 2002; O'Donnell and Warren, 2002)

These observations introduce the question of whether they are relevant to FMRP function in the mammalian brain. Technical difficulties in sedimentation studies using CNS tissue have, until recently, prevented researchers from being able to definitively show that FMRP is present on polyribosomes from brain. The most promising evidence showed that FMRP may co-sediment with polyribosomes in synaptosomal preparations from rat cortex, however, this data included neither an RNA absorbance profile nor

sufficient biochemical controls for synaptosomal preparation purity (Feng et al., 1997b). In addition, a recent study reported that FMRP is in fact not associated with polyribosomes in brain extracts, but rather that it cosediments with the 80S ribosome and with heterogeneous complexes of smaller size. (Zalfa et al., 2003)

We devoted a significant effort to improving existing methodologies for the purification of polyribosomes from mouse brain. The essential differences in our technique are the dissection and removal of underlying white matter tracts from the cerebral cortex, and the use of 0.3% to 1% NP40 as a detergent instead of sodium deoxycholate. Our method of polyribosome fractionation allows us to demonstrate that, indeed, FMRP is present on polyribosomes in brain in both juvenile (postnatal day 9, Figure 3.3A) and adult (5 months old, Figure 3.3B) mice. OD₂₅₄ absorption profiles and Western blots are shown for polyribosome gradients treated with either 5mM magnesium chloride or 30mM EDTA. EDTA treatment entirely disrupts polyribosomal complexes into the small and large ribosomal subunits, as shown by the shift in rpS6 distribution. It also results in a significant and similar shift in FMRP distribution, from association with the largest polyribosomes to co-sedimentation with, or slightly larger than, the 60S ribosomal subunit. To examine whether the cosedimentation of FMRP with polyribosomes in the brain was RNA-dependent, extracts were treated with RNases A and/or T1, and analyzed by gradient centrifugation. This treatment resulted in complete disruption of polyribosomes, as shown by the shift of ribosomal protein P0, and also shifted FMRP to the lighter fractions of the gradient (Figure 3.3C). (Stefani et al., 2004) In addition, when extracts are treated with both RNase and EDTA, there is a small additional shift to the lightest fractions of the gradient (Figure 3.3D). This suggests that

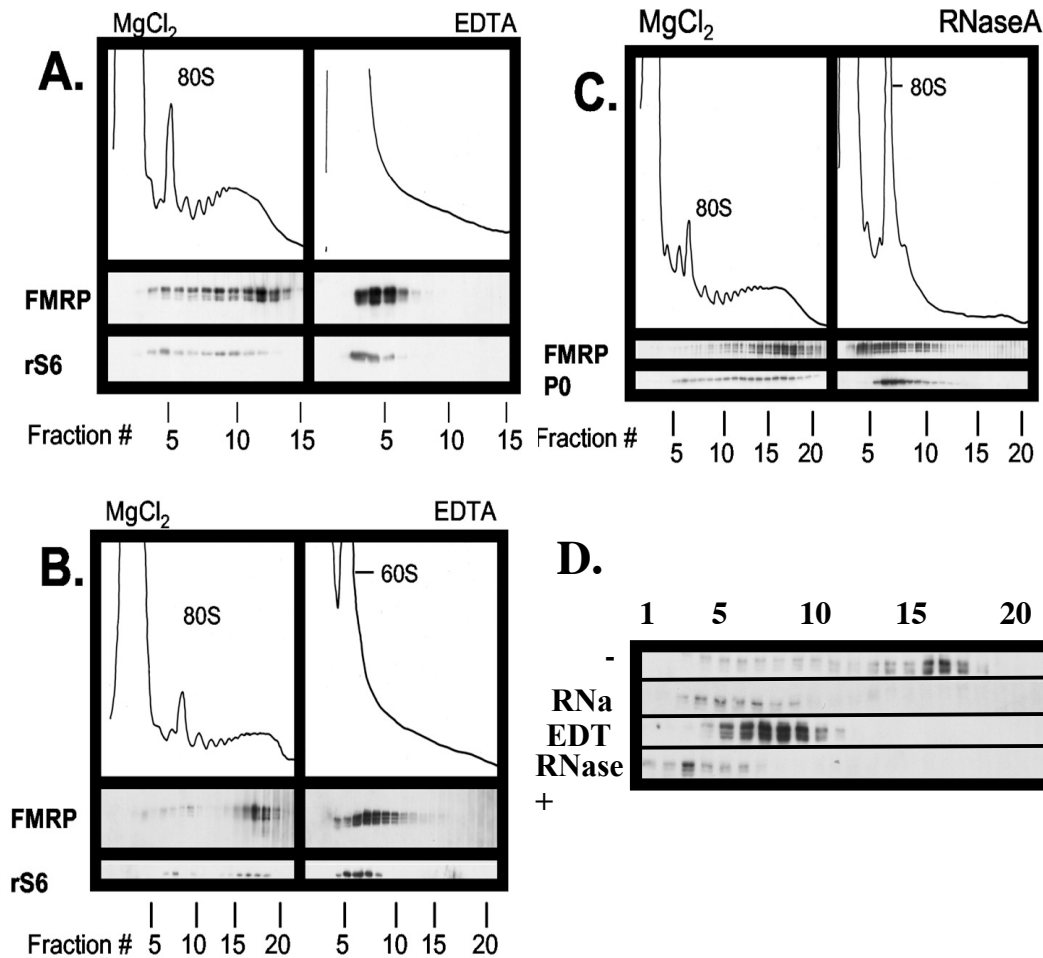


Figure 3.3 FMRP is associated with polyribosomes in mouse cerebral cortex. UV absorbance profiles and Western blots for FMRP and ribosomal protein S6 (rS6) of polyribosome fractionations from cerebral cortices of juvenile (postnatal day 9, **A**) and adult (five months of age, **B**) mice. A comparison between control conditions (MgCl₂) and EDTA treatment shows that both FMRP and polyribosomes (profiles, rS6) are disrupted by chelation of magnesium for both juvenile and adult mice. (**C**) Treatment of juvenile (postnatal day 9) mouse cortical lysate with RNase A also causes FMRP and ribosomal protein P0 (P0) to shift into the upper fractions of the gradient. (**D**) Treatment with both RNase A and EDTA (bottom panel) results in a supershift of EDTA-released FMRP complexes. Western blots for FMRP of polyribosome gradients from mouse cortical lysate with the indicated treatments.

FMRP may exist in complexes of multiple sizes, which are differentially dependent on both magnesium and RNA. These results are in agreement with previous observations in non-neuronal cell lines. (Ceman et al., 2003; Eberhart et al., 1996)

One variable used in previous polyribosome profiles from brain has been the presence or absence of ionic detergent such as deoxycholate. (Bagni et al., 2000; Zalfa et al., 2003) To rule out the possibility that the observed sedimentation pattern of FMRP might relate to the presence or absence of detergents, we analyzed gradients of brain extracts prepared in the presence of deoxycholate, Nonidet P-40, or in the absence of any detergent. After elimination of the insoluble material with a 20,000 x g centrifugation step, we obtained clearly discernible polyribosome peaks in sucrose gradients (Figure 3.4A). In agreement with our previous results, FMRP cosediments with polyribosomes in this preparation, eliminating the possibility of technical artifacts caused by the presence of detergents in the extract. Conversely, we find that FMRP association with polyribosomes is completely disrupted by the presence of deoxycholate (Figure 3.4B). (Stefani et al., 2004) These results demonstrate that FMRP is associated with polyribosomes in the brain. Khandjian and colleagues have reported similar findings on the impact of deoxycholate on FMRP distribution. (Khandjian et al., 2004)

The molecular mechanism through which FMRP might modulate the expression of its target mRNAs is unknown. Even tissue-culture studies have failed to convincingly show whether FMRP associates with actively translating polyribosomes or with translationally arrested ribosome-associated messenger ribonucleoprotein complexes

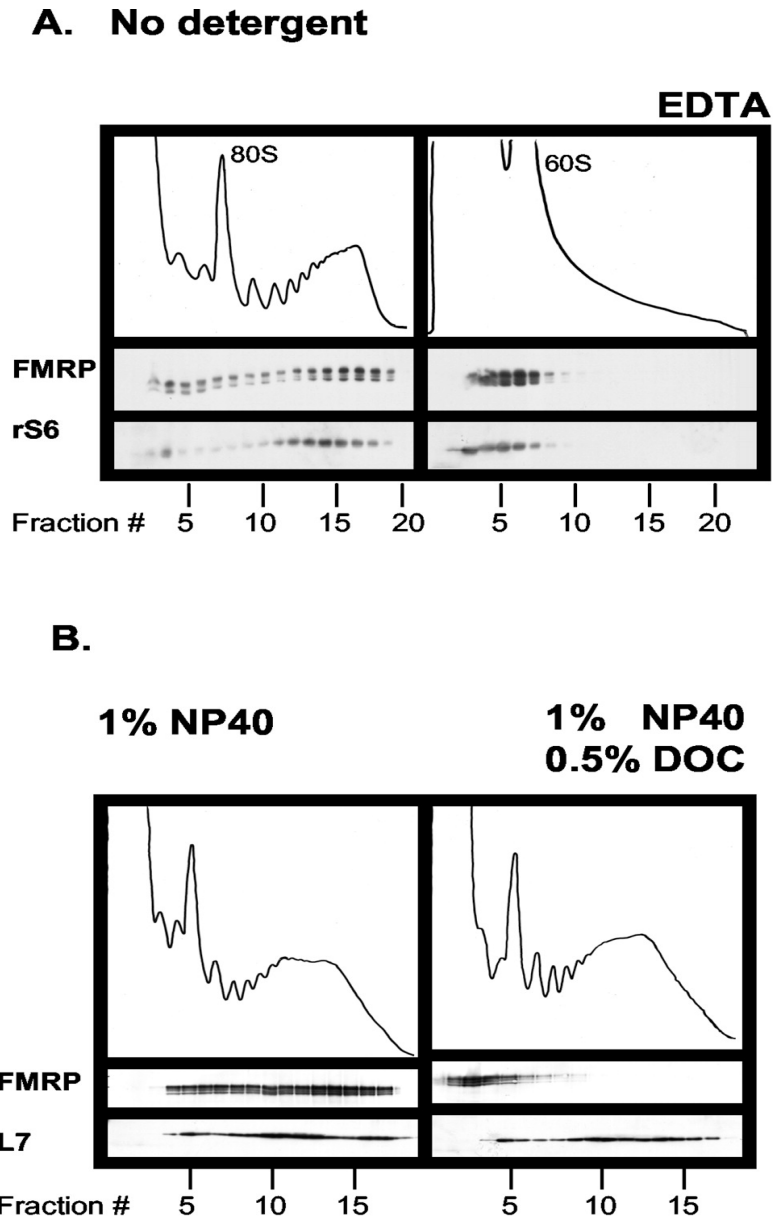


Figure 3.4 FMRP associates with polyribosomes in the absence of detergent. Post-mitochondrial supernatants from juvenile mouse cortices were prepared without detergent (A) and subjected to control (left panel) and EDTA (right panel) treatments, or in the presence of 1% Nonidet P40 (NP40) (B, left panel) or NP40 plus 0.5% sodium deoxycholate (DOC, right panel). Western blots of polyribosome gradient fractions are shown for FMRP and the ribosomal proteins S6 (rS6) and L7.

(RNPs). It has been suggested that the phosphorylation state of FMRP may regulate whether it is associated with active or stalled polyribosomes. (Ceman et al., 2003)

In tissue-culture studies, the FMRP-polyribosome association has been disrupted by treatment with pleiotropic inhibitors of translation reinitiation such as sodium fluoride and sodium azide (Ceman et al., 2003; Feng et al., 1997a), but it is unknown whether the FMRP-polyribosome association can be disrupted with these agents in a cytoplasmic lysate from mouse cerebral cortex. Figure 3.5 shows the distribution of FMRP in control, EDTA-treated and translation re-initiation-inhibited conditions. These data demonstrate that, when ribosomes are allowed to undergo run-off translation, 14% of polyribosomal FMRP shifts from fractions 12-21 into fractions 5-10. Further evidence for the association of FMRP with actively translating polyribosomes is shown in Figure 3.6. (Stefani et al., 2004) To assess whether FMRP is associated with actively translating polyribosomes in cells of neuronal lineage, we treated Neuro-2a cells, a murine neuroblastoma cell line, with the translational inhibitor puromycin. Puromycin is an analog of amino-acyl transfer RNA that binds to the acceptor site of the ribosome, blocking elongation and causing premature release of the growing polypeptide chain. (Davis et al., 1974) Therefore, the drug specifically targets actively translating polyribosomes. We attempted to assay FMRP sensitivity to puromycin treatment *in vivo* by injecting puromycin into the intraperitoneal space of mice. We were not able to obtain usable polyribosome gradients from the brains of these mice due to the lipid insolubility of puromycin. As a relatively hydrophilic molecule, puromycin has difficulty crossing the blood-brain barrier. We were unable to achieve sufficient puromycin concentrations in the brain before the mice died due to systemic toxicity (data not shown.) For these

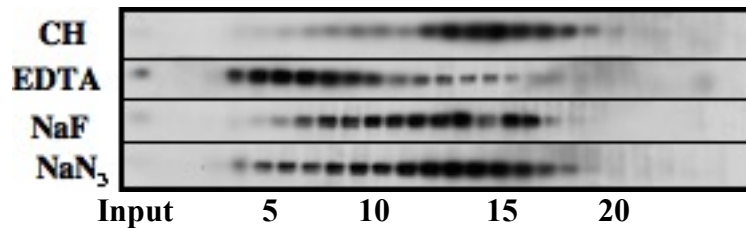


Figure 3.5 FMRP cosediments with translating polyribosomes in mouse cortex. Western blots for FMRP from polyribosome gradient fractions in Figure 3.1 show that 65% of FMRP is bound to polyribosomal complexes containing at least 5 ribosomes (top panel, fractions 12-20). 14% of this population of FMRP shifts under run-off conditions when translation initiation is inhibited by NaF or NaN_3 .

reasons we have used a neuroblastoma cell line (Neuro2a) to test whether FMRP is bound to actively translating polyribosomes.

FMRP cosediments with the larger polyribosomes in Neuro-2A cells treated with cycloheximide (Figure 3.6 top panel), as observed in the brain. After treatment of Neuro-2A cells with 1mM puromycin for 3 hr, virtually all polyribosomes containing multiple (i.e., >4-6) ribosomes were fully disrupted, whereas the peak corresponding to the 80S ribosome monomer was markedly increased, as judged by the OD254 profile and the Western blot for ribosomal protein S6 (middle panel). Under these conditions, the sedimentation of FMRP shifts from fractions harboring large polyribosomes (more than six ribosomes) to less dense fractions. These FMRP-containing fractions present after puromycin treatment correspond either to non-translating monosome-size particles or to residual polyribosomes containing 2-4 ribosomes bound to mRNA. Ribosomal protein S6 and Poly-A Binding Protein (PABP) shift to smaller fractions, mostly corresponding to 80S ribosomal monomers and 60S large subunits. Taken together, these results

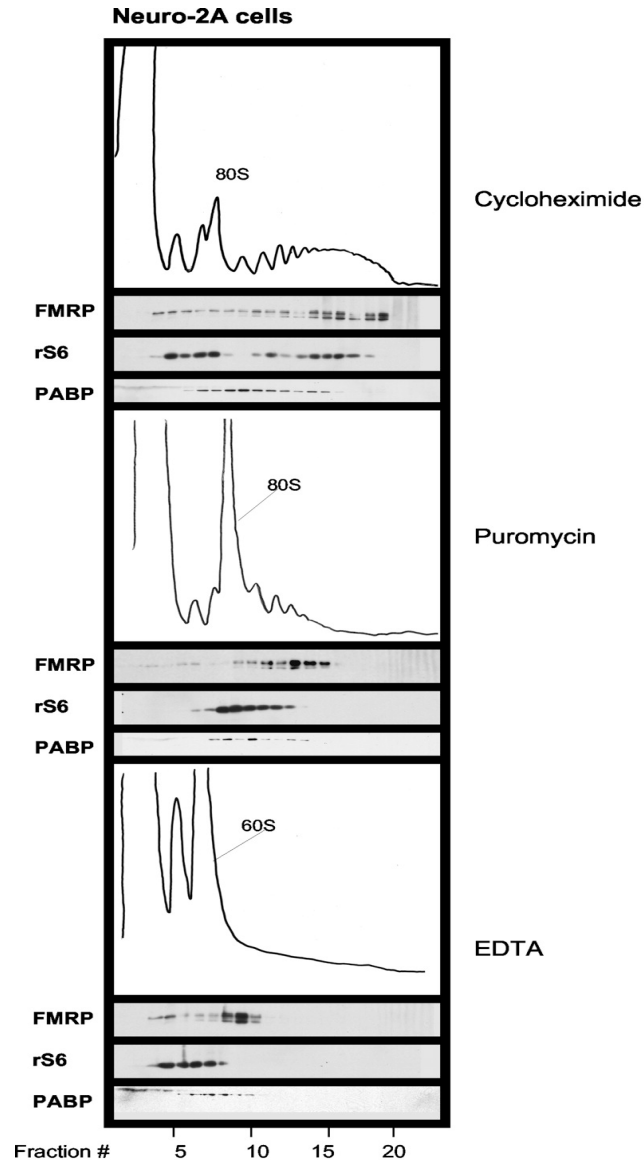


Figure 3.6 FMRP associates with functional polyribosomes in neuronal cells. Neuro-2A cells treated with 100ug/ml cycloheximide (top), puromycin (middle) or 30mM EDTA and analyzed by sucrose gradient centrifugation. Western blots are shown for FMRP, ribosomal protein S6 (rS6), and poly(A)-binding protein (PABP).

demonstrate that FMRP is associated with functional polyribosomes in neuronal cells. (Stefani et al., 2004)

Discussion

We have optimized a reliable procedure for the analysis of polyribosomes from differentiated neural tissues, based on the careful dissection of cerebral cortex with removal of underlying white matter, followed by homogenization and lysis in mild conditions that preserve the interaction between various neuronal RNA-binding proteins and polyribosomes. Data from these experiments demonstrate that neuronal proteins can exhibit varying degrees of polyribosomal association when analyzed by gradient centrifugation. Here we have looked at several proteins identified as target antigens for autoimmune responses initiated by the ectopic expression of these antigens in somatic cancer cells. The results of these paraneoplastic autoimmune activations are neurologic syndromes whose constellation of characteristics can inform studies on the normal function of these proteins in neurons. Trim9, identified as a target antigen of sera from a patient with paraneoplastic cerebellar symptoms (W. Y. Park, unpublished results), is partially located in heavy gradient fractions in a magnesium-dependent manner. This association is resistant to treatment with translation reinitiation inhibitors, suggesting that Trim9 may be a component of a large complex that may include stalled polyribosomes. The Hu antigens, first described as targets of paraneoplastic subacute sensory neuropathy, encephalomyelopathy, and/or cerebellar dysfunction, are entirely associated with actively translating ribosomal complexes when present in the post-mitochondrial supernatant.

Approximately 15-20% of non-nuclear paraneoplastic opsoclonus-myoclonus ataxia (POMA) targets Nova1 and Nova2 are associated with polyribosomes in cerebral cortex.

Although not a known target antigen for a paraneoplastic neurologic disorder, FMRP is related to Nova by means of their KH-type RNA-binding domains. FMRP is widely expressed, with highest expression observed in brain and testis. (Abitbol et al., 1993; Devys et al., 1993) Despite the essentially ubiquitous distribution, the human syndrome caused by the absence of FMRP is most notably characterized by cognitive impairments. (O'Donnell and Warren, 2002) Therefore, a detailed understanding of FMRP biology in the cerebral cortex is of great interest. In addition, the clinical and biochemical data from Fragile-X patients suggest that a defect in mRNA processing may be a critical component of this syndrome. We provide definitive evidence that FMRP is associated with polyribosomes in mammalian brain. We reproducibly observed EDTA and RNase-sensitive association of FMRP with polyribosomes in extracts from cerebral cortex from young and adult animals. Furthermore, we demonstrate that the complexes formed by FMRP with polyribosomes are translationally active in neuronal cells, because treatment with puromycin, sodium fluoride or sodium azide sufficient for disruption of polyribosomes decreases the rate of sedimentation of FMRP. Association of FMRP with polyribosomes has been described in non-neuronal cell lines, but contradictory reports exist in the literature about the existence of such interaction in neural tissues. One previous report has suggested a possible association of FMRP with polyribosomes in neural tissue. (Feng et al., 1997a) However, the evidence presented was not conclusive, in part because the absence of an absorbance profile makes the integrity and purity of the polyribosomes difficult to assess. In contrast, a recent report suggests that FMRP is not

associated with polyribosomes specifically in mouse brain, but instead cosediments with RNPs smaller than a single ribosome, (Zalfa et al., 2003) The results presented in this study definitively demonstrate that FMRP cosediments with polyribosomes purified from cerebral cortex. The purity of the polyribosomes from cortex is comparable to preparations from cultured cells, as judged by absorbance profiles.

Although the observed migration of FMRP on sucrose gradients and its sensitivity to EDTA and RNase are highly suggestive of an interaction between FMRP and translating polyribosomes, other interpretations cannot be ruled out *a priori*. Krichevsky and Kosik (Krichevsky and Kosik, 2001) have reported evidence for large molecular weight RNP granules in neurons. However, these data were obtained from primary neuronal cultures, and no such granules were seen in cells grown for 3 d in vitro (only after 7 d in vitro), suggesting a phenomenon closely related to culture conditions. In our work, we see no evidence for such RNP granules on OD₂₅₄ profiles of either young or adult mouse brain. In other studies, FMRP has been reported in large cytoplasmic complexes similar to stress granules in tissue culture after various treatments (Mazroui et al., 2002). Again, we have not observed a measurable amount of FMRP in such complexes in untreated mouse brain.

The association of FMRP with actively translating polyribosomes suggests that the protein may itself play a role in translational regulation. Previous studies have suggested that FMRP may promote mRNA translation. (Brown et al., 2001; Miyashiro et al., 2003; Todd et al., 2003) Although in principle an association with actively translating polyribosomes might be consistent with a positive role on translation, a number of reports also suggest that FMRP may inhibit the translation of its target RNAs.

(Laggerbauer et al., 2001; Li et al., 2001b; Mazroui et al., 2002; Zhang et al., 2001)

Interestingly, whereas puromycin clearly disrupts large FMRP-polyribosome complexes, FMRP still cosediments with the largest remaining polyribosomes containing three to four ribosomes rather than with the much more prominent peak of monomeric ribosomes (Figure 3.6). The less than complete shift of FMRP may be consistent with relative translational inhibition of some FMRP-associated transcripts. Alternatively, since FMRP is generally associated with larger polyribosomal complexes (compared to the distribution of ribosomal proteins, see Figures 3.3-3.6), its continued association with the largest remaining complexes may simply reflect incomplete treatment with puromycin, leaving a percentage of translating ribosomes intact. A third possibility is that FMRP is bound to mRNAs that are longer than average, and can therefore carry a greater number of ribosomes. Finally, FMRP binding may inhibit the rate of translation elongation, rather than initiation. This causes the number of ribosomes on a transcript to increase, since the ribosomes are packed more closely together. The association of FMRP with "stalled" polyribosomes has been shown previously to correlate with phosphorylation in non-neuronal cells treated with sodium azide. (Ceman et al., 2003) Although unusual, examples of translational repression of mRNAs associated with polyribosomes have been reported. Hac1 mRNA expression in yeast is regulated on translationally stalled polyribosomes by means of an RNA-RNA interaction between the 5' UTR and a retained intron in the 3' region of the message. (Chapman and Walter, 1997; Ruegsegger et al., 2001) Translationally suppressed nanos mRNA is associated with puromycin-sensitive polyribosomes in *Drosophila*, and is subject to translational runoff in vitro. (Clark et al., 2000) Moreover, it has been reported that lin-14 and lin-28 mRNAs, whose translation is

blocked by miRNA lin-4, are nonetheless found in the polyribosomal fraction. (Krichevsky and Kosik, 2001; Olsen and Ambros, 1999; Seggerson et al., 2002) Our puromycin results suggest that translation is occurring on FMRP-bound mRNAs; however, taken together, all of our data is consistent with a slow elongation rate. By modulating the efficiency of translation of mRNAs that are fully engaged in polyribosomal complexes, FMRP might be suitable to mediate particularly rapid and reversible regulatory events, consistent with its proposed role in the regulation of protein synthesis-dependent synaptic plasticity. (Huber et al., 2002)

Acknowledgements

This work would not have been possible without the insight and expertise of Giovanni Stefani. As a master of all things biochemical, he developed our method for polyribosome fractionation from mouse brain and spinal cord. In addition, he performed the critical experiments using deoxycholate and puromycin to disrupt the interaction of FMRP with polyribosomes.

CHAPTER IV: MICROARRAY ANALYSIS OF POLYRIBOMAL MESSENGER RNAS

Introduction

Identification of affected mRNA targets is critical to an investigation of the role of an RNA-binding protein in translational regulation. While target mRNAs have been identified by co-immunoprecipitation and/or bioinformatics screen for a known binding sequence (Brown et al., 2001; Darnell et al., 2001), one can also look at the general contribution of an RNA-binding protein on the translational status of expressed mRNAs. As for the transcriptional state of a tissue, microarrays can be used to examine whether the absence of an RNA-binding protein changes the mRNAs engaged by multiple ribosomes. This scheme for high-throughput expression screening based on mRNA translation states was first employed by Zong et al to compare translational profiles of resting and mitogenically-activated fibroblasts (Zong et al., 1999). Briefly, mRNAs isolated from polyribosome fractions corresponding either to association with less than two or to association with two or more ribosomes were used to interrogate cDNA arrays. Polyribosomal profile patterns of mRNAs were compared between resting and activated cells. A number of groups have used similar methods to assay the mRNA content of polyribosomes purified from conditions including poliovirus-infected HeLa cells (Johannes et al., 1999), human Fragile X lymphoblastoid cell lines (Brown et al., 2001), rapamycin-treated T cells (Grolleau et al., 2002), rapidly growing and rapamycin-treated *Saccharomyces cerevisiae* (Arava et al., 2003; Preiss et al., 2003), renal carcinoma cells expressing von Hippel-Lindau tumor suppressor protein (Galban et al., 2003), mouse glial progenitor cells with activation of the Ras and/or Akt signaling pathways (Holland

et al., 2004; Rajasekhar and Holland, 2004), cultured embryonic rat cortical neurons treated with BDNF +/- rapamycin (Schratt et al., 2004), *Arabidopsis thaliana* leaves under normal conditions and mild dehydration stress (Kawaguchi and Bailey-Serres, 2005), and prepuberal and adult mouse testis extracts (Iguchi et al., 2006). We have combined our method for polyribosome preparation with knockout animals for two RNA-binding proteins, Nova-1 and FMRP.

The identification of mRNA targets of FMRP is a goal of many laboratories in the field. FMRP has been shown to negatively regulate the translation of several mRNAs, both in an *in vitro* rabbit reticulocyte lysate system and *in vivo* in cell culture (Laggerbauer et al., 2001; Li et al., 2001b; Mazroui et al., 2002; Schaeffer et al., 2001). However, in our hands, these data seem to result from a global inhibitory effect of FMRP on translation, rather than specific inhibition of a target mRNA (JC Darnell, unpublished data.) FMRP has been shown to bind its own mRNA through a G quartet motif (Ashley et al., 1993; Brown et al., 1998; Ceman et al., 1999; Schaeffer et al., 2001). Other target mRNAs for which there is some evidence of translational regulation by FMRP include elongation factor 1A (EF-1A) (Sung et al., 2003), MAP1B (Brown et al., 2001; Darnell et al., 2001; Zalfa et al., 2003), and PSD-95 (Todd et al., 2003). Many possible targets of FMRP have been identified through a number of screening methods. Using co-immunoprecipitation of FMRP-RNA complexes from mouse brain followed by oligonucleotide microarray screening, 432 mRNAs were identified with the ability to preferentially co-IP with FMRP (Brown et al., 2001). 251 mRNAs differing in the polyribosomal fractions of human normal and fragile X lymphoblastoid cell lines were also identified by microarrays. Of these, 136 were increased in fragile X polyribosomes

and 115 were decreased. 14 of the co-IP-identified mRNAs were present in the polyribosome-identified group. A G quartet sequence was identified in 8 of these mRNAs. MAP1B was present in both groups, and was shown to be increased in polyribosomes from fragile X cells. However, this study may have been biased toward identification of RGG box-binding mRNAs, since the co-immunoprecipitation buffer contained 30mM EDTA. It is possible that mRNA targets bound in a magnesium dependent manner by the KH2 domain of FMRP have not been identified by this study. In addition, mRNA targets differentially associated with polyribosomes in the presence or absence of FMRP were identified from lymphoblastoid cells, not brain. The lymphoblastoid cell lines used for these experiments were derived from 5 human subjects in each group, introducing more biological variation than is present in inbred mice. Finally, there is evidence that RNA-protein complexes can be falsely co-immunoprecipitated when the two components have been expressed in separate cell lines, then lysed and pooled, suggesting that there may be a significant number of false positives in the co-IP data (Mili and Steitz, 2004). Thus, screens for differentially polyribosome-associated mRNAs in the brain of the *Fmr1* KO mouse may reveal further information.

Antibody Positioned RNA Amplification (ARPA) has identified another set of possible FMRP targets from primary rat hippocampal neuronal cultures (Miyashiro et al., 2003). Of the 83 cDNA clones identified by this method, 25% contain G quartets, although it is not stated what percentage of all neuronal mRNAs contain G quartets using the sequence definition particular to this paper. *In vitro* RNA selection from transcribed sequences in brain (cDNA-SELEX) was used to identify 57 cDNAs, of which 2 contain

G quartets (Chen et al., 2003). MAP1B was identified by this method and shown to have 23% decreased expression in *Fmr1* KO cerebellum. Finally, total RNA from pooled whole brains from adult wild type and *Fmr1* KO mice were used to probe a cDNA microarray (D'Agata et al., 2002). 73 genes were identified with greater than 2-fold differences between WT and KO signal. However, real-time PCR validation did not support the magnitude of the differences observed on the array. Others have indicated that there are few deviations from normal steady-state expression levels for most genes in fragile X patient cells or *Fmr1* knockout mouse brain (Jin and Warren, 2003).

While the role of FMRP in translational regulation is being extensively studied, that for the Nova proteins has not yet been considered. Although Nova-1 and Nova-2 are present in the cytoplasm of neurons, and do bind some mRNA targets in exonic regions (Dredge et al., 2005; Ule et al., 2003; Ule et al., 2005b), no translational targets of either Nova protein have yet been validated. Like the related RNA-binding proteins hnRNP K and hnRNP E1/E2 (Ostareck-Lederer and Ostareck, 2004), the Nova proteins may have a significant role in translational regulation. It is generally thought that mRNAs associated with polyribosomes are being actively translated. Our lab has preliminary data to suggest that 11.8-22% of Nova may be associated with polyribosomes in the mouse CNS at approximately one month of age (Racca et al., submitted). Thus, it is an open question whether Nova inhibits translation of mRNAs in Nova-associated mRNPs, or whether Nova may promote or inhibit translation by binding directly to polyribosomes. If Nova acts in a similar manner to that which has been suggested for hnRNP K (Ostareck-Lederer and Ostareck, 2004), we expect to see an increase in association with polyribosomes of target mRNAs in Nova-1 null mouse spinal cord. Or, we may see a

more complex set of data, with some mRNAs increased and others decreased in Nova-1 knockout polyribosomal RNA, as has been seen for the lymphoblastoid cell lines from patients with Fragile X mental retardation syndrome (Brown et al., 2001). Thus both Nova and FMRP are candidate model systems to assay changes in translational status for mRNAs.

Results

Nova-1 wild type versus knockout mouse spinal cord

Nova RNA-binding proteins, Nova-1 and Nova-2, are target antigens of paraneoplastic opsoclonus-myoclonus ataxia (POMA), a neurodegenerative disorder of autoimmune etiology (reviewed in Darnell, 1996). Nova-1 is expressed in the ventral spinal cord, brainstem, deep nuclei of the cerebellum, and midbrain (Buckanovich et al., 1993). Nova proteins contain three hnRNP K-homology (KH) domains with RNA-binding activity. Although the Nova proteins are predominantly nuclear, they are present in significant quantities in the cytoplasm and Nova-1 has been co-localized with GlyR α 2 mRNA in post-synaptic regions of the dendrite (Racca et al., submitted). While there is evidence that Nova proteins act to influence alternative splicing in neurons (Jensen et al., 2000a; Ule et al., 2003; Ule et al., 2005b), they may also have a role in the local regulation of protein synthesis in neurons. Both Nova-1 and Nova-2 are associated with polyribosomes in mouse brain and spinal cord in a magnesium-dependent manner (See Figures 3.1-3). For this reason we have used gradient density centrifugation, fractionation and microarray (Affymetrix MGU74v2ABC GeneChips) hybridization to identify mRNAs that have an altered distribution on polyribosomes in Nova-1 knockout

spinal cord from four day-old mice. To control for transcriptional differences between Nova-1 knockout and wild type spinal cord we have also assayed total RNA from the same samples. Three biological replicates of four pooled spinal cords each were used for each genotype. A representative polyribosome profile and Western blots are shown in Figure 4.1. Signal values for each probeset were calculated using Microarray Suite 5.0 (Affymetrix). Further data analysis was accomplished using Genespring 5.0 (Silicon Genetics). To analyze the differences between total mRNA and polyribosomal RNA populations, these signals were compared irrespective of Nova-1 genotype. The probesets that exhibited a change between total RNA and polyribosomal RNA ($p < 0.001$) are shown in Figure 4.2. Of note, these probesets include mRNAs for 21 ribosomal proteins (Table 4.1) containing oligopyrimidine tracts (TOP) in their 5'UTR regions and known to be translationally repressed in post-mitotic cells (Meyuhas, 2000). mRNAs showing an increased representation in polyribosomes include GAPDH and actin.

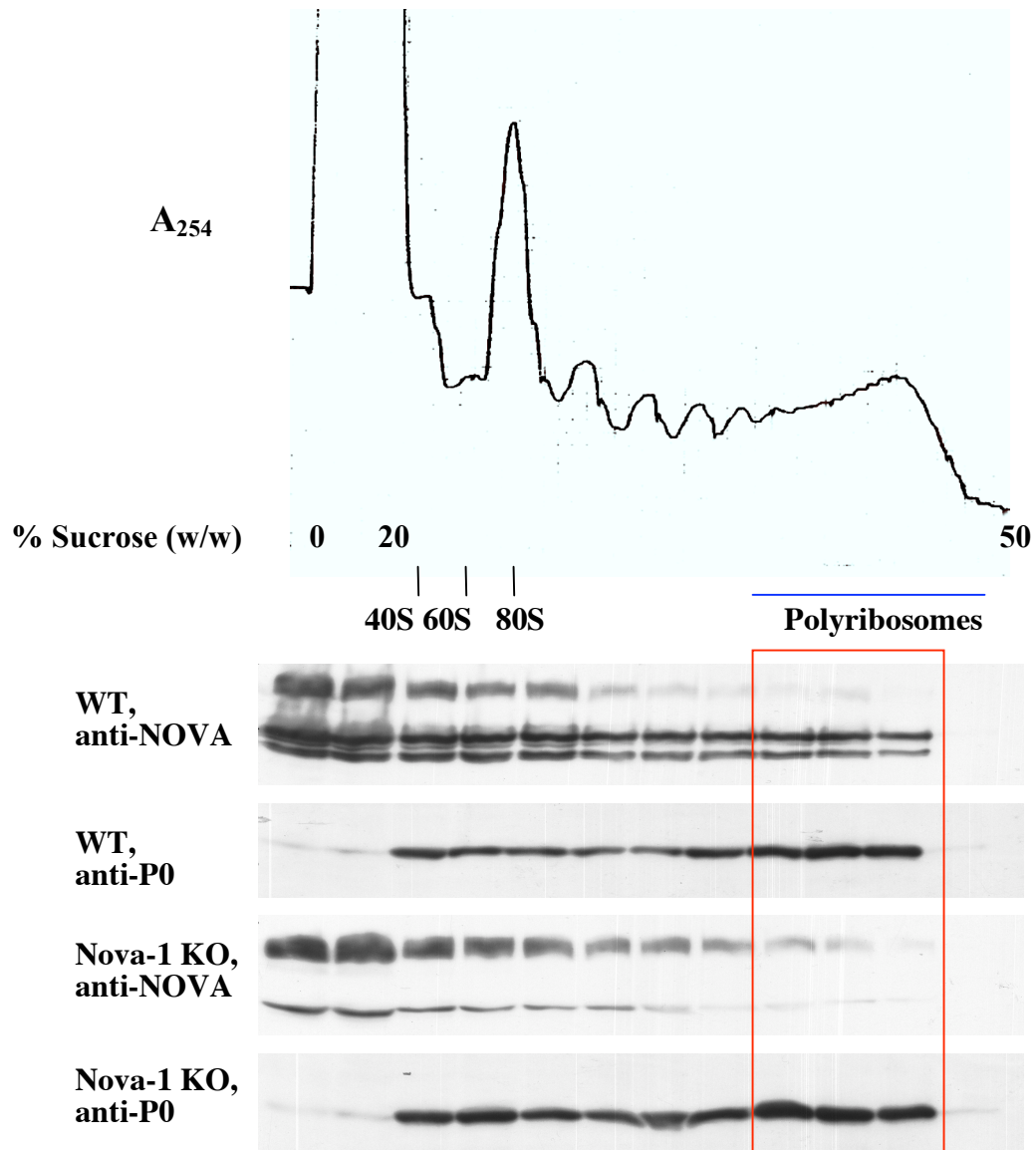


Figure 4.1 Polyribosome Fractionation from Nova-1 wild type and knockout mouse spinal cord. Four spinal cords from postnatal day 4 mice were used for each 20-50% sucrose gradient centrifugation. A representative OD254 profile is shown in the top panel. Positions of the ribosomal subunits and polyribosomes are indicated. Gradients were fractionated and aliquots assayed for protein by Western blot. Polyribosomal distributions of Nova-1 and Nova-2 proteins (anti-Nova) and ribosomal protein P0 (anti-P0) are shown for wild type (WT) and Nova-1 knockout (KO) spinal cords. Fractions 9-11 (red box) from each gradient were pooled to isolate polyribosomal RNA.

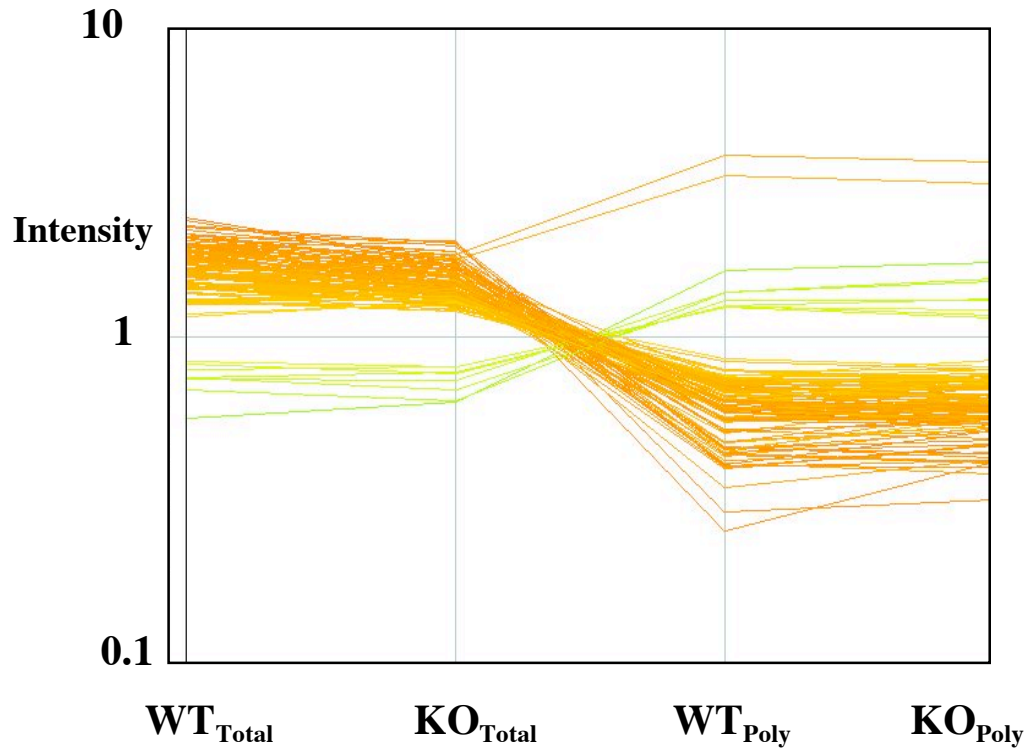


Figure 4.2 mRNAs that differ between Total and Polyribosomal RNA, $p \leq 0.001$. Normalized intensities (log scale) of probeset signals were compared by t-test between total RNA and polyribosomal RNA, irrespective of genotype. Probeset signals are displayed for all four conditions on a discontinuous graph.

mRNA	p-value
ribosomal protein S12	3.48E-05
ribosomal protein L13*	4.97E-05
ribosomal protein S26	4.97E-05
ribosomal protein L41	1.01E-04
acidic ribosomal phosphoprotein PO*	1.22E-04
ribosomal protein, large, P1	1.22E-04
ribosomal protein L27a (x2)	1.31E-04
ribosomal protein S28 (x2)	1.55E-04
ribosomal protein S8*	2.24E-04
ribosomal protein L28	2.57E-04
mitochondrial ribosomal protein L52	2.71E-04
ribosomal protein P23*	3.60E-04
ribosomal protein S2	3.60E-04
ribosomal protein L23a,	3.60E-04
ribosomal protein P40*	3.79E-04
ribosomal protein L30*	4.24E-04
ribosomal protein L27A	5.17E-04
ribosomal protein L21	5.55E-04
ribosomal protein L31	5.69E-04
ribosomal protein S17*	6.34E-04
ribosomal protein S11*	8.11E-04

Table 4.1 Genes encoding ribosomal proteins are translationally repressed.

*Indicates a TOP mRNA; mRNAs interrogated by multiple Affymetrix probesets are indicated in parentheses

Figure 4.3 shows a graphical representation of RNAs that showed a difference between wild type and Nova-1 knockout polyribosomes with $p < 0.05$. Signals for each gene on the polyribosomal chips were normalized to the corresponding signal from total RNA by genotype and sample, thus all signals equal one for both wild type and Nova-1 knockout total RNA data points. Table 4.2 lists the mRNAs that exhibit a greater than twofold difference between wild type and Nova-1 knockout polyribosomal RNA. We were unable to confirm these data by real-time quantitative PCR and Western blotting for changes in steady state protein levels between Nova-1 wild type and knockout spinal cords (data not shown.) Of note, Nova-2 is also expressed in mouse spinal cord, albeit in dorsal rather than ventral areas. Since Nova-1 and Nova-2 have similar RNA binding specificities, slight changes in the polyribosomal distributions of a Nova target mRNA could be further obscured by the presence of Nova-2 in Nova-1 knockout spinal cord.

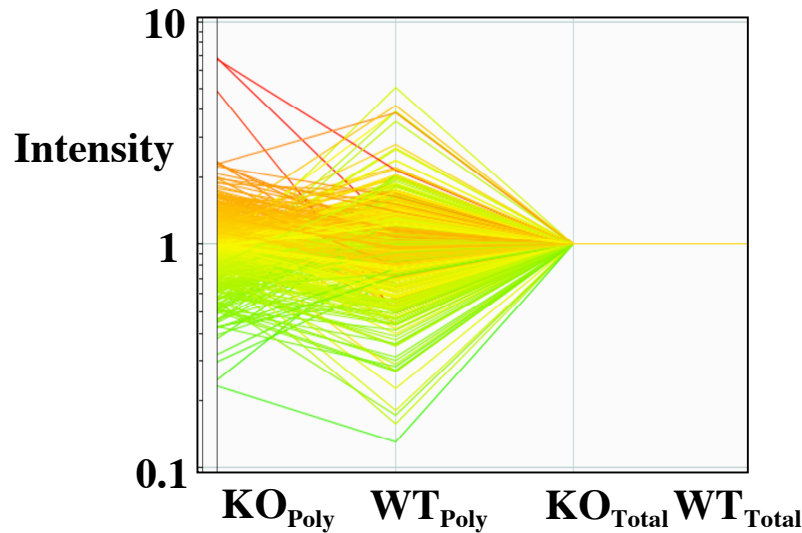


Figure 4.3 Comparison of mRNAs sedimenting with wild type versus Nova-1 knockout polyribosomes. Probeset signals are normalized to Total RNA signal for each gene by genotype, thus all probesets have a normalized intensity of 1 in KO Total and WT Total conditions. KO Polyribosome compared to WT Polyribosomes by t-test, $p \leq 0.05$.

Increased in KO Polysomes

	Fold
selenium binding protein 2	9.92
polynucleotide kinase 3'-phosphatase (PNKP)	5.87
VACM (vasopressin activated calcium mobilizing)	4.73
epithelial membrane protein 3	4.51
homeodomain interacting protein kinase 1	3.91
RIKEN cDNA 2300004H16 gene	3.89
similar to PDZ protein interacting with TC10	3.44
18S rRNA	3.19
Filamin	3.12
ESTs	2.95
ESTs	2.92
putative membrane protein	2.76
chloride channel regulator lcln (pseudogene)	2.64
EST, Weakly similar to RIKEN cDNA 2510008H07 [Mus musculus]	2.43
similar to testis expressed sequence 27, clone IMAGE:4950648, mRNA	2.42
BALC (brain and acute leukemia, cytoplasmic)	2.40
RIKEN cDNA 4933426K21 gene	2.40
RIKEN cDNA 2510005D08 gene	2.36
Cluster Incl U62674; histone H2a.2-615, and histone H3.2-615 genes	2.35
neural stem cell derived neuronal survival protein	2.30
RIKEN cDNA C330026P08 gene	2.29
clone IMAGE:4507459, mRNA, partial cds	2.27
ESTs	2.14
vw95f07.r1 Stratagene mouse skin cDNA clone IMAGE:1262725 5', mRNA	2.09
exostoses-like 1	2.08
RIKEN cDNA 5830417I10 gene	2.07
EST, Weakly similar to NED4_MOUSE NEDD-4 protein [M.musculus]	2.07
ESTs, Moderately similar to COTE_HUMAN COTE1 PROTEIN [H.sapiens]	2.02

- Functions in synaptic densities
- Metalloproteinases
- Transcription factors

Decreased in KO Polysomes

	Fold
ESTs	6.13
EST (transcription factor?)	5.29
Mouse CNK (connector enhancer of ksr)	4.79
RIKEN cDNA 4933439F18 gene	4.3
Damage-induced neuronal endopeptidase (DINE)	4.16
RIKEN cDNA 5830415F09 gene	4.01
cell division cycle 45 homolog-like	3.92
Rho GTPase activating protein 6	3.53
ESTs	3.42
RIKEN cDNA 1110038G02 gene	3.38
ESTs sim to JC5629 mullerian-inhibiting substance type II receptor	3.35
Janus kinase 3	3.19
endothelin converting enzyme 2	3.12
RIKEN cDNA 1810031K17 gene	3.07
expressed sequence AI448652	2.95
ESTs	2.63
Ngfi-A binding protein 1 (NAB1)	2.63
Helicase-like transcription factor	2.56
ADAMTS15	2.55
ras homolog gene family, member J	2.5
ESTs	2.49
ESTs	2.44
melastatin 1	2.43
ESTs	2.43
Fas-activated serine/threonine kinase	2.41
acid phosphatase 2, lysosomal	2.4
RIKEN cDNA 2010002L15 gene	2.36
expressed sequence AI315192	2.32
RIKEN cDNA 1110012J22 gene	2.3
ESTs	2.28
RIKEN cDNA 1110021E09 gene	2.26
neuronal PAS domain protein 1 (nPAS 1)	2.19
RIKEN cDNA 2410018G23 gene	2.19
phospholipase A2, group IID	2.19
ESTs	2.19
RIKEN cDNA 5830401B18 gene	2.18
similar to cleavage stimulation factor subunit 3	1.15
Structural protein of Rauscher oncovirus p10	2.05
DAP-1	2.04

Table 4.2. Nova1-dependent changes in polyribosomal mRNAs. mRNA targets increased or decreased in Nova-1 KO polyribosomes relative to wild type polyribosomes and normalized to total RNA signal for each gene and genotype. Genes are boxed by color according to function.

More recently, our lab has identified and validated an extensive network of Nova-1 and Nova-2 RNA targets through two novel methods, cross-linked immunoprecipitation (CLIP, (Ule et al., 2005a)) and a custom Affymetrix splicing microarray (Ule et al., 2005b). For 1143 validated Nova targets, 1480 unique Affymetrix probesets were identified on the MGU74v2ABC GeneChips. While this list of validated Nova target RNAs does not overlap with those in Table 4.2, using this validated target list, the complements of Nova-1 wild type and knockout polyribosomal mRNAs were compared anew. Figure 4.4 illustrates the mean signal values each gene in Nova-1 knockout and wild type polyribosomes for each of the three GeneChips (A, B, and C) for the MGU74v2 mouse genome set. For each chip type, gene signals from polyribosomal and total RNA samples are shown for wild type and Nova-1 knockout (left panels), and after normalization to the corresponding signal from total RNA by genotype (right panel). These gene expression patterns are clustered by similarity. It is interesting to note that while the majority of these RNAs show a great change between polyribosomal and total RNA distributions, none show a wild type vs. Nova-1 knockout fold-change of 2.0 or greater using either normalization scheme (Figure 4.4). Validation of possible translational targets of Nova-1 identified in Table 4.2 bears further investigation with methods designed to maximize the signal: noise ratio.

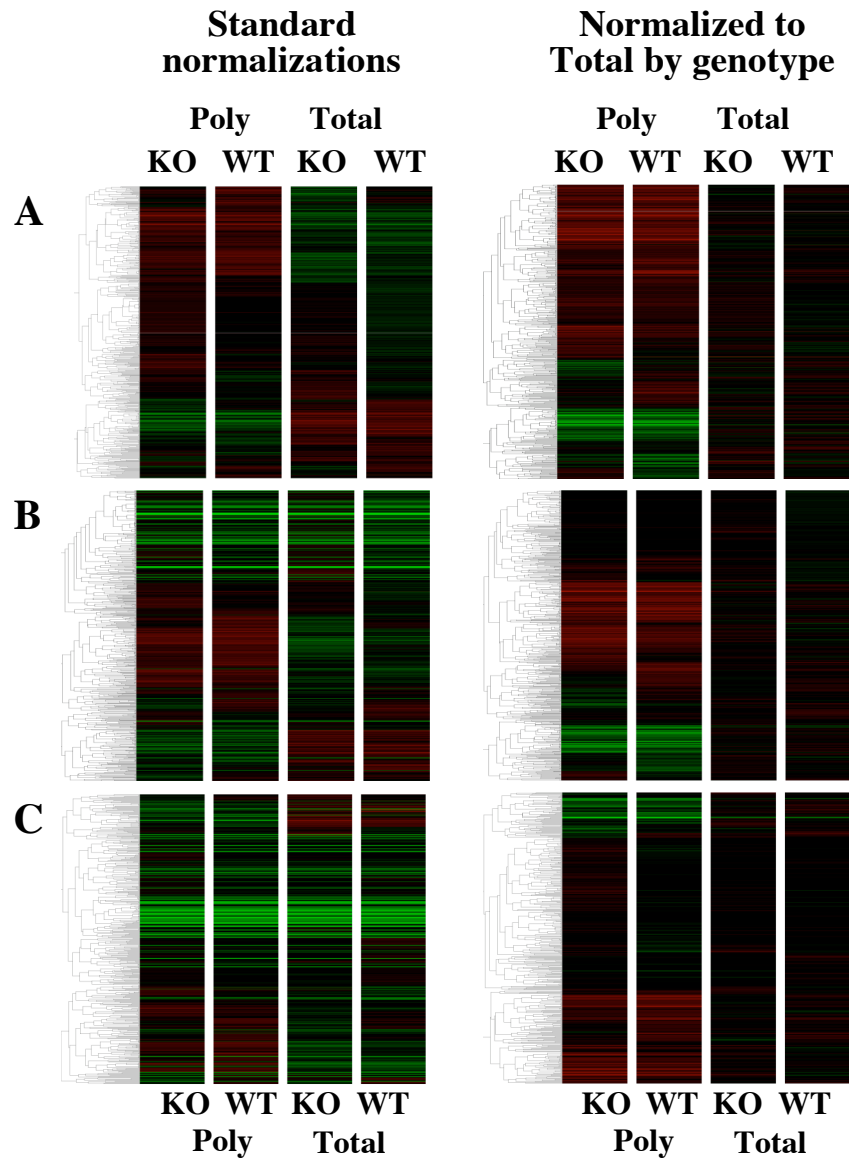


Figure 4.4. Polyribosomal profiles of Nova CLIP and splicing target mRNAs. Validated RNA targets identified by CLIP or splicing microarray methods (Ule et al., 2003; Ule et al., 2005b) were interrogated by MGU74v2 A, B, and C GeneChips (A, B, and C panels, respectively.) Left panels show dendrograms of probeset signals normalized by the standard GCRMA algorithm. Right panels show the same polyribosomal RNA probesets normalized to total RNA signal by genotype after GCRMA processing.

Fmr1 wild type versus knockout cerebral cortex

The identification of mRNA targets of FMRP is a goal of many laboratories in the field. Several laboratories have identified putative G-quartet-containing mRNA targets of FMRP. Because additional KH2-binding mRNA targets may not have been identified by these studies, we have performed a microarray screen to identify mRNAs that are differentially associated with polyribosomes in the cerebral cortex of P7 *Fmr1* null mice (KO) compared to their wild type littermates (WT). To control for sex differences, only male mice were used. Briefly, cytoplasmic lysates from cerebral cortices (two per gradient) were prepared in buffer containing 1% NP40 and 5mM MgCl₂. 20% of total lysate was taken for total RNA. The remaining sample was fractionated by gradient centrifugation on a 20-50% sucrose gradient. Fractions were collected and 15% of each fraction analyzed by Western blot. RNA was extracted from the remaining 85% of each fraction. RNA from fractions 10-13, corresponding to association with 6 or more ribosomes, was pooled as polyribosomal RNA. Both total and polyribosomal RNAs were labeled by standard methods and used to interrogate Affymetrix Mouse 430 2.0 GeneChips. Six biological replicates from each genotype were prepared (12 mice from each genotype, 24 microarrays encompassing four groups with six replicates each). A representative profile and Western blots are shown in Figure 4.5. Data was analyzed using the Robust Multi-Array Average (GCRMA) method (Irizarry et al., 2003), GeneTraffic (Iobion/Stratagene), and GeneSpring 7.3 (Agilent). Comparisons between WT and *Fmr1* KO polyribosomal mRNA levels directly (Figure 4.6A), or after normalization of each probeset to its mean signal in Total RNA by genotype (Figure 4.6B), show that few probesets lie outside the two-fold difference cutoff (black lines).

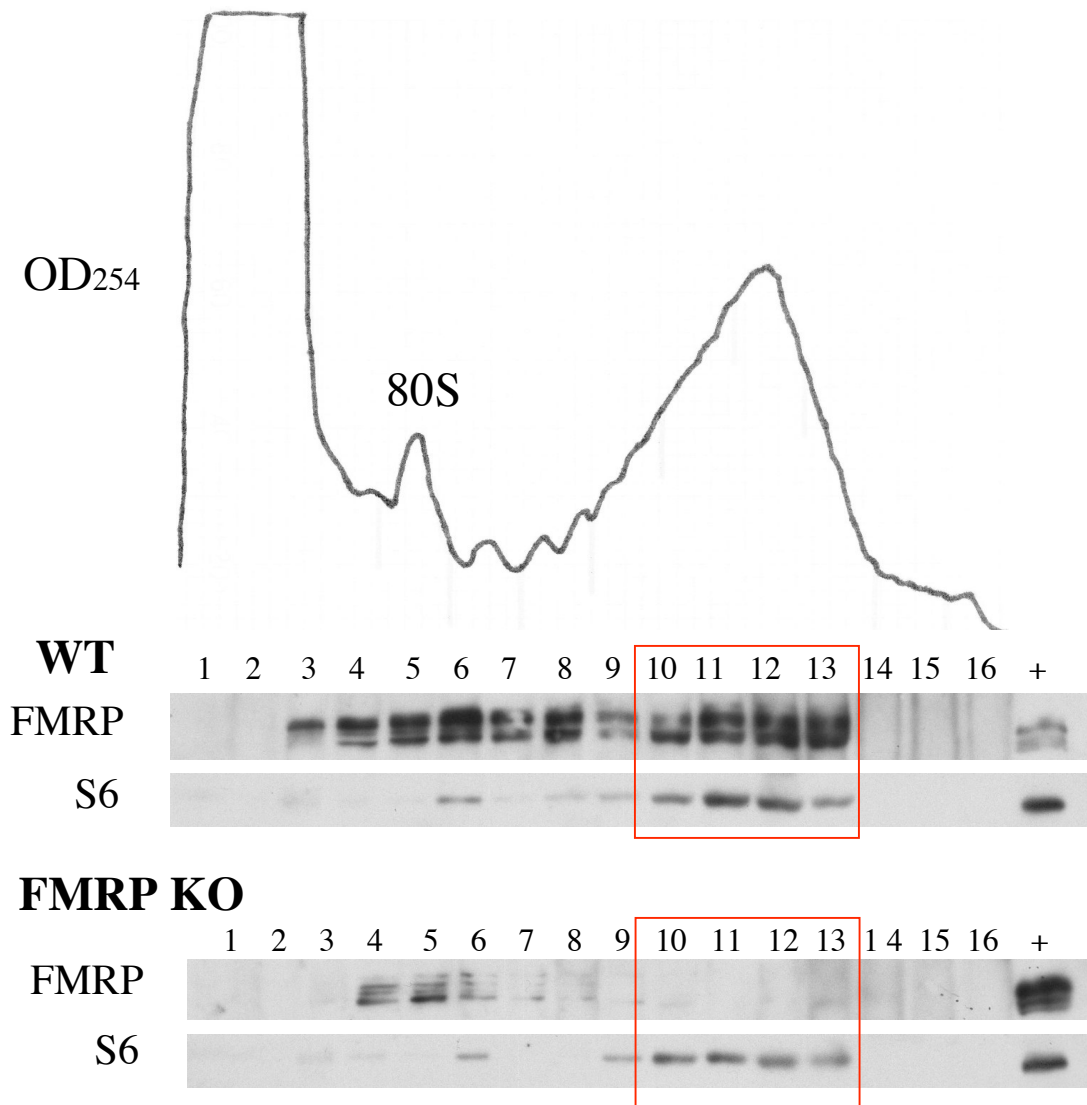


Figure 4.5 Polyribosomal fractionation of wild type and FMRP KO mouse brain. Two cerebral cortices from postnatal day 7 mice were used for each 20-50% (w/w) gradient. Fraction 1 corresponds to the 0% sucrose brain lysate loaded on top of the 20-50% gradient. Fraction 16 is the heaviest 0.75ml fraction from each 12ml gradient. A representative RNA absorbance profile is shown at top. Western blots are shown for anti-FMRP (1C3, Chemicon) and ribosomal protein S6. Fractions 10-13 (boxed) were pooled from each gradient for extraction of polyribosomal RNA. Signal for FMRP in the FMRP KO sample is due to 1C3 cross-reaction with FXR1. (+: positive control for FMRP and S6 proteins)

Notably, *Fmr1* is the only gene with a signal difference of greater than 2-fold (3 probesets show 6.4-fold, 4.1-fold, and 1.7-fold increases in WT polyribosomal RNA.) Genes with signal differences greater than 1.27 are listed in Figure 4.6C and Table 4.3 (of these, the highest fold change is a 1.42-fold decrease). Of these 39 genes, only three are increased in KO polyribosomes, while 36 are decreased. In addition, three genes, gelsolin, proteolipid protein (Plp), and tuberlin-like protein 1 were exhibited similar changes in two probesets on the chips. Of note, FMRP has previously been shown to bind Plp mRNA (Wang et al., 2004). A comparison of total RNA from WT and KO animals (Figure 4.7) reveals decreases of 4.9-fold, 3.25-fold, and 1.45-fold for the three *Fmr1* probesets in KO total RNA. Again *Fmr1* is the only gene with a fold change above 2. No genes show an increase greater than 1.27-fold in KO total RNA. 38 genes exhibit signal decreases between 1.27-fold and 1.8-fold in KO total RNA, and are listed in Table 4.4. Of these, receptor (calcitonin) activity modifying protein 2 (Ramp2) exhibits a greater than 1.27 fold decrease in 3 probesets, and calbindin 1 and cartilage link protein 1 show decreases in 2 probesets each. Calbindin 1 and RGS13 have been previously identified as FMRP targets by APRA (Miyashiro et al., 2003).

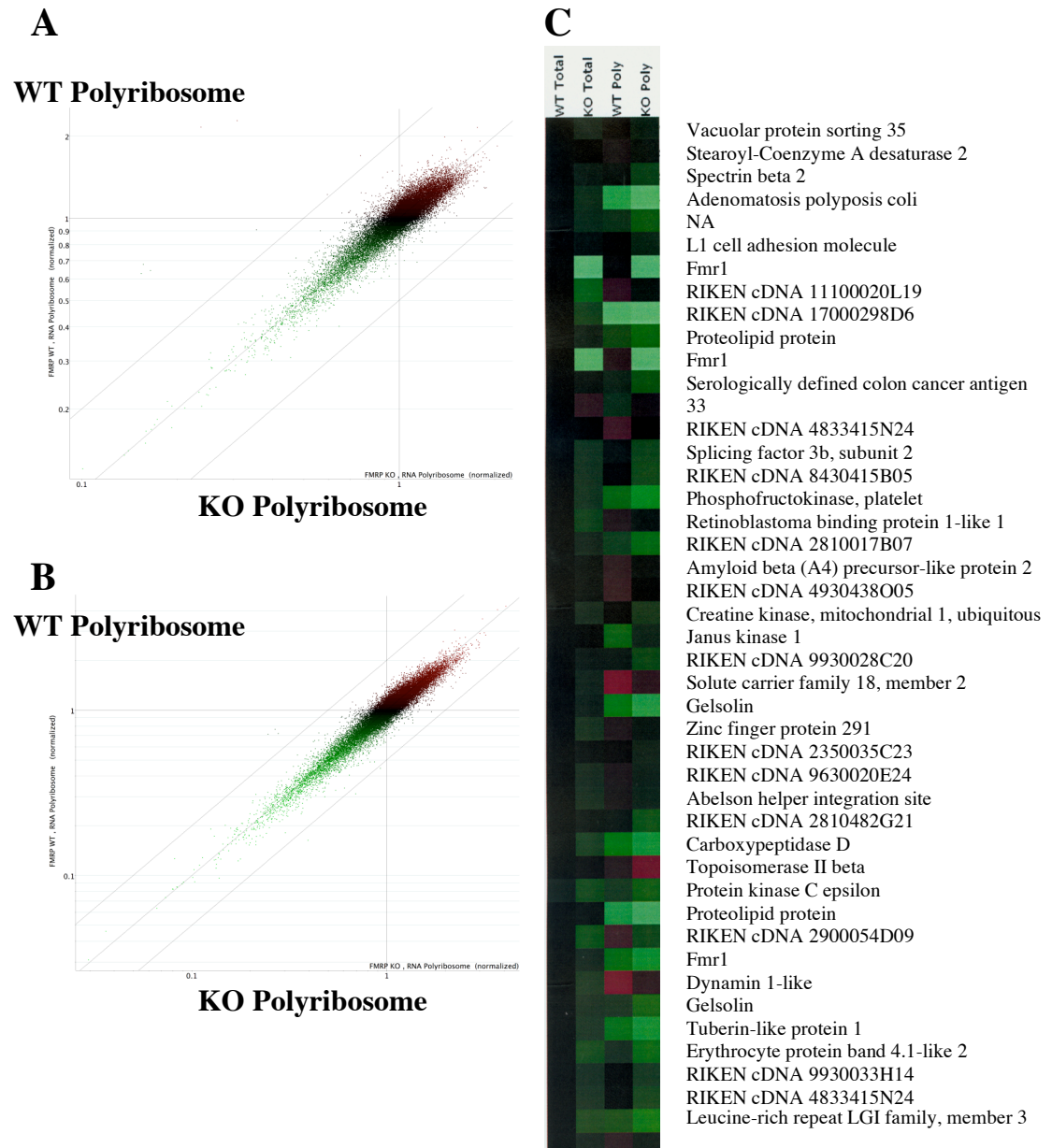


Figure 4.6 Wild type versus FMRP knockout polyribosomal mRNAs. (A) Mean signals from wild type polyribosome chips are compared to signals from FMRP knockout polyribosome chips for all probesets interrogated (MG430 2.0 GeneChip). Black lines indicate a two-fold change. (B) Comparison as in (A), except mean polyribosomal signals have been normalized to total mRNA levels by genotype for each probeset. (C) Probesets exhibiting a fold-change greater than 1.27 between wild type and knockout polyribosomes. Mean signals shown as a comparison to a baseline WT Total signal for each probeset.

mRNAs changed in <i>Fmr1</i> knockout polyribosomal RNA	
Increased:	Decreased:
Protein Kinase C epsilon	Gelsolin
2 RIKEN cDNAs	Proteolipid protein (myelin)
	amyloid beta (A4) precursor-like protein 2
	Janus kinase 1
	Stearoyl-CoA desaturase 2
	Spectrin beta 2
	L1 cell adhesion molecule
	Splicing factor 3b, subunit 2
	Serologically defined colon cancer Ag 33
	Adenomatosis polyposis coli
	Vacuolar protein sorting 35
	Carboxypeptidase D
	Topoisomerase (DNA) II beta
	Phosphofructokinase, platelet
	Erythrocyte protein band 4.1-like 2
	Retinoblastoma binding protein 1-like 1
	Abelson helper integration site
	Creatine kinase, mitochondrial 1
	Solute carrier family 18
	Leucine-rich repeat LGI family 3
	Zinc finger protein 291
	Tuberin-like protein 1
	Dynamin 1-like
	12 RIKEN cDNAs

Table 4.3 Probesets with fold-change ≥ 1.27 between wild type and FMRP knockout polyribosomes.

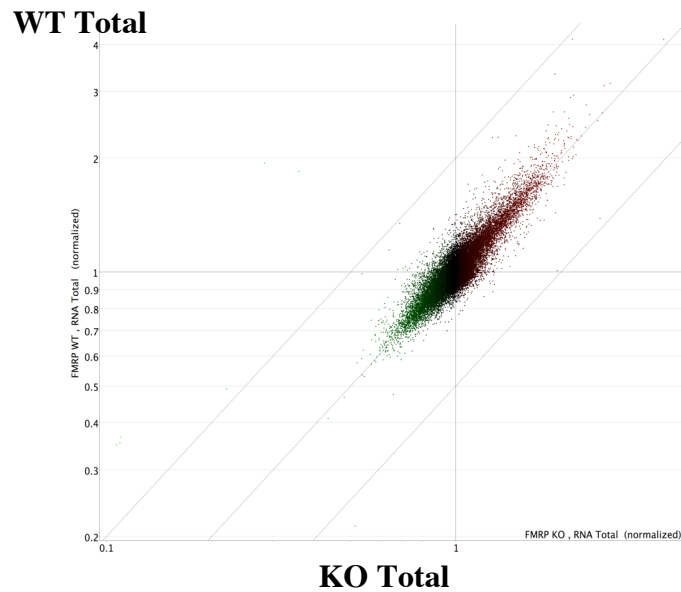


Figure 4.7 Wild type versus knockout total RNAs. Mean signals from wild type and FMRP knockout total RNA chips are compared for all probesets. Black lines indicate a two-fold change.

mRNAs changed in *Fmr1* knockout total RNA

Cartilage link protein 1
Rho GTPase activating protein 15
Rho GTPase activating protein 5
Regulator of G-protein signaling 13
Purinergic receptor P2Y, G-protein coupled 12
Discs, large homolog 2 (*Drosophila*)
Plexin C1
Chloride channel calcium activated 2
Calbindin 1
Receptor (calcitonin) activity modifying protein 2
Topoisomerase (DNA) II beta
Hemoglobin, beta
Hemoglobin, alpha
Alpha-thalassemia/ mental retardation syndrome X-linked homolog (human)
X-linked lymphocyte-regulated 3a
Tuberin-like protein 1
ROD1 regulator of differentiation 1 (*S. pombe*)
Enigma homolog (*R. norvegicus*)
15 RIKEN cDNAs

Table 4.4 Probesets with fold-change ≥ 1.27 between wild type and FMRP knockout total RNAs.

To assess the quality of this microarray data, we can look at several positive controls incorporated into the experimental design. The significant decrease in *Fmr1* on all KO chips confirms the genotypes of the mice used and the ability of these arrays to detect a change of this magnitude. The quality of the polyribosome preparations can be assessed by comparing polyribosomal RNA to total RNA for wild type mouse cortex. This comparison identifies 18 probesets that increase greater than 2-fold in polyribosomal RNA, and 878 probesets that decrease greater than two-fold (Figure 4.8), none of which show significant changes in FMRP knockout mouse brain. Of the 878 probesets that decrease, at least 32 assay for ribosomal protein mRNAs and identify a minimum of 24 different ribosomal proteins. This is consistent with work showing that many mRNAs encoding ribosomal proteins also contain a 5' terminal oligopyrimidine tract (TOP

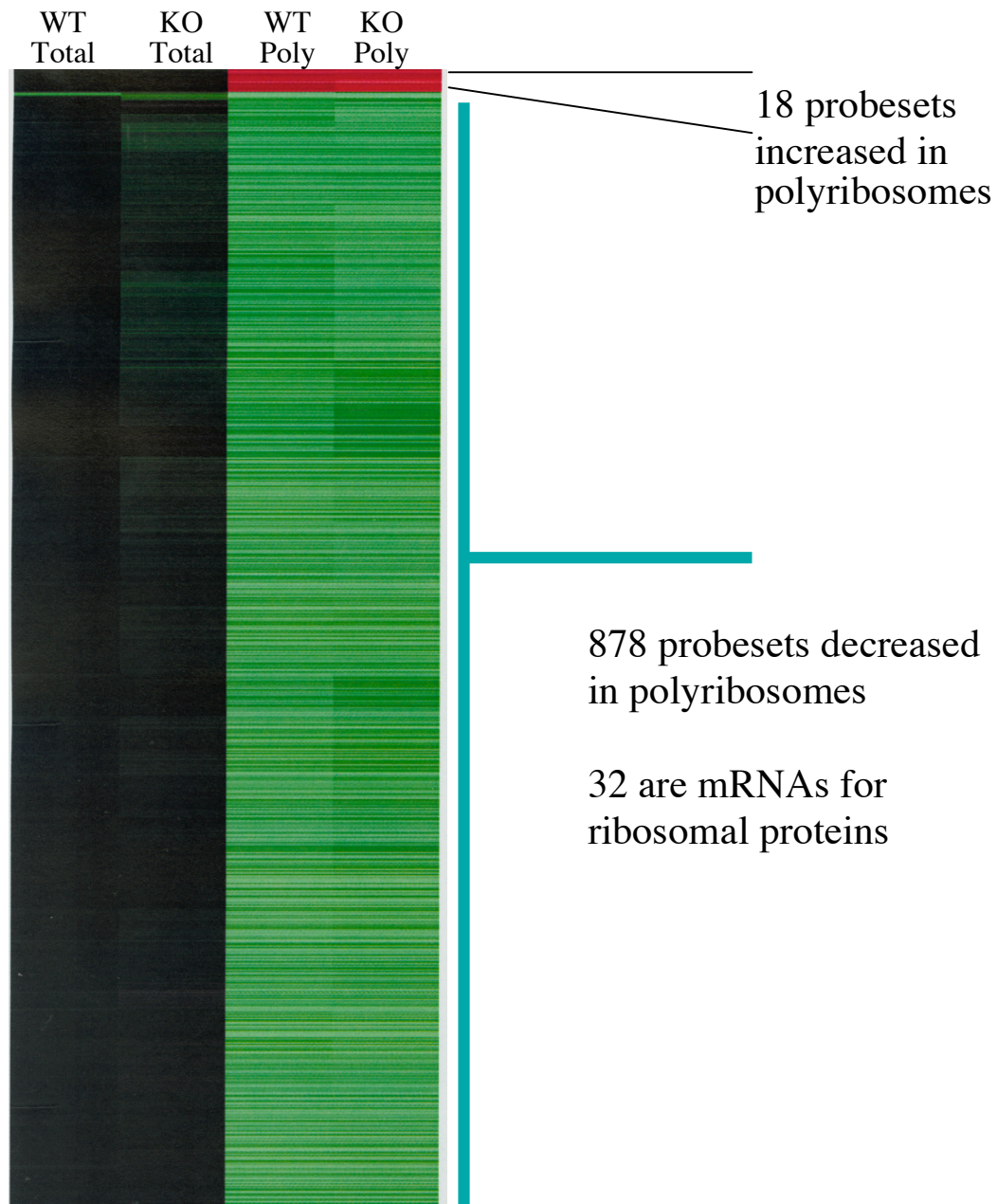


Figure 4.8 mRNAs that differ between wild type polyribosome and wild type total with fold change ≥ 2 . GCRMA-normalized intensities of probeset signals were compared by t-test between WT total RNA and WT polyribosomal RNA (knockout groups were not included for this comparison.) Probesets are grouped by signal pattern similarity and are displayed for all four conditions.

mRNAs), and that such mRNAs are translationally repressed in non-dividing cells (Meyuhas, 2000).

Recent work by Julie Zang has identified mRNAs that UV cross-link to FMRP *in vivo*. To look specifically at these FMRP target mRNAs, 1451 Affymetrix probesets were identified for 1254 FMRP CLIP tag sequences. A direct comparison of WT and KO polyribosomal signals for these mRNAs is shown in Figure 4.9A. None exhibit greater than two-fold differences between groups. Likewise, none exhibit greater than two-fold difference between WT and KO total samples (Figure 4.9B). FMRP target mRNAs are grouped by polyribosomal profile pattern between WT and KO mice in Figure 4.9C. To correct for transcriptional changes between WT and KO mice, these analyses were repeated after normalization of polyribosomal signals to total signals by genotype and probeset (Figures 4.9D,E). Our polyribosomal pattern profile data does not indicate that there is an obvious, reproducible change in polyribosomal association of target mRNAs in the absence of FMRP.

Given the lack of robust FMRP-dependent polyribosomal association of target mRNAs, together with the confirmation of expected results for *Fmr1* and TOP mRNAs, we can hypothesize that perhaps the presence of FMRP shifts a target mRNA in a subtle fashion between polyribosome fractions (for example, a shift from 15 ribosomes to 8 ribosomes). Thus it may be worthwhile to use real-time PCR to compare the distribution of the identified mRNAs (from the polyribosomal RNA comparison between WT and KO) over all individual fractions of the polyribosomal gradients of WT and KO cortices, thereby confirming, for example, a decrease in polyribosome association by a corresponding increase in mRNP representation for a given target. This method may

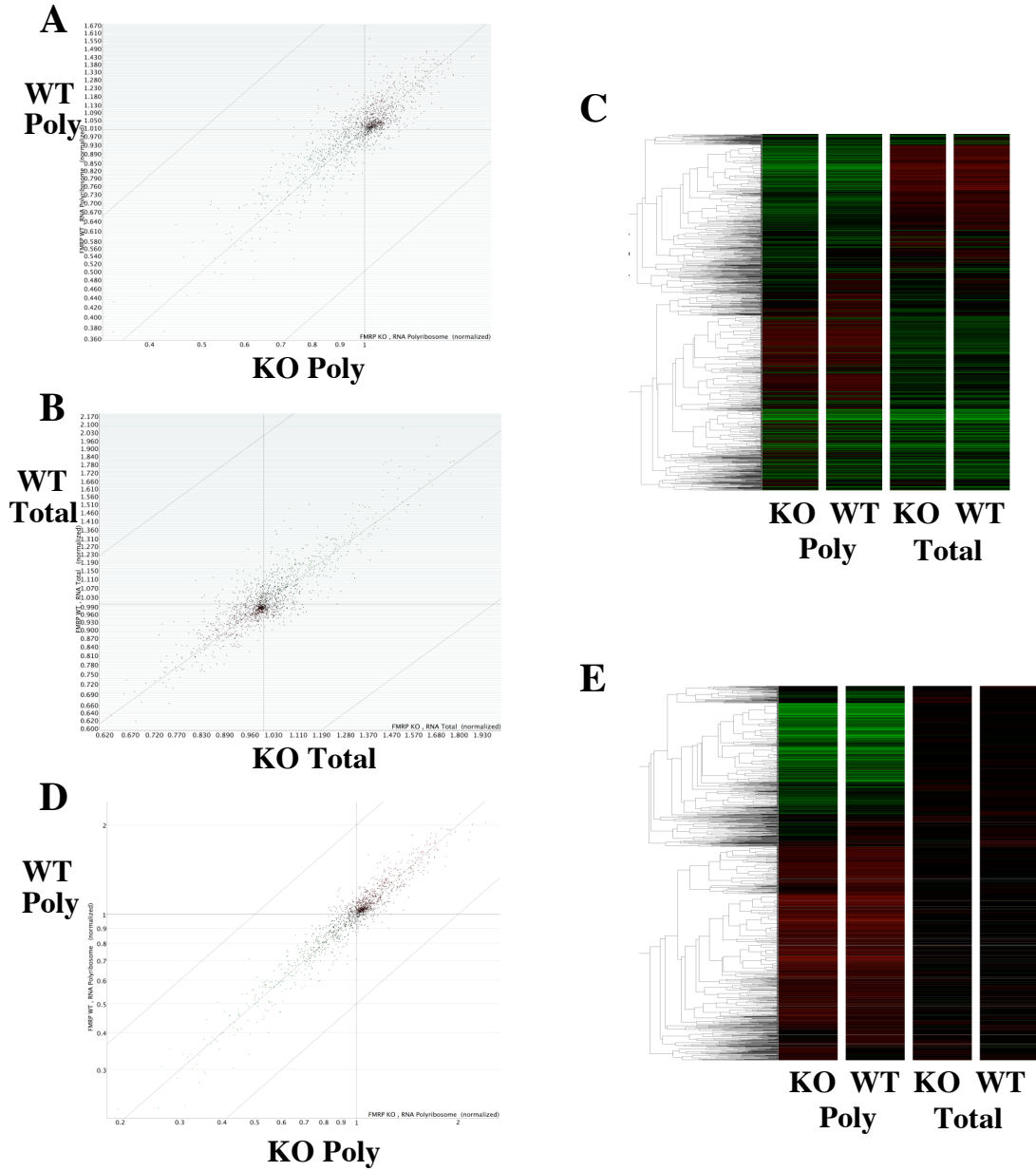


Figure 4.9 Polyribosomal distributions of FMRP CLIP target mRNAs. mRNAs that UV-crosslink with FMRP *in vivo* are interrogated by probesets on MG430 2.0 GeneChips. (A, B) Scatter plots of probeset signals compared between WT and FMRP KO polyribosomal (A), and total RNA (B), standard GCRMA normalization, black lines indicate two-fold change. (C) Dendrogram of probesets normalized by the standard GCRMA algorithm. (D) Scatter plot of probeset signals for WT vs. KO polyribosomes normalized to respective total signals. (E) Dendrogram of probesets: polyribosomal signals normalized to total RNA signal by genotype after GCRMA processing.

further elucidate the mechanism of FMRP, since data from previous studies is inconsistent as to whether FMRP increases or decreases translation of target mRNAs (Brown et al., 2001; Chen et al., 2003; Darnell et al., 2001). Confirmation of translational regulation at the protein level by Western blot remains a more definitive method of validation, but presents its own difficulties in achieving an adequate signal to noise ratio. Other possible issues in this work include the influence of neurotransmitter stimulation for FMRP action, and/or FMRP function at a small subset of polyribosomes in the cerebral cortex. Only a handful of mRNA targets have validated FMRP-dependent changes in protein level (Chen et al., 2003; Hou et al., 2006; Lu et al., 2004; Miyashiro et al., 2003; Wang et al., 2004; Zhang et al., 2001).

Since FMRP is consistently associated with the most rapidly sedimenting polyribosomes in neurons, these data present three possibilities for a role of FMRP in translational regulation. First, FMRP may be associated with population of RNAs that are particularly lengthy. This is not the case in this data set, since the mRNAs in Table 4.3 average 2570nt in length, with a distribution of 626 – 5995nt. Second, FMRP may be associated with a population of mRNAs that are translated at a slower rate. Finally, mRNAs associated with FMRP may be stalled in translation, with many ribosomes bound but little translation occurring.

Discussion

These data present a picture of the steady-state translational profiles of mRNAs in the central nervous systems of two mouse models of human disease. What is most remarkable is the very lack of robust changes in polyribosomal associations of mRNA

targets of these disease-related RNA-binding proteins. The expected number of false positive results exceeds the number of probesets with significant changes identified in each experiment. For example, of the 36,000 probesets included on the MGU74v2 GeneChips, 1,800 probesets are expected to show a false positive change between conditions at $p < 0.05$ (without multiple testing correction.) In the comparison between Nova-1 wild type and knockout polyribosomal RNA, using three biological replicates per condition, only 67 probesets show a fold change greater than 2 with $p < 0.05$ (Table 4.2). Furthermore, these differences are not reproducible by real-time PCR in our hands. However, expected results for TOP mRNAs are seen with high significance ($p < 0.001$, Figures 4.2, Table 4.1). This contrast between a comparison by genotype (wild type versus knockout) and by fractionation (total RNA versus polyribosomal RNA) is even more striking when six biological replicates are used for each condition. When FMRP wild type total RNA is compared to wild type polyribosomal RNA, 896 probesets are identified with a fold change greater than two. Yet, when wild type mouse cortex is compared to FMRP knockout cortex for either total or polyribosomal mRNA populations, the only probesets showing a difference greater than two-fold hybridize to the *Fmr1* mRNA itself. These data stand in stark contrast to the 426 mRNAs identified previously as increasing or decreasing greater than two-fold in fragile X lymphoblastoid cell lines (Brown et al., 2001). There are a number of reasons for this contrast. Our experiments used inbred mouse cerebral cortices with six biological replicates per condition, compared to the five independently transformed lymphoblastoid cell lines from non-genetically identical humans for each condition. Furthermore, we were able to use later generation microarray technology and analytic software, which may further decrease the

statistical impact of outlier data points. Taken together, these data indicate that the steady-state transcriptional and translational profiles do not exhibit significant changes in the absence of Nova-1 or FMRP.

Efforts to maximize the signal to noise ratio in polyribosomal profile pattern analysis by microarray are ongoing in our laboratory. For the Nova proteins, double Nova-1 and Nova-2 knockout mouse brains show increased splicing affects for target RNAs (M. Ruggiu, J. Ule, H. Wang, and A. Mele, unpublished data.) It is likely that any affect of Nova on polyribosomal association of target mRNAs will be amplified in double knockout mice.

Similarly, two neuronal proteins closely related to FMRP, FXR1 and FXR2, may share redundant functions with FMRP. A mouse model that eliminates the contributions of all three of these proteins in neurons would be an ideally suited for polyribosomal mRNA interrogation of microarrays. Furthermore, recent evidence indicates that FMRP can contribute to activity-dependent, rapid fluctuations in protein levels in hippocampal neurons (Hou et al., 2006). The magnitude of these DHPG-induced protein changes is at most a 50% change, as assayed by Western blot. Thus, real changes in polyribosomal association that occur in response to activity in specific neurons are likely to be overwhelmed by the steady state levels of mRNA translation that exist in the unaffected neurons and glia in mouse cerebral cortex. Other methods could include chemical or electrical stimulation of neuronal activity to increase the FMRP-dependent change in polyribosomal mRNAs. A more rigorous dissection of polyribosomal profile patterns for FMRP target mRNAs under a variety of conditions including synaptic activity and/or

abrogation of redundant function of FMRP-related proteins may provide a foundation for future research evaluating the mechanism of translational regulation by FMRP.

Acknowledgments

The Nova-1 knockout polyribosome microarray experiment was a joint effort with Giovanni Stefani. The Rockefeller University GeneArray facility performed all post-hybridization GeneChip processing.

CHAPTER V: KISSING COMPLEX RNA MEDIATES INTERACTION BETWEEN FMRP AND BRAIN POLYRIBOSOMES

Introduction

Fragile-X Mental Retardation Protein (FMRP), the product of the FMR1 gene, is characterized by a number of protein-protein interaction domains, and three RNA-binding domains, specifically two tandem KH-type RNA-binding domains and a third RGG-type RNA-binding domain (RGG box). Although transcriptional silencing of FMR1 due to trinucleotide repeat expansion and hypermethylation is the most frequent cause of the disorder, patients expressing mutations or deletions within FMR1 have been described (Jin and Warren, 2003), emphasizing that loss of FMRP function leads to the Fragile-X syndrome. Given that the loss of FMRP activity leads to complex behavioral and cognitive deficits, understanding FMRP function has the potential to provide a link between molecular neurobiology and higher brain function.

FMRP is believed to regulate mRNA translation in the brain, although the mechanism for this action, and the RNA targets of this regulation, are unknown. We and others have demonstrated that FMRP is present on polyribosome fractions from both brain and tissue culture cells. (Corbin et al., 1997; Feng et al., 1997a; Feng et al., 1997b; Khandjian et al., 1996; Khandjian et al., 2004; Stefani et al., 2004) Additional data suggests that there may be a link between FMRP function and microRNA (miRNA) regulation of translation, since miRNAs are associated with polyribosomes (Bartel, 2004), and FMRP co-purifies with components of the RNA-induced silencing complex (RISC) (Caudy et al., 2003; Caudy et al., 2002; Ishizuka et al., 2002; Jin et al., 2004).

Previous studies in cell lines have examined the requirement of various domains of FMRP for association with polyribosomes. EBV-transformed lymphoblastoid cell lines from a patient with a missense mutation (I304N) in FMRP have been used to study the impact of this mutation on the association of FMRP with polyribosomes. Located in KH2, the I304N mutation does not impair capture of FMRP via association with poly(A) RNA, so mRNA binding is still possible for this mutant. However, association of FMRP with polyribosomes is abolished by I304N, and mRNPs containing I304N are abnormally small. (Siomi et al., 1994) FXR2P, an FMRP ortholog and interacting protein, remains associated with polyribosomes in the presence of I304N FMRP. (Feng et al., 1997a) Transfections of FMRP deletion constructs into an FMRP-null cell line reveal that the protein-protein interaction (PPI_d), KH1 and KH2 domains are all required for association with polyribosomes. Point mutations in either KH1 (I241N) or KH2 (I304N) abrogate binding of FMRP to polyribosomes and show a diffuse cytoplasmic distribution. In contrast, deletion of the RGG box results in a wild type distribution of FMRP on polyribosomes and punctate cytoplasmic distribution. (Darnell et al., 2005b) Interestingly PPI_d and KH1, but not KH2, are required for FMRP interaction with FXR1P, in an RNA-independent manner. Finally, the RGG box seems to be required for incorporation of FMRP into temperature-sensitive stress granules in the cytoplasm of cells. (Mazroui et al., 2003) In the phosphorylation domain (Phd) of FMRP, Ser499 can be phosphorylated by casein kinase II (CKII). Phosphorylation of FMRP at this site has been shown to be associated with stalled polyribosomes in cell culture, while Ser499Ala FMRP is associated with actively translating polyribosomes. Phosphorylation seems to have no effect on the magnesium- and RNA- dependence of FMRP association with

polyribosomes, and hasn't shown an effect on FMRP binding to known targets *in vivo* (although an effect of phosphorylation on dFMR binding to ribohomopolymers has been demonstrated.) (Ceman et al., 2003; Siomi et al., 2002) Thus it seems that the KH2 and/or KH1 domains of FMRP, but not the RGG box, are required for association with actively translating polyribosomes.

The finding that the I304N mutation can reduce FMRP binding to ribohomopolymers offers a clue to the function of the KH2 domain. (Siomi et al., 1994) Structure-studies of KH domains from analogous proteins indicate that the FMRP I304N mutation maps to the RNA binding pocket and is critical for stabilizing sequence-specific RNA-protein interactions. (Lewis et al., 2000; Ramos et al., 2003b) RNA selection with full-length FMRP has led to the identification of high-affinity FMRP RNA ligands that harbor a signature structural motif termed a G-quartet. (Brown et al., 2001; Darnell et al., 2001) G-quartets are stacked coplanar arrays of 4 guanine bases each, stabilized by cyclic Hoogsteen bonding between guanines. Of the three RNA-binding domains in FMRP, G-quartet RNA binding maps to the RGG box. (Darnell et al., 2001) Evaluation for G-quartets has been widely used to screen candidate FMRP RNA targets, identified through a variety of approaches, including bioinformatics, immunoprecipitation and DNA chip analysis, antibody-positioned RNA amplification, and biochemical approaches. (Brown et al., 2001; Darnell et al., 2001; Miyashiro et al., 2003; Schaeffer et al., 2001; Todd et al., 2003) Biochemical studies have suggested that FMRP may associate with specific mRNAs on polyribosomes; several mRNAs were found to have altered polyribosome distributions in lymphoblastoid cells obtained from Fragile X patient cells and to harbor G-quartet motifs. (Brown et al., 2001; Darnell et al., 2001)

Subsequent analysis of one of these targets, MAP1B, suggests that the *Drosophila* homolog of this protein (*futsch*) may be regulated at the translational level by the *Drosophila* FMRP homolog, dFMR. (Zhang et al., 2001)

Subsequent RNA selection with the KH1 and KH2 domains of FMRP identified a loop-loop pseudoknot, or “kissing complex” motif as a high affinity ligand for KH2. (Darnell et al., 2005a) The nature of the kissing complex structure, containing two stem-loops that form a third stem and an additional inter-loop base pair, has prevented easy identification of endogenous kissing complex RNAs that may be regulated by FMRP. Detailed analysis of the FMRP KH domain-RNA interaction revealed that binding to the kissing complex is KH2-specific, and is abrogated by the I304N mutation. Binding is dependent in part on the RNA structure and on several specific nucleotides, as indicated by Mg⁺²-dependence, chemical modification, and mutational analysis. (Darnell et al., 2005a)

Identification of the kissing complex as a KH2 target allowed us to re-examine the nature of FMRP association with polyribosomes. We find that the association of FMRP with brain polyribosomes is entirely and specifically competed by kissing complex RNAs. These results suggest that mental retardation associated with the I304N mutation, and likely the Fragile-X syndrome more generally, may relate to a crucial role for RNAs harboring kissing complex motifs as targets or mediators of FMRP translational regulation.

Results

FMRP RNA-binding domains bind different high affinity RNA ligands

We have tested the hypothesis that the RNA-binding ability of KH2 may mediate polyribosome association with a novel method that forces endogenous FMRP in mouse brain lysate to choose between binding a high affinity RNA ligand and binding its endogenous RNA targets, in a domain-specific manner. Our lab has identified high affinity RNA target ligands through *in vitro* RNA selection (SELEX) of a pool of random 96-mers for FMRP binding. (Darnell et al., 2005a; Darnell et al., 2001) Two groups of target sequences have been identified. Those that bind preferentially to the RGG box of FMRP contain a G quartet structure that is required for binding (gqRNAs), and those that bind to KH2 exhibit a loop-loop pseudoknot “kissing complex” structure. This binding is sequence- and structure- specific, as mutations in the RNAs and or protein domains can abrogate binding. (For a thorough treatment, see details in (Darnell et al., 2005a; Darnell et al., 2001)). Figure 5.1 shows the an experimentally derived structure for high affinity RNA ligands of the G-quartet type (sc1; Figure 5.1A; adapted from (Darnell et al., 2001)) an M-Fold predicted structure for an RNA of the kissing complex type (kc2; Figure 5.1B; (Zuker, 2003)). A mutation that abrogates binding to FMRP is indicated for each. A consensus structure from experimental data for kcRNA folding is shown in Figure 5.1C and illustrates the requirement for an inter-loop stem and a Watson-Crick base pair “kiss” between the opposite sides of the loops (Darnell et al., 2005a).

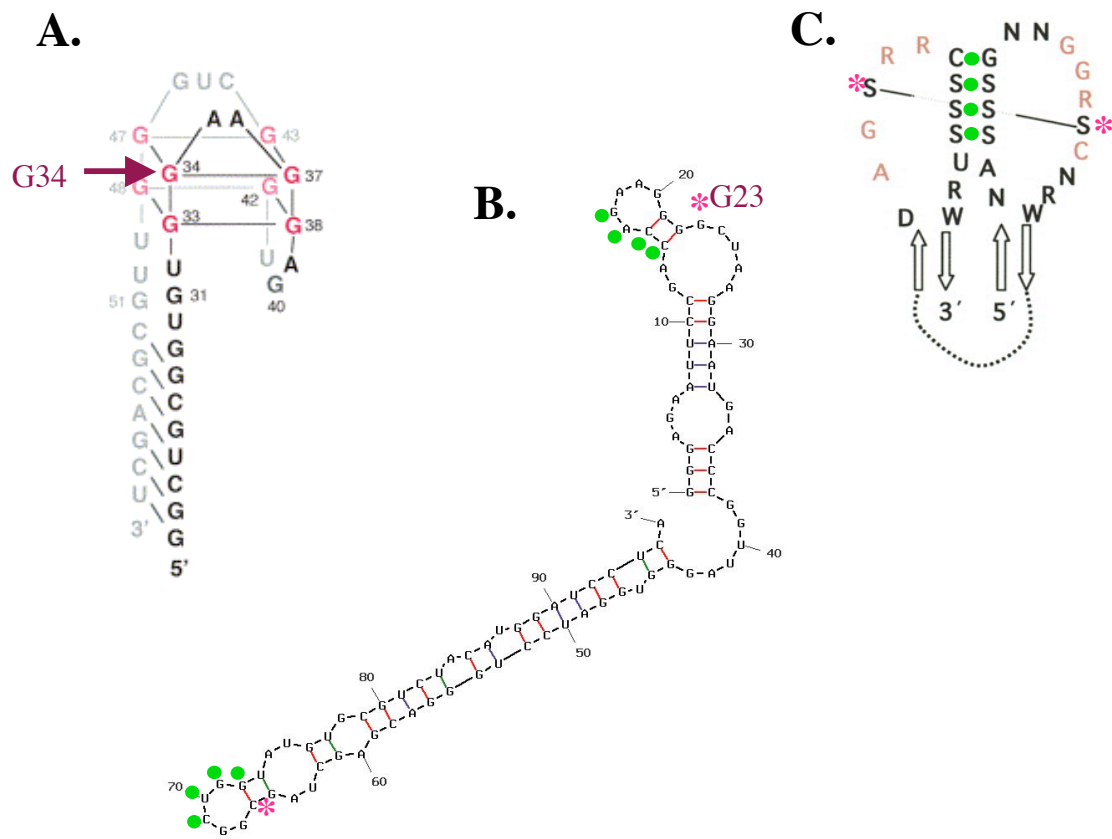


Figure 5.1 Experimental and predicted structures of FMRP RNA ligands. (A) Experimentally derived G-quartet RNA structure of sc1 RNA (Darnell et al., 2001), arrow indicates a mutation that abrogates binding to FMRP. (B) Mfold-predicted structure of kc2 RNA folding (Zuker, 2003). Green dots indicate nucleotides that form a stem between loops. Pink asterisks indicate the base-pair “kiss” between opposing sides of the loops. The G23C mutation prevents this “kiss” and binding by FMRP. (C) An experimentally derived model of the consensus kissing complex RNA motif (Darnell et al., 2005a).

kcRNA can shift FMRP off polyribosomes in brain lysate

The first suggestion that FMRP might be involved in translational regulation was based on findings that it associated with polyribosomes in tissue culture cell lines, and that this association was abrogated by the I304N mutation. We have used both kcRNAs and gqRNAs to compete with endogenous RNAs for FMRP binding as a tool to analyze the importance of KH2 and RGG RNA-binding domains on FMRP association with polyribosomes. As an initial experiment, post-mitochondrial supernatant from postnatal day 9 mouse cerebral cortex was prepared using standard conditions for polyribosome analysis. This lysate was incubated with either 500nM *in vitro* transcribed kc1 RNA or an equal volume of RNA folding buffer (1X SBB) for 15 minutes at room temperature. Samples were then loaded on 20-50% (w/w) sucrose gradients and analyzed using standard methods. The resulting Western blots are shown in Figure 5.2. While the distributions of ribosomal protein S6 (rpS6) and gamma tubulin remain consistent between samples, the distribution of FMRP changes dramatically in the sample treated with kc1 RNA. While FMRP signal peaks in fractions 13-20 in the control sample, all FMRP in the kc1 RNA-treated gradient is located in fractions 3-6, a complete shift to the lighter area of the gradient. Thus, it appears that in the presence of kcRNA, FMRP dissociates from a fast-sedimenting complex into a smaller particle. Since kcRNA does not disrupt the distribution of the small ribosomal subunit (as shown by rpS6), and since FMRP has been shown to associate with actively translating polyribosomes (Stefani et al., 2004), it is a reasonable hypothesis that the presence of kcRNA results in the specific dissociation of FMRP from polyribosomes in brain lysate, leaving previously FMRP-bound polyribosomes intact. This is consistent with previously published data showing

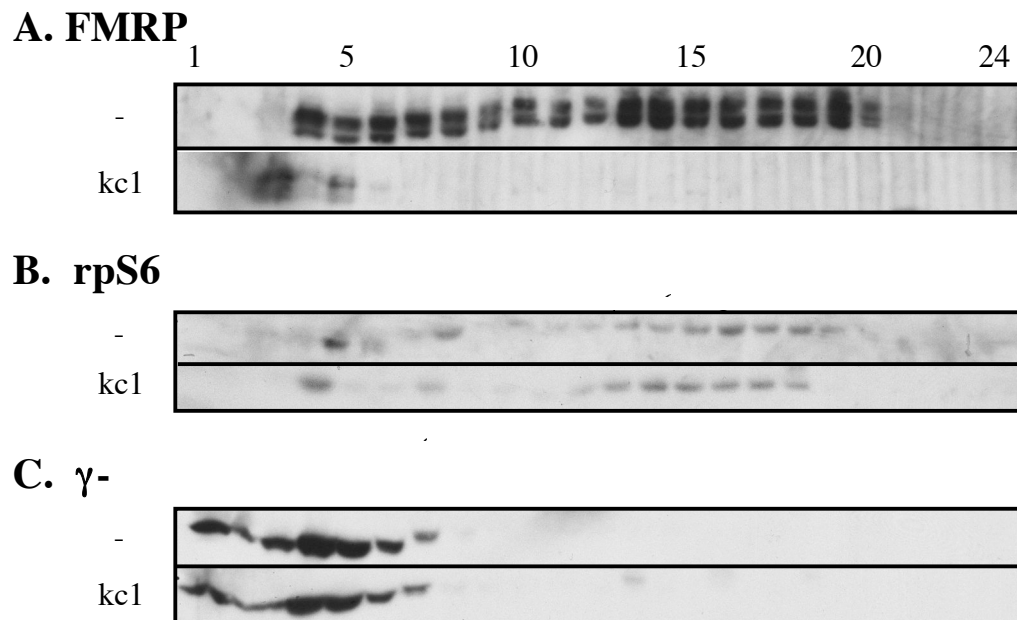


Figure 5.2 Kissing complex RNA competes FMRP off polyribosomes. Extracts from mouse cerebral cortices were incubated with 500nM kc1 RNA (kc1) or an equal volume of buffer (-) before polyribosome gradient fractionation on 20-50% sucrose gradients. Western blots of gradient fractions are shown for FMRP (**A**), ribosomal protein S6 (**B**), and gamma-tubulin (**C**).

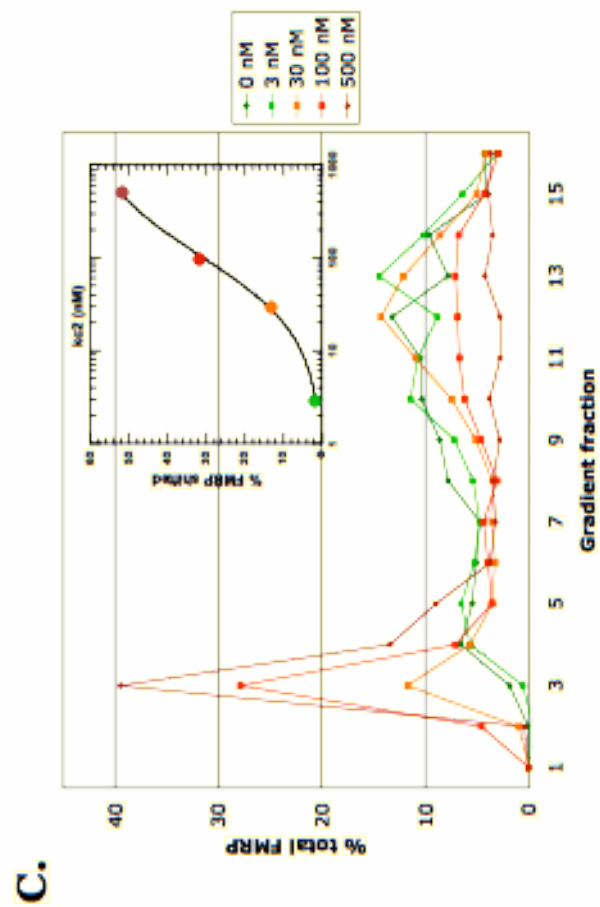
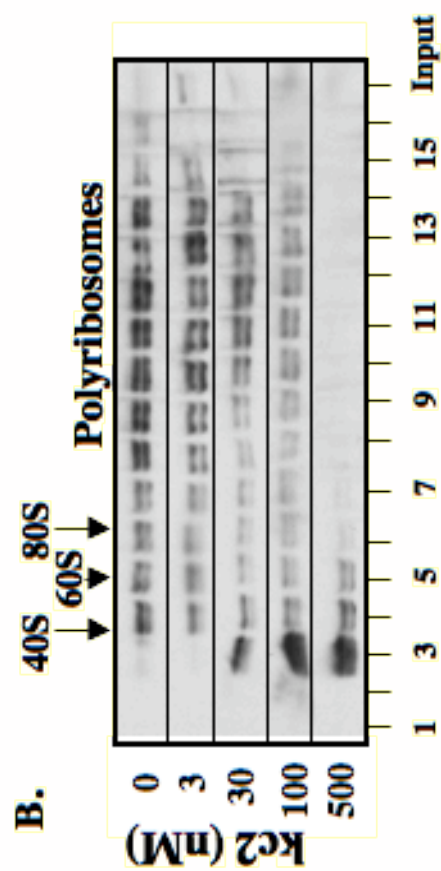
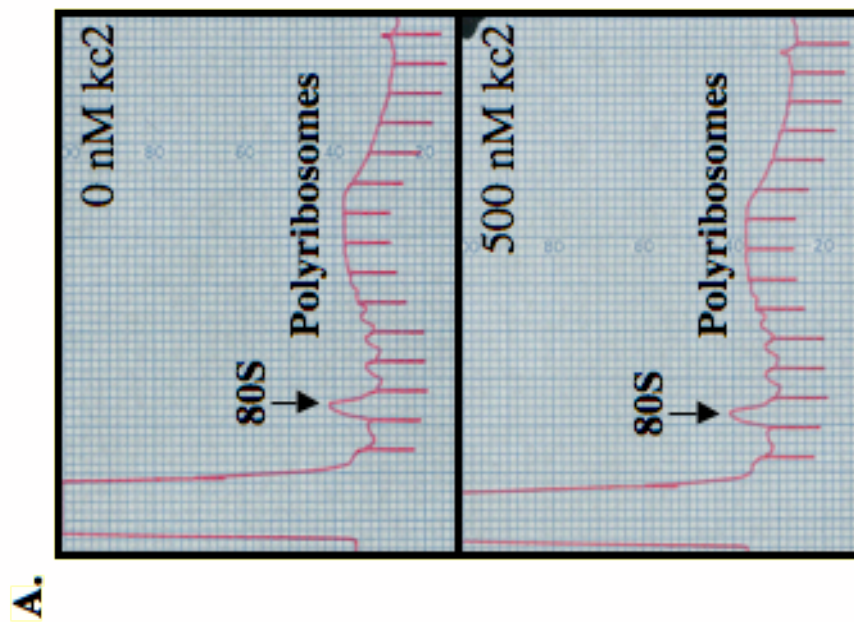
that the KH2 domain is required for FMRP cosedimentation with polyribosomes. (Darnell et al., 2005b; Mazroui et al., 2003)

To look for a dose-dependent effect on competition of kcRNA with endogenous targets that mediate the FMRP:polyribosome complex, and to define the approximate affinity of this interaction, we tested increasing concentrations of kcRNA. When brain polyribosomes were incubated with 3-500nM kc2 RNA, we observed a dramatic dose-dependent shift in FMRP sedimentation from the polyribosomal fractions to light fractions corresponding to a size smaller than the 40S ribosomal subunit (Figure 5.3B). As seen with kc1 RNA, FMRP shifts from fractions 10-14 (of 16) to fractions 2-4 when in the presence of 500 nM kc2 RNA. The global polyribosomal profile was unchanged, as shown by UV absorbance tracings for 0nM and 500nM kc2 RNA (Figure 5.3A). Quantitation reveals that the half-maximal FMRP shift off polyribosomes occurred at an RNA concentration of approximately 100nM (Figure 5.3C), similar to the K_D for FMRP binding kc2 RNA in vitro (~66nM). The ability of kissing complex RNA ligands to compete endogenous FMRP off polyribosomes suggests that this ligand mimics the site FMRP uses to regulate translation in neurons.

FMRP:polyribosomes are specifically dissociated by kcRNA

To assess the specificity of these results, and to further probe the role of RGG box-mediated RNA binding in FMRP polyribosome association, the experiment was repeated with sc1 RNA, a G-quartet ligand (gqRNA) for the RGG box. Sc1 RNA binds purified FMRP with an affinity of approximately 8nM in vitro. (Darnell et al., 2001) As a critical control, we tested mut kc2, a point mutant in kc2 that destroys a critical base

Figure 5.3 Exogenous kc2 RNA dissociates FMRP from polyribosomes in a dose-dependent manner. (A) P8 mouse cerebral cortical extracts were separated by sucrose density gradient (20%-50%) centrifugation; positions of the 80S monosome and polyribosomes are indicated. Indicated amounts of kc2 RNA were added to brain lysates and incubated for 15 min at room temperature prior to centrifugation. A254 profile of sucrose density gradients for the lowest and highest kc2 RNA concentrations are shown; A254 profiles for all kc2 concentrations were indistinguishable (data not shown). (B) Western blots of indicated fractions from sucrose density gradients incubated with the indicated kc2 concentration were probed with the FMRP monoclonal antibody 1C3. Fractions corresponding to 40S, 60S, and 80S ribosomal peaks are indicated. Ribosomal protein S6 was unshifted at all kc2 concentrations (data not shown). (C) Quantitation by chemiluminescence of the Western blot data in B (Versadoc imaging); the percentage of FMRP present in each fraction as a function of the total FMRP signal in that gradient is indicated. Inset shows a plot of the percent of FMRP shifted off the polyribosomes plotted against kc2 concentration (nanomolar). The half-maximal concentration of kc2 able to compete FMRP off of polyribosome fractions is ~100 nM kc2 RNA.



pair in the loop-loop interaction (G23C, see Figure 5.1, (Darnell et al., 2005a)), and mut sc1, a point mutant in sc1 that destroys the G-quartet (G34C, (Darnell et al., 2001)). In each case, the association of FMRP with polyribosomes was unaffected (Figure 5.4). As an additional control, we evaluated sedimentation of the neuronal Hu RNA-binding proteins, since the nonneuronal Hu isoform is known to be polyribosome associated. (Gallouzi et al., 2000; Okano and Darnell, 1997) We found that the neuronal Hu proteins are polyribosome associated in mouse brain, but that neither kcRNA nor gqRNA was able to disrupt this association (Figure 5.4). These results demonstrate that kcRNA specifically disrupts FMRP polyribosome association. Taken together, these experiments suggest that the binding of KH2 to kissing complex RNA ligands may mediate polyribosomal association in the brain, while association of the RGG box with G-quartets is not required for stability of the complex.

The ability of kcRNA to fold correctly and bind KH2 is dependent on the presence of a magnesium ion coordinated between the two loops of the pseudoknot. As a result, KH2 is unable to bind kcRNA in the presence of EDTA. (Darnell et al., 2005a) However, the interaction between the RGG box and gqRNA is unaffected by EDTA. (Darnell et al., 2001) To confirm that even an increased concentration of gqRNA is not able to change the sedimentation rate of polyribosome-associated FMRP complexes, brain lysate was incubated with 5 μ M sc1 RNA (Figure 5.5). Although there is some variation between the FMRP distributions of 500nM sc1 and mut sc1, 5 μ M sc1 RNA and control samples, there is no consistent effect of gqRNA, even at a concentration approximately 500 times that of its *in vitro* affinity for FMRP. To analyze whether competition with gqRNA is able to change the sedimentation rate of smaller FMRP

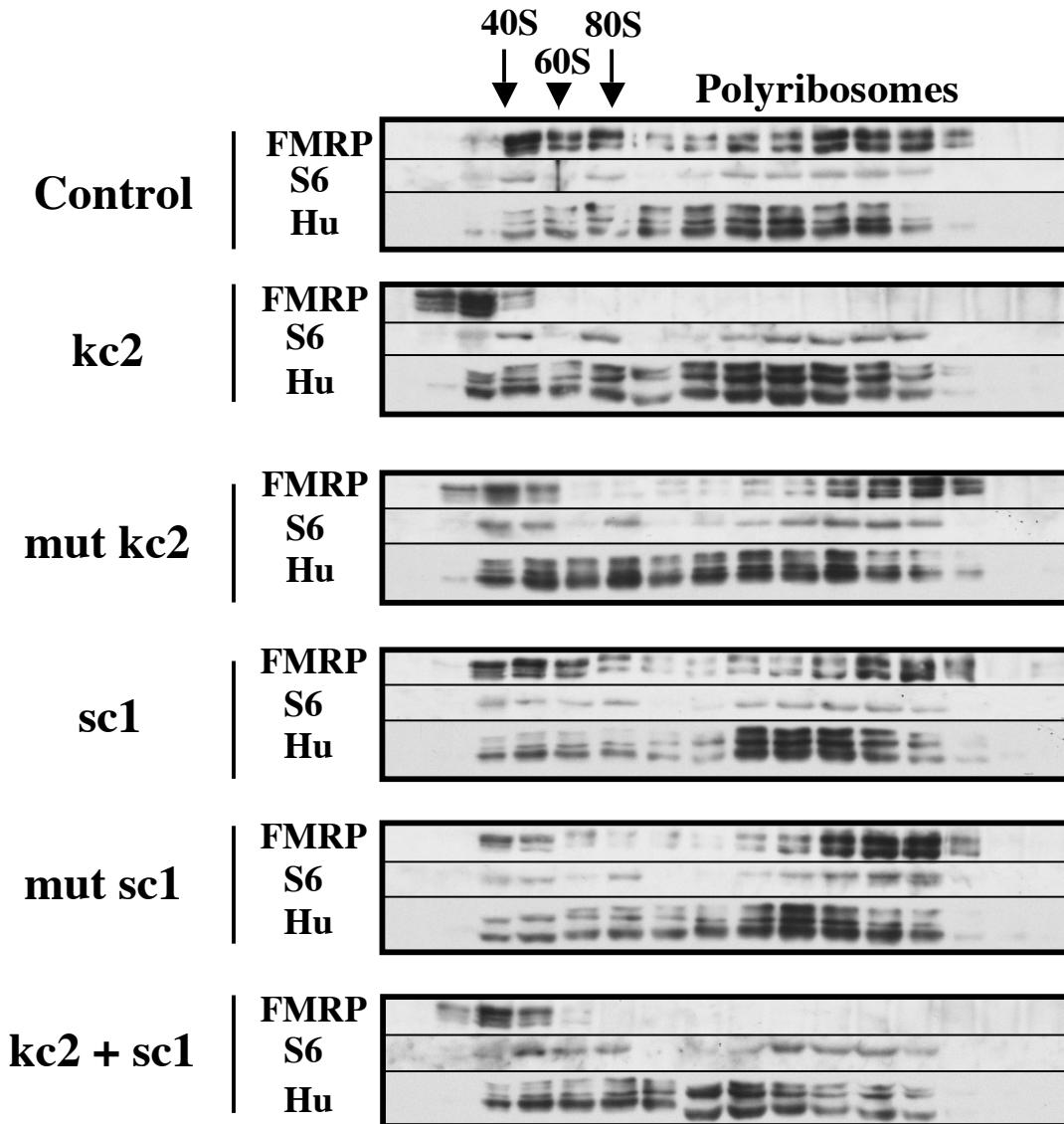


Figure 5.4 Kissing complex, but not G-quartet RNA, competes FMRP off polyribosomes. Western blot as in Figure 5.3 of sucrose density gradients in which indicated RNAs were used to compete FMRP polyribosomal association; mut kc2 harbors a single point mutation in kc2 RNA, and mut sc1 harbors a single point mutation that destroys G-quartet formation and FMRP binding (indicated in Figure 5.1). Western blots were probed with antibodies to the indicated proteins, including FMRP, ribosomal protein S6, and the Hu family of RNA-binding proteins.

mRNPs that have been dissociated from polyribosomes (see Figure 3.4D), brain lysate was incubated with 500nM sc1 or mut sc1 RNA plus 30mM EDTA. UV absorbance profiles for RNA+EDTA-treated samples do not differ from a profile of a sample treated with EDTA alone (Figure 5.5A). Western blots for these gradients reveal that FMRP is located in fractions 2-7, as expected since EDTA disrupts polyribosomes. (See Chapter 3; (Stefani et al., 2004)) However, as shown in Figure 5.5B, there is no significant difference between the FMRP distributions for sc1 + EDTA and mut sc1 + EDTA. In addition, incubation of lysate with both kcRNA and gqRNA produces an FMRP distribution that is identical to that of treatment with kcRNA alone (Figure 5.4, bottom panel). While these data suggest that gqRNA targets are not involved in regulating whether FMRP is associated with large complexes, this result does not rule out the possibility that FMRP mRNPs (that have been dissociated from polyribosomes by EDTA) contain G-quartet mRNAs. This question warrants further investigation using 5-25% sucrose gradients for a finer assessment of FMRP sedimentation rates under these conditions, and is being pursued by another PhD candidate in the lab.

We also examined whether an irrelevant RNA could disrupt the FMRP:polyribosome interaction. Nova proteins contain KH-type RNA-binding domains that bind *in vitro* selected RNAs harboring a stem-loop structure and a YCAY motif. “10021” RNA binds Nova KH domains with an affinity of ~500nM. (Jensen et al., 2000b) Competition for KH-mediated RNA binding by 30uM 10021 RNA does not disrupt the interaction of Nova with large complexes (Figure 5.6A). Furthermore, 10021 RNA does not affect FMRP distribution, as shown in Figure 5.6B. Thus, a high affinity

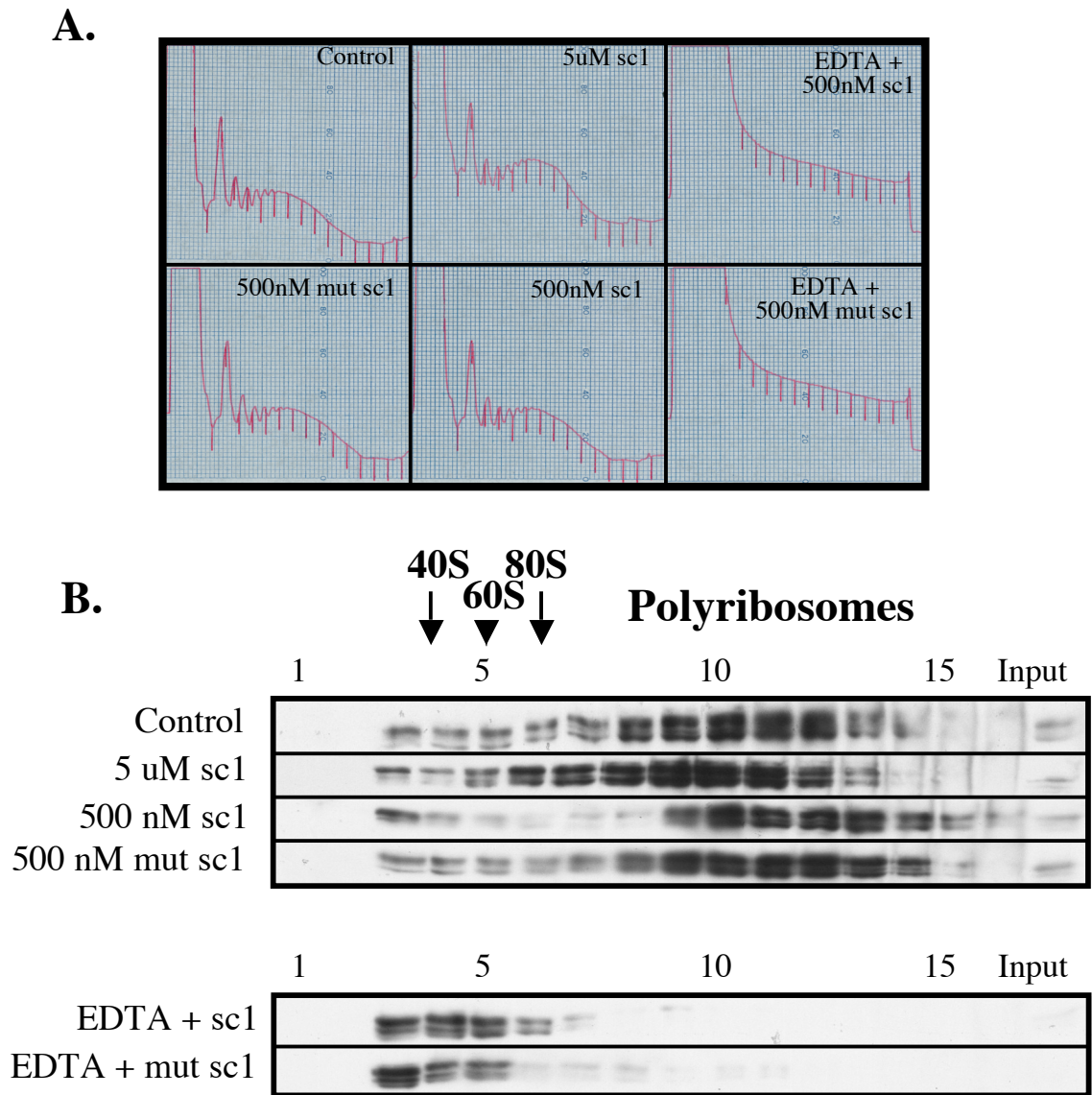


Figure 5.5 *sc1* RNA does not change FMRP distribution on polyribosomes or in EDTA-liberated mRNPs. A254 profiles (A) and Western blots (B) for mouse cerebral cortex treated with *sc1* RNA, mutant *sc1* RNA and/or EDTA before sucrose centrifugation. The *in vitro* binding affinity of the FMRP RGG box for *sc1* is approximately 8nM (Darnell et al., 2001).

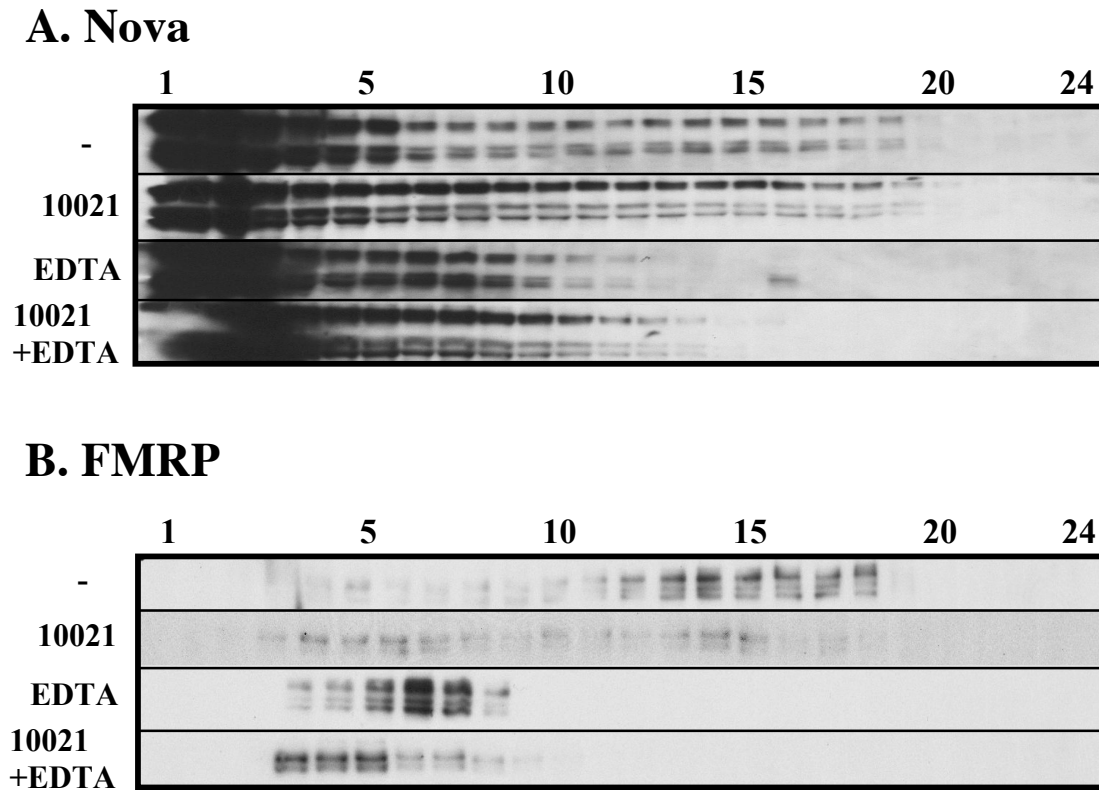


Figure 5.6 Sucrose gradient distributions of Nova-1, Nova-2 and FMRP are unaffected by the presence of 10021, an RNA ligand for the Nova KH domains. Western blots for Nova (A) and FMRP (B) from TCA-precipitated sucrose gradient fractions analyzing mouse cerebral cortex lysate under treatment with exogenous 10021 RNA and/or EDTA.

RNA ligand that binds a KH domain of another RBP is not able to disrupt the interaction of FMRP with polyribosomes.

BC1 RNA and tRNA do not compete for KH2 binding

It has recently been reported that FMRP binding to BC1, a neuron-specific, dendritically localized nontranslatable RNA (Brosius and Tiedge, 2001), may play a role in modulating mRNA translation (Zalfa et al., 2003). It has also been reported that FMRP binds to highly structured RNAs such as tRNA. (Gabus et al., 2004) We tested whether an excess of BC1 RNA or tRNA was able to compete FMRP off polyribosomes (Figure 5.7). Addition of up to 5 μ M BC1 (Figure 5.7A) or tRNA (Figure 5.7B) to cortical lysates fails to compete with the *in vivo* binding site of FMRP on polyribosomes and displace it. Moreover, although BC1 has been shown to be involved in the global regulation of translation (Wang et al., 2005), the polyribosome profile and rpS6 distribution do not change in the presence of a vast excess of this RNA. Other researchers have found no significant interaction between FMRP and BC1 (Wang et al., 2005). Our results demonstrate that FMRP is specifically associated with brain polyribosomes, and that this interaction is effectively competed by kissing complex RNAs.

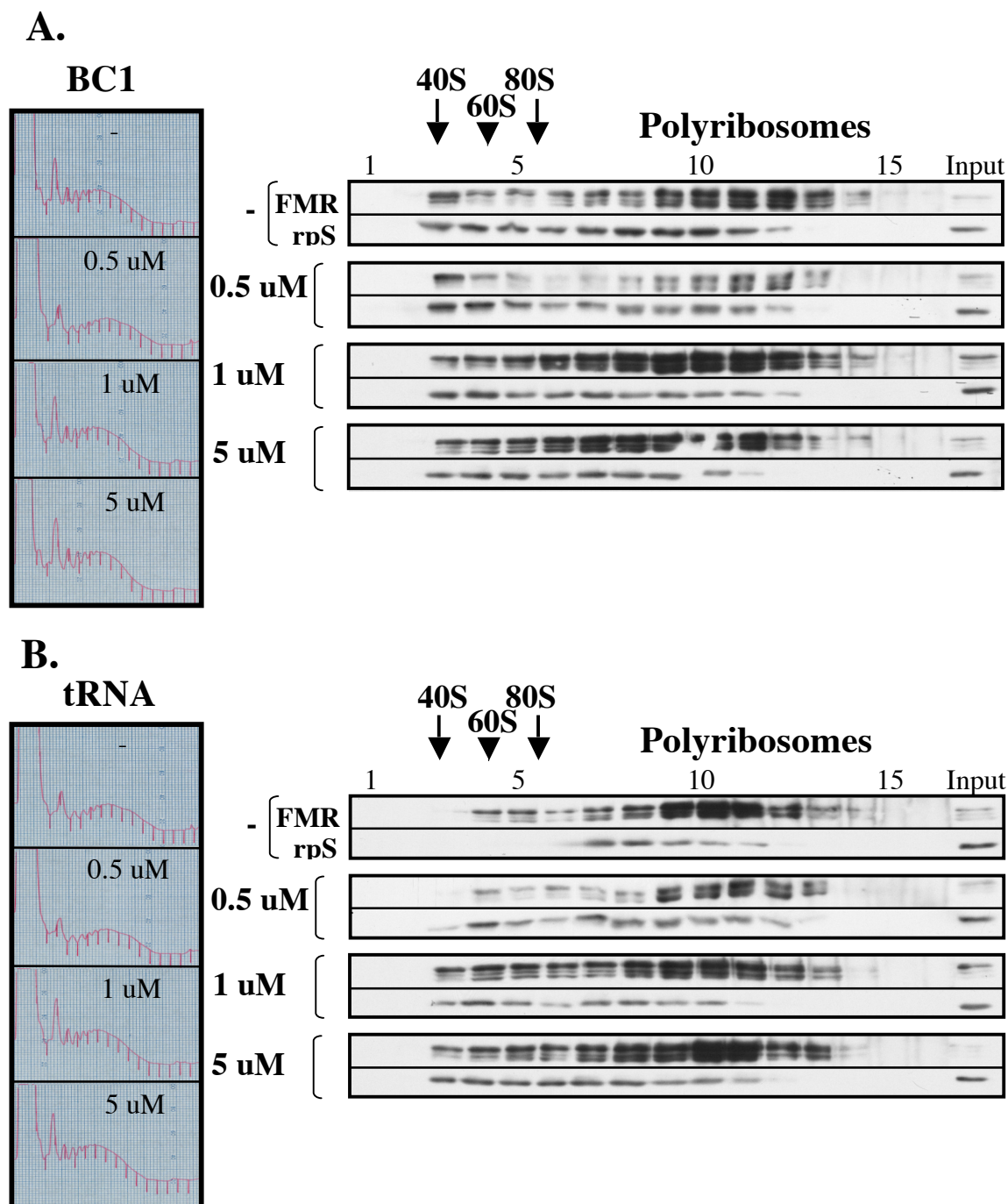


Figure 5.7 BC1 RNA and yeast tRNA do not disrupt FMRP interaction with polyribosomes. A254 traces (A) and Western blot analysis for FMRP or ribosomal S6 protein (B), as in Figure 6. No RNA was added to the control lysates (control). BC1 RNA (produced by in vitro transcription and gel purified) and yeast tRNA at the indicated concentrations fail to compete with FMRP binding to polyribosomes.

kcRNA cross-links directly to FMRP in brain lysate

Despite the fact that FMRP binds kcRNA with high affinity *in vitro*, the possibility remains that kcRNA disrupts FMRP:polyribosome complexes by some mechanism that does not involve stable binding to FMRP. For example, in brain lysate, kcRNA could act as a “toxic” aptamer, transiently binding FMRP and releasing a denatured protein. Alternatively, kcRNA could interfere with the FMRP binding site in translating mRNAs. In order to address this question and confirm FMRP:kcRNA complex formation in brain lysate, we used cross-linking immunoprecipitation (CLIP) to assess whether there is a direct interaction between protein and RNA. CLIP has been developed and optimized by our lab as a method for rapid identification of *in vivo* targets of RNA-binding proteins. (Ule et al., 2005a) FMRP-specific modifications to this protocol have been developed by J. Zang and J. C. Darnell (unpublished data.) For this experiment, a post-mitochondrial supernatant was prepared from wild type or FMR1 knockout mouse cerebral cortex, and divided into two tubes. One set of wild type and knockout lysates was incubated with 500nM kc7 RNA for 15 minutes at room temperature. Each sample was then loaded on a 20-50% sucrose gradient, centrifuged and collected into 16 fractions using standard polyribosome protocols. After fraction collection, the first 6 fractions of each gradient were divided equally, and protein from an aliquot precipitated for Western blot analysis. One half of each fraction was brought to 30mM EDTA, mixed, and placed on ice. Both sets of gradient fractions (intact and EDTA-treated) were exposed to UV cross-linking (6 X 4000J) while kept on ice and swirled before each UV exposure. Fractions 1-3 from each gradient and condition were pooled and FMRP:RNA complexes were immunoprecipitated using mixed monoclonal

and bridging antibodies with Protein A Dynabeads (see Chapter II for details). After 1:100 digestion with RNase A and a series of stringent washes, radio-labeled RNA linkers were ligated to RNAs that remained cross-linked to FMRP. These RNAs were isolated by SDS-PAGE and nitrocellulose blotting (Figure 5.8B), eluted, subjected to PCR (Figure 5.8C) and cloned. Figure 5.8A shows the FMRP distribution in fractions 1-6 of the wild type (WT) and kc7 RNA treated-wild type (WTkc7) gradients. As expected, there is no signal for FMRP in the knockout (KO) and kc7 RNA treated-knockout (KOkc7) gradients. Of the eight sets of fractions (each gradient +/- EDTA), we have immunoprecipitated from four: WT+EDTA, WTkc7, WTkc7+EDTA, and KOkc7+EDTA. After immunoprecipitation and RNA ligation, FMRP-RNA complexes electrophorese more slowly than non-cross-linked FMRP. Specifically, we can expect FMRP cross-linked to a RNA of ~100 nucleotides to migrate at about 33 kilo Daltons larger than that of FMRP alone (68-80kDa) ((Ule et al., 2005a); J. Zang, J. Ule, and A. Mele, unpublished data.) Figure 5.8B is an autoradiograph from labeled FMRP:RNA complexes that have been transferred to nitrocellulose after electrophoresis. Specific bands are visible which correspond to specific RNA species. A band between approximately 120kDa and 140kDa (asterisk) is just visible in the WT+EDTA and KOkc7+EDTA lanes. This is a non-specific band that is neither kc7- nor FMRP-dependent. (It represents contaminating immunoprecipitation of Ago2:miRNA complexes by the 7G1-1 monoclonal antibody against FMRP that cross-reacts with Ago2 (J. Zang, unpublished data.))

A dark band of ~90-100kDa (§) is visible in the WTkc7 and WT kc7+EDTA lanes. When cloned and sequenced, 17 of 26 tags from the WTkc7 band are pieces of

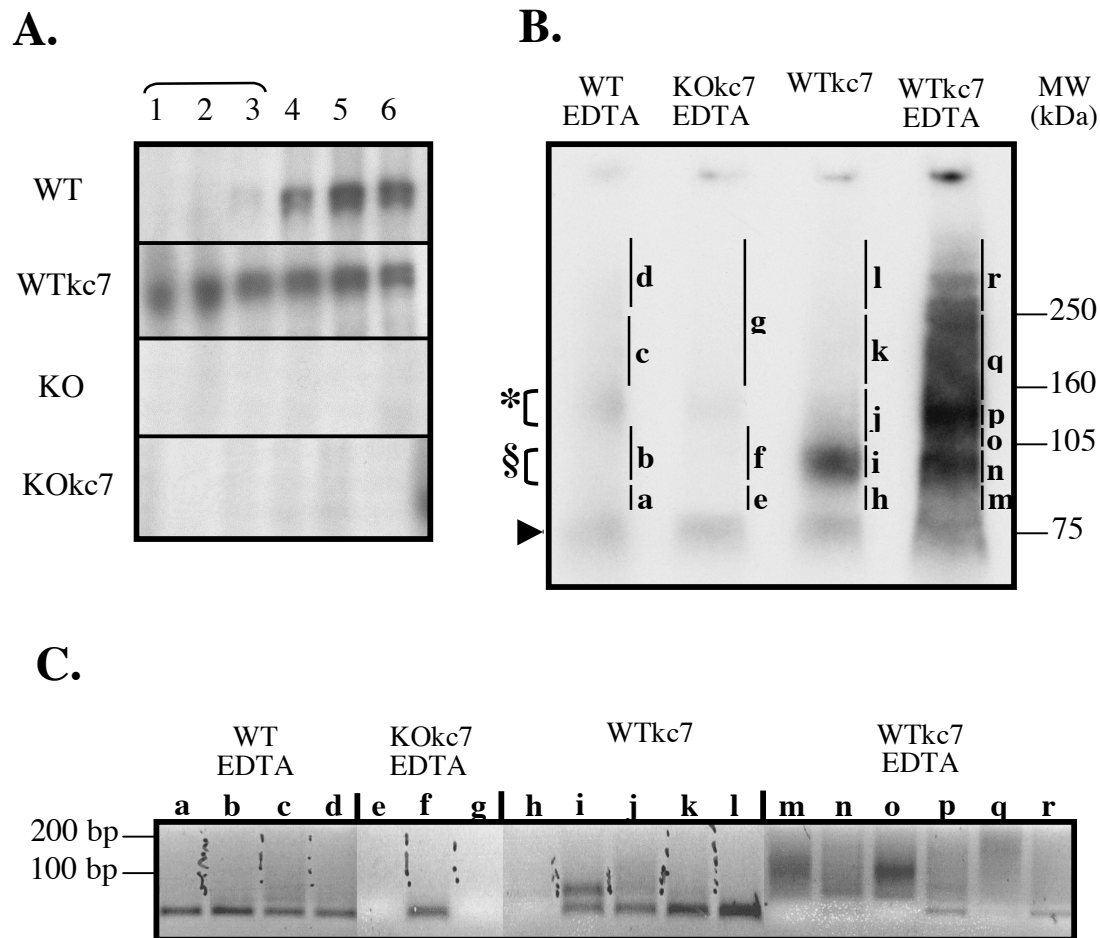


Figure 5.8 kc7 RNA cross-links to FMRP in sucrose gradients. (A) Western blots against FMRP of fractions 1-6 (of 16) of FMRP wild type and knockout mouse cortex polyribosomal gradients. Fractions 1-3 from each gradient are pooled for immunoprecipitation in (B), an autoradiogram of a nitrocellulose membrane showing radiolabeled RNA crosslinked to immunoprecipitating proteins. Arrowhead indicates the expected migration of non-crosslinked FMRP. Lettered bands were cut out of the membrane, amplified by PCR and gel-purified (C).

kc7 RNA. In fact, a closer look at these sequences reveals that every sequence includes either of the two “kissing” nucleotides (G23 and C64 in kc7, the equivalent of G23 and C66 in kc2 and G20 and C45 in Δ kc2), as shown in Table 5.1. This data from an unbiased screen supports previous biochemical data describing the sequence and structure specificities of kissing complex RNA folding and interaction with FMRP. (Darnell et al., 2005a) The remaining nine sequences from this band are six ribosomal RNAs, one miRNA, one intronic sequence, and one clone of repeat sequence from genomic DNA. Notably, kc7 sequences were not found among the KOkc7+EDTA and WTkc7+EDTA CLIP tags. This indicates that magnesium can be chelated by addition of EDTA when FMRP has first been incubated with kc7 RNA, and that this chelation disrupts the FMRP:kc7 interaction, preventing subsequent cross-linking.

The WTkc7+EDTA autoradiograph in Figure 5.8B while very dark, seems to contain many bands of different sizes, all of which were cloned and sequenced. 18 of 23 CLIP tags from this lane were identified as ribosomal RNAs. These sequences map to many regions of the full-length 18S and 28S rRNAs. It is possible that FMRP may interact with ribosomal RNA *in vivo*. Indeed there is a report that FMRP associates with the large subunit of the ribosome (Siomi et al., 1996), and there are 6 rRNA CLIP tags identified from the intact WTkc7 CLIP. However, it is more likely that as a “sticky” RNA-binding protein newly dissociated from its RNA cargoes by the combination of kc7 followed by EDTA, it is now able to bind to rRNAs, present in great abundance in the cell. Moreover, EDTA treatment also disrupts the ribosome, exposing surfaces of rRNAs that are normally inaccessible to FMRP. In addition, rRNAs are the most frequent non-specific “contaminating” RNAs identified by CLIP for several RNA-binding proteins (J.

Zang, A. Mele, and J. Ule, unpublished data.) Thus, in the absence of a particular over-enriched sequence from either the 18S or 28S species in the resulting CLIP tags, this interaction may be an artifact and not biologically relevant. Taken together, these data indicate that FMRP interacts directly with the kcRNA pseudoknot in brain lysate, that this interaction is stable enough to persist through a two-hour centrifugation and fraction collection, and that EDTA disrupts this interaction.

	WT EDTA	KOkc7 EDTA	WTkc7	WTkc7 EDTA
kc7 sequence	-	-	17	-
3' hairpin (includes C64)			16	
5' hairpin (includes G23)			1	
rRNA	6	-	6	18
18S rRNA	0		2	16
28S rRNA	6		4	2
mRNA	2 [†]	-	-	4 [†]
miRNA	1	-	1	1
snRNA	-	1	-	-
other*	2	1	2	-
# sequenced	32	4	46	53
Total CLIP tags	11	2	26	23

Table 5.1 Classification of kc7-shifted FMRP CLIP tags.

Sequences cloned from bands in Figure 5.8C were identified by BLAT (UCSC) and NCBI BLAST. Of the total number of clones sequenced, many represented vector religation or primer-primer ligation events. As such the total number of identifiable sequences is given as Total CLIP tags. *other sequences include: mitochondrial DNA (1), genomic DNA (3), intron RNA (1). [†]mRNAs: cat eye syndrome chromosome region candidate 6 (Cecr6), cytidine monophospho-N-acetylneuraminic acid synthetase (Cmas), Fucosyl transferase 10, UDP-Gal β galactosyl transferase 6, RIKEN cDNAs 4930500O05 and G730014J15

Recombinant KH2 as a competitor for polyribosome association

FMRP:polyribosome complexes can be dissociated by an RNA competitor for KH2 binding. Therefore, the converse question is relevant: can increased concentrations of recombinant KH2 compete for endogenous kissing complex-type binding sites on polyribosomes and thereby displace endogenous FMRP? If so, KH2 could act as a dominant negative protein that could potentially serve as a tool to inhibit FMRP function *in vivo*. To test this hypothesis, we purified two types of recombinant FMRP KH2, a construct encoding the minimal sequence necessary to bind kcRNA that lacks exons 11 and 12 (KH2 -e11, 12), and a protein that includes the variable loop of exon 11 within the KH2 domain (KH2 -e12). Incubation of either of these KH2 isoforms at concentrations from 500nM to 18 μ M does not alter the distribution of FMRP (Figure 5.9B, C). As a control, these protein preparations were tested for *in vitro* kcRNA-binding ability by filter binding assay. The affinity of KH2 for kc7 RNA has previously been measured as approximately 35nM (Darnell et al., 2005a). From the binding curves in Figure 5.9A, the K_d for KH2 (-e11,12) is ~27nM, and the K_d for KH2 (-e12) is ~45nM. Thus, these preparations of both KH2 isoforms are able to bind kc7 with the expected affinity, and the presence of exon11 does not affect this binding. While this experiment shows that using recombinant KH2 as a spiked-in competitor for endogenous KH2 target RNAs does not disrupt FMRP:polyribosome complexes, there are many caveats to this result. First, kcRNA is an *in vitro* selected aptamer that, by definition, will have the highest possible affinity for KH2. Therefore, it is a reasonable possibility that endogenous targets of KH2 have lower affinities and are easily displaced by kcRNA in

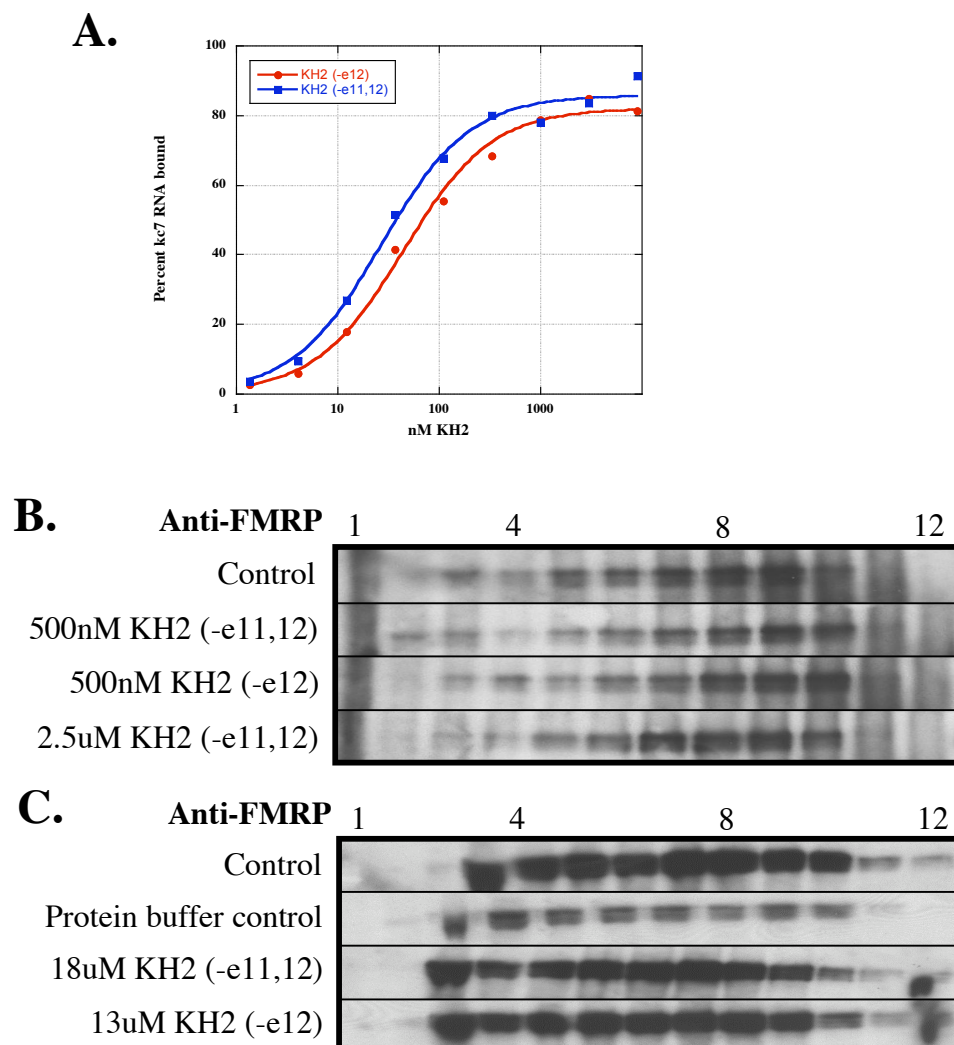


Figure 5.9 Recombinant KH2 does not shift endogenous FMRP off polyribosomes.

(A) Filter binding assays of two preparations of recombinant KH2 (-e11, 12) and (-e12) show that these preparations bind kc7 RNA with the expected affinity *in vitro* (~30nM). (B, C) Western blots for FMRP from mouse cortex polyribosome gradients that have been treated with various concentrations of recombinant KH2 isoforms.

our assay. However, recombinant KH2 and full-length FMRP have the same *in vitro* affinity for kcRNA. Thus, when competing with endogenous FMRP in brain lysate, recombinant KH2 does not have the advantage of a higher affinity for RNA targets. Second, there are as yet no data describing the mechanism by which FMRP binds *in vivo* mRNA targets. This process may very well involve other domains in FMRP as discussed earlier, perhaps in a helicase-type manner that allows another domain to bind an otherwise hidden site. Or, multiple domains may be required for enhanced stability of the FMRP:mRNA interaction, perhaps involving several binding sites on a single mRNA. As a single RNA-binding domain spiked into a cytoplasmic lysate, recombinant KH2 does not see the same opportunities for binding mRNAs during the course of normal processing events. Finally, KH2 has been shown to be important for heterodimerization of FMRP with FXR2. (Feng et al., 1997a) This suggests that spiked-in KH2 may be sequestered by protein-protein interactions and therefore fail to bind endogenous mRNA targets. An alternative scenario hinges on the likelihood that FMRP may bind its mRNA targets in the nucleus and participate in their export, transport, and translation. All of these issues bear investigation. Further study is warranted to more precisely elucidate the role of the KH2 domain in FMRP function.

Discussion

Fragile-X mental retardation results from mutations that lead to loss of FMRP function. In particular, the substitution of an arginine for a conserved isoleucine in the second KH domain results in a severe form of Fragile-X mental retardation syndrome, and abrogates polyribosome association (De Boulle et al., 1993; Feng et al., 1997a).

However, there have been various interpretations of the latter observation, including suggestions that the I304N mutation interferes with protein homodimerization (Laggerbauer et al., 2001), disrupts heterodimerization with FXR2 (Feng et al., 1997a), causes protein instability (Musco et al., 1996), or abrogates sequence-specific RNA binding (Lewis et al., 2000; Ramos et al., 2003a). To specifically target the contributions of KH2 to normal FMRP function in neurons, we have used high affinity RNA ligands to compete for binding to either the KH2 or RGG RNA-binding domain. The kissing complex ligand for KH2 is a unique structure motif that offers clues to the mechanism of FMRP interaction with polyribosomes. The I304N mutant FMRP is associated with abnormally small mRNPs rather than polyribosomes (Feng et al., 1997a), and does not bind kissing complex RNAs in vitro (Darnell et al., 2005a). We find that the association of FMRP with brain polyribosomes is specifically competed by the KH2 kissing complex RNA, but not by G-quartet RNAs that bind to the FMRP RGG domain. Therefore, the FMRP:kissing complex RNA interaction links the neurologic disease of Fragile-X syndrome and the potential role of FMRP in translational regulation.

Biologically relevant kissing loop-loop interactions were first described in tRNA. (Kim et al., 1974; Moras et al., 1980) This subset of loop-loop pseudoknots can be intramolecular, as for tRNA, or intermolecular, such as the HIV RNA genome, which dimerizes via the stem loop of the DIS region. (Paillart et al., 1996) The bicoid mRNA homodimerizes via loop-loop interactions between 3' UTRs of two bicoid messages. This dimerization is required for interaction with Staufen and localization to the anterior pole of the *Drosophila* embryo. (Ferrandon et al., 1997) It is possible that FMRP could bind to two stem-loops on different RNA molecules present in high enough local

concentration. However, our data indicate that an intramolecular kissing complex is required for interaction with KH2.

There are several examples of loop-loop interactions that suppress translation *in vivo*. In the yeast Hac1 mRNA, a site in the 3'UTR forms a duplex with a stem loop in a retained intron, causing translating polyribosomes to stall. (Chapman and Walter, 1997; Ruegsegger et al., 2001) In prokaryotes, two small non-translated RNAs, OxyS and CopA, are complementary to 5' noncoding sequences of target mRNAs, and form intermolecular kissing complexes that suppress translation. (Argaman and Altuvia, 2000; Kolb et al., 2000) The FMRP:kissing complex may stabilize the interaction of two stem-loops in a single mRNA or in distinct messages. It is also possible FMRP is involved in the mechanism of translation inhibition by miRNAs by means of this type of structure.

These results predict that the *in vivo* RNA target(s) of FMRP KH domains harbor kissing complex motifs. Ongoing efforts in our lab and others aim to develop a bioinformatic screen for this complex structure- and sequence- specific motif. In addition, it is possible that previously identified FMRP targets may contain one or both stem-loops of a functional kissing complex.

FMRP interacts with a number of RNA-binding proteins, some of which are also associated with polyribosomes (see Chapter 6 for discussion and references.) As such, it has been suggested that FMRP may bind to polyribosomes indirectly, perhaps through another protein:RNA complex or through interaction with components of the RISC complex and/or miRNAs. However, our data support the direct association of FMRP with RNAs that contain a kissing complex motif, and that are bound by ribosomes.

FMRP may independently bind RNAs harboring G-quartets or kissing complex motifs, or may interact with both motifs at the same time. Such interactions could be in trans, such that FMRP binds both motifs in independent RNA molecules, facilitating interaction between different RNA-protein complexes. Alternatively, FMRP may bind single transcripts harboring both G-quartet and kissing complex RNA targets. Furthermore the combination of these distinct binding sites may be key to the overall function of FMRP. While KH2 binding to kissing complex RNA allows FMRP to interact with polyribosomes, the RGG:G-quartet complex may influence translational regulation by an additional or complementary mechanism. Moreover, G-quartet RNA binding by FMRP may also be involved in other aspects of the export and localization of target mRNAs.

We have found that kissing complex RNA is able to displace FMRP from polyribosomes at ~100-nM concentration. This disruption is very specific for the kcRNA ligand, since FMRP polyribosome association is not affected by kcRNA harboring a single point mutation, nor by 50-fold higher concentrations of G-quartet RNA, tRNA, or BC1 RNA. These observations clearly indicate that the ability of the KH2 domain to specifically bind kcRNA is critical for the association of FMRP with brain polyribosomes and may be central to the molecular pathophysiology of Fragile X syndrome.

Acknowledgements

Jennifer Darnell identified and characterized both high affinity RNA ligands for FMRP. Without this meticulous and ground-breaking work, I would not have been able to do the experiments discussed here. In addition, Jennifer's suggestions, reagents,

protocols, and general enthusiasm throughout much of this work have been invaluable. Julie Zang and Jennifer optimized the CLIP protocol for FMRP. Jennifer provided the recombinant KH2 isoforms used in Figure 5.9.

CHAPTER VI: FXR1 AND FXR2 BIND POLYRIBOSOMES VIA KISSING COMPLEX RNA

Introduction

Local control of protein synthesis in specific sub-cellular domains is an important aspect of the regulation of gene expression in neurons. In particular, regulation of translation at post-synaptic sites plays a role in activity-dependent synaptic changes. A number of RNA-binding proteins have been shown to regulate the translation of their target mRNAs. One family of candidate RNA-binding proteins for this regulatory role in neurons is the Fragile X related (FXR) family of FMRP, and the Fragile X mental retardation-related proteins 1 and 2 (FXR1 and FXR2). The absence of FMRP or FXR2 produces a neurologic phenotype in mouse models, and these proteins have been localized to sub-synaptic areas of dendrites.

The phenotype of fragile X syndrome includes mild to severe cognitive deficits, attention-deficit hyperactivity disorder, autistic-like behavior, abnormal facial features such as a prominent jaw and large ears, macroorchidism in postpubescent males, and some connective tissue abnormalities. *Fmr1* knockout mice have subtle defects in behavior, learning and motor coordination, as well as an increased sensitivity to audiogenic epileptic seizures, and increased anxiety. FMRP is normally expressed in most tissues at variable levels, although the highest expression is observed in brain and testis. Despite the presence of an NLS, the majority of FMRP is located in the cytoplasm. FMRP has been shown to shuttle between the nucleus and cytoplasm and has been localized to polyribosomes at synapses in neurons. Since FMRP is associated with polyribosomes at synapses, and since the FMRP null phenotype includes cognitive

impairment and synaptic abnormalities, FMRP may be involved in the regulation of local translation in neurons. (Reviewed in (O'Donnell and Warren, 2002; Willemsen et al., 2004).)

FMRP has two autosomal paralogs, FXR1 and FXR2, which share >60% amino acid identity. Like FMRP, both FXR1 and FXR2 include two N-terminal tandem KH-type RNA-binding domains, as well as an RG-rich region in the C-terminal region. However, if one considers only the two KH domains, the percentage of conserved similar residues rises to 90% (Figure 6.1). (The extended variable loop for FMRP is dispensable for RNA binding by KH2 and has been excluded from this comparison (Darnell et al., 2005a).) In contrast, the RG-rich regions of FXR1 and FXR2 are less than 40% identical to that of FMRP (Figure 6.2). Both FXR1 and FXR2 have been shown to associate with polyribosomes in the presence or absence of FMRP. (Corbin et al., 1997; Feng et al., 1997a) In addition, FMRP can heterodimerize with FXR1 and FXR2, an interaction that is not RNA-dependent, but may involve the KH domains of both partners. (Siomi et al., 1994)

Like FMRP, FXR2 is expressed in the brain and testes, with the majority of protein in the cytoplasm. *Fxr2*-null mice display increased hyperactivity, impaired motor coordination, impaired fear conditioning, and decreased ability to locate the platform in the Morris water maze. (Bontekoe et al., 2002) In contrast, FXR1 is the only family member with a critical role in skeletal and cardiac muscle development and function. FXR1 is localized to costameres within muscle fibers, and is important for proper localization of vinculin, dystrophin and alpha-actinin. It has been suggested that FXR1 may have a primary role in the regulation of transport and translation of costameric

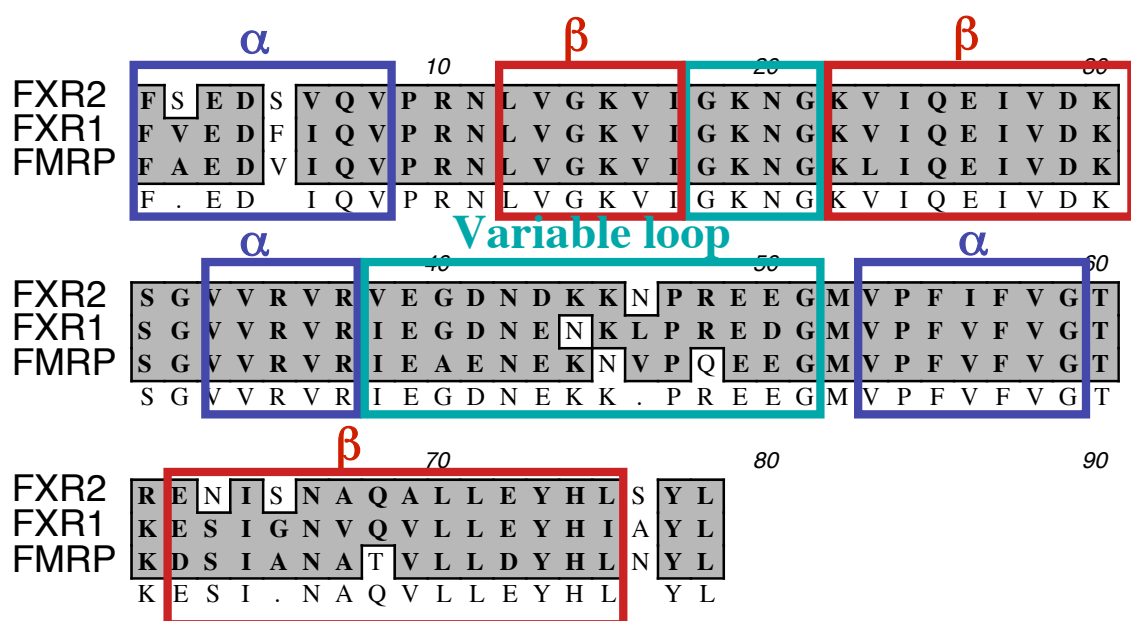


Figure 6.1 Alignment of minimal KH domains of mammalian FXR family members FMRP, FXR1 and FXR2.

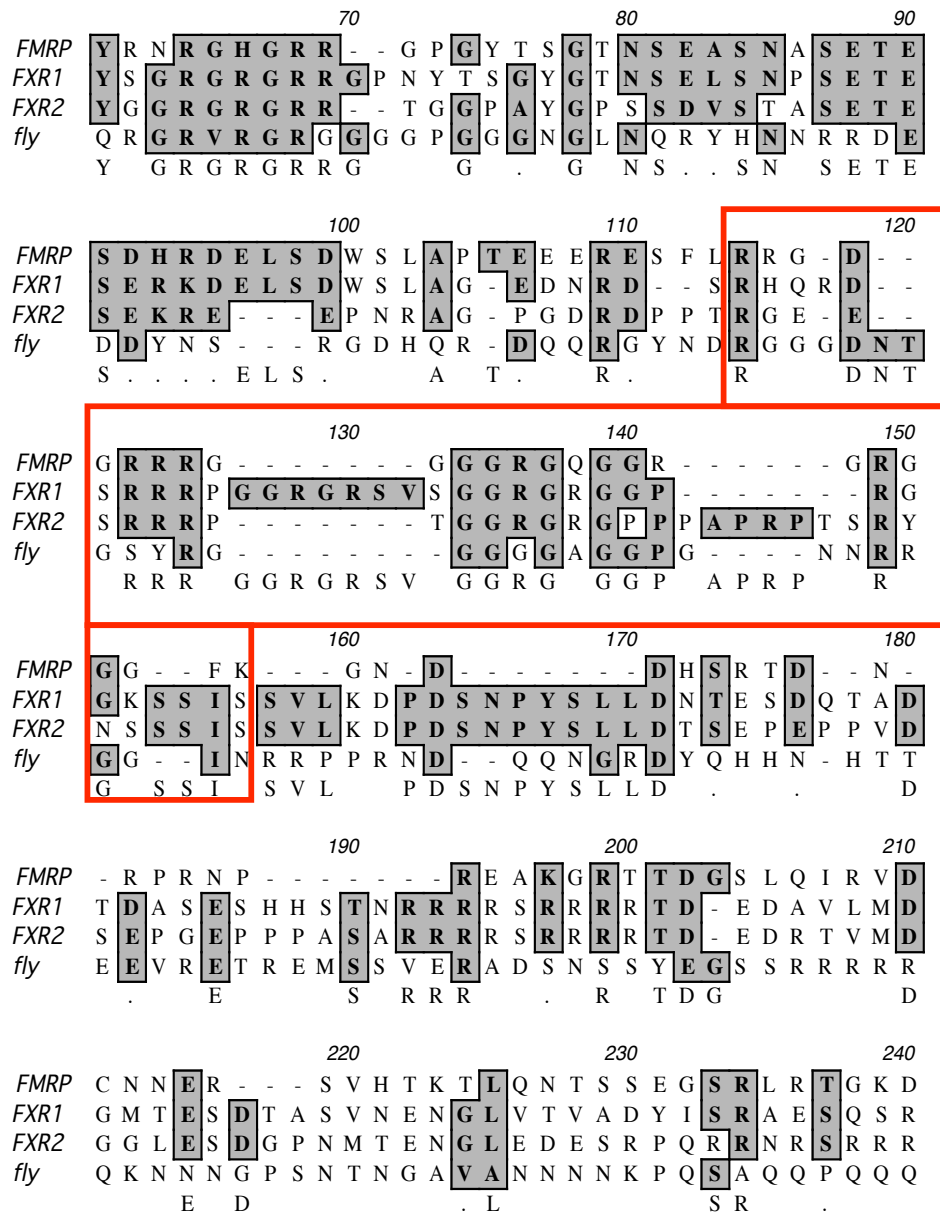


Figure 6.2 Alignment of C-terminal regions from mammalian and fly FXR family proteins. Red box outlines RGG box domain of FMRP.

mRNAs. In addition to skeletal and cardiac muscle, FXR1 is highly expressed in the central nervous system and gonads. *Fxr1*-knockout mice die shortly after birth, likely due to cardiac or respiratory failure. Mice homozygous for a hypomorphic allele of *Fxr1*, *Fxr1*+neo, show reduced expression of FXR1 protein, and live for several months after birth. Due to severely impaired muscle development, behavioral analysis of these mice has not been attempted. (Mientjes et al., 2004) An investigation of the CNS role of FXR1 requires a tissue-specific conditional knockout. There are no known human diseases due to mutations in either *Fxr2* or *Fxr1*.

It has been suggested that the FXR family members may display significant functional redundancy in the cells where they are co-expressed. This is supported by the subtle neurologic phenotypes observed in mice that lack FMRP or FXR2. Thus, a double knockout may reveal a more obvious phenotype, and provide additional clues to the roles of both proteins. *Fmr1/Fxr2* double-knockout mice have been generated by the Nelson and Oostra laboratories. Compared to single knockout littermates, these mice exhibit increased hyperactivity in the open field test, decreased habituation to a novel environment, decreased prepulse inhibition to an acoustic startle, and impaired fear conditioning. (Spencer et al., 2006)

The *Drosophila* genome contains a single homolog of the FXR family, *dfmr*. This protein does not contain a functional RGG box (J. C. Darnell, manuscript in preparation); however, the two KH-type RNA-binding domains of dFmr are highly conserved through mouse and human homologs. *dfmr* mutant flies exhibit defects in neurite extension, branching and guidance. (Michel et al., 2004; Morales et al., 2002) In addition, they display arrhythmic circadian activity (Dockendorff et al., 2002; Inoue et al., 2002) and an

inability to maintain courtship interest. (Dockendorff et al., 2002) The alterations in circadian rhythm of *dfmr* mutant flies echo the sleep disturbances characteristic of some patients with Fragile-X syndrome (Gould et al., 2000). Thus, there is evidence that, in addition to a highly conserved sequence, the three FXR family members in mammals may share similar functions in neurons.

We have recently shown that FMRP binds to kissing complex RNA (kcRNA) via KH2, and that the FMRP:polyribosome complex can be disrupted by excess amounts of kcRNA. Since the KH domains of all three FXR family members are so highly conserved, it is of interest to test whether FXR1 and FXR2 behave in the same manner. FXR1 and FXR2 heterodimerize with FMRP, and may be dissociated from polyribosomes by kcRNA for three reasons. First, they may be shifted off polyribosomes when their protein-protein binding partner, FMRP, loses its association with an mRNA undergoing translation. In this case, the heterodimer would remain intact, but RNA binding by FMRP KH2 would be disrupted. Second, kcRNA may disrupt the protein-protein interaction between FMRP and FXR1 or FXR2. Here, both proteins would shift off polyribosomes, but their ability to interact would be abrogated. Third, if the KH2 domain of FXR1 and/or FXR2 is able to bind kcRNA directly, kcRNA will disrupt the association of these proteins with polyribosomes. More importantly, this effect will be observed in brain lysates of both wild type and *Fmr1* knockout mice. Our data support the third possibility, and suggest that the KH2 domains of FMRP, FXR1 and FXR2 associate with polyribosomes via kissing complex RNA motifs.

In addition to FXR1 and FXR2, FMRP has been identified as a binding partner for many proteins, some of which also bind RNA and/or are associated with

polyribosomes. Notable interactors include: NUFIP1 (Bardoni et al., 1999; Bardoni et al., 2003b); CYFIP1 and CYFIP2 (Schenck et al., 2001); 82-FIP (Bardoni et al., 2003a); Nucleolin (Ceman et al., 1999); YB1 (Ceman et al., 2000); Pur α , mStaufen and Myosin Va (Ohashi et al., 2002); microspherule protein 58 (Davidovic et al., 2006); PAR6, mLgl and aPKC ζ (Zarnescu et al., 2005); ribosomal proteins L5 and L11; Dmp68 (Ishizuka et al., 2002); and the Argonaute protein Ago2 (eIF2C2) (Caudy et al., 2002; Ishizuka et al., 2002; Jin et al., 2004). Our high-affinity RNA ligands for the KH2 and RGG domains of FMRP are useful tools to study whether these domains are required for interaction with the above proteins, and whether any of these interacting proteins remain associated with FMRP even after it has been dissociated from polyribosomes by *kcRNA*.

Finally, our domain-specific high-affinity RNA ligands, *kcRNA* and *gqRNA*, represent unique tools to address the contributions of each RNA-binding domain for the FXR protein family. By selectively targeting the RNA-binding ability of KH2, *in vivo* expression of these ligands as “RNA decoys” can overcome the overlapping roles of FMRP, FXR1 and FXR2, creating a KH2-domain functional hypomorph for all three genes.

Results

kcRNA dissociates FXR1 and FXR2 from polyribosomes

The KH domains of FMRP, FXR1 and FXR2 are highly conserved, as shown in Figure 6.1, whereas the RG-rich C-terminal regions of these proteins display little similarity (Figure 6.2). In addition FMRP has been shown to interact with both FXR1 and FXR2. We have previously shown that FMRP dissociates from polyribosomes when

treated with 30mM EDTA or 500nM kcRNA, but not 500nM gqRNA (Chapter 5 and (Darnell et al., 2005a)). For these reasons, we probed Western blots from Figure 5.4 with an antibody to FXR2 (1G2). As shown in Figure 6.3, when a post-mitochondrial supernatant from wild type mouse brain is fractionated on a 20-50% sucrose gradient, FXR2 cosediments with polyribosomes. Treatment with EDTA shifts FXR2 to fractions 3-8 (of 16). When brain lysate is incubated with kcRNA (500nM kc2), but not gqRNA (500nM sc1), FXR2 no longer cosediments with intact polyribosomes. Thus, the effect of an excess of kissing complex RNA is not specific to FMRP, but may extend to other FMRP-interacting proteins and/or other proteins able to bind kcRNA.

The KH domains of FXR family members are highly conserved from *Drosophila melanogaster* through *Homo sapiens*. (Morales et al., 2002) As such, we investigated whether human FXR family members also were susceptible to competition by kcRNA. IMR-32 cells, originally derived from a human neuroblastoma were lysed with 0.3% NP40 in a hypotonic buffer. A post-mitochondrial supernatant was incubated with

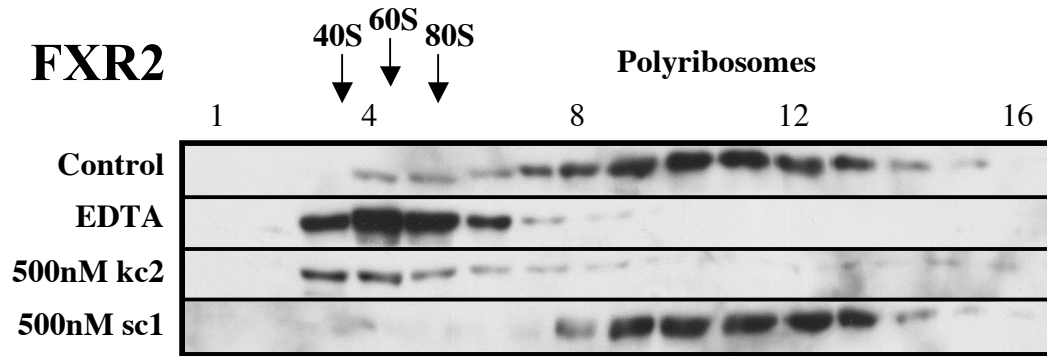


Figure 6.3 FXR2 is shifted off polyribosomes by EDTA and kissing complex RNA. Polyribosome gradient separations were performed from wild type mouse cerebral cortex and subjected to incubation with 30mM EDTA, 500nM kc2 RNA or 500nM sc1 RNA. Western blots of TCA-precipitated gradient fractions are shown for FXR2.

500nM RNA or an equal volume of RNA-folding buffer, and loaded onto a 20-50% sucrose gradient. Figure 6.4 shows polyribosome profiles (A) and Western blots (B) from samples treated with kc2 RNA, mutant kc2 RNA or sc1 RNA. Both FMRP and FXR1 fail to cosediment with polyribosomes in the presence of 500nM kcRNA, but not mutant kcRNA or gqRNA. Therefore, the ability to interact with polyribosomes via kcRNA is conserved in mouse and human FXR family members.

FXR1 and FXR2 associate with kcRNA and polyribosomes independently of FMRP

Additional experiments by J. C. Darnell show that recombinant KH2 domains of FXR1 and FXR2 bind kcRNA *in vitro*, and that this interaction is abrogated by mutations in FXR1 and FXR2 that are analogous to the FMRP I304N mutation in KH2 (data not shown). Moreover, the dfmr KH2 domain also binds kcRNA with a similar affinity. The KH1 domains of all three family members do not bind kcRNA. Interestingly, while FMRP binds gqRNA with high affinity (Darnell et al., 2001), the C-termini of FXR1 and

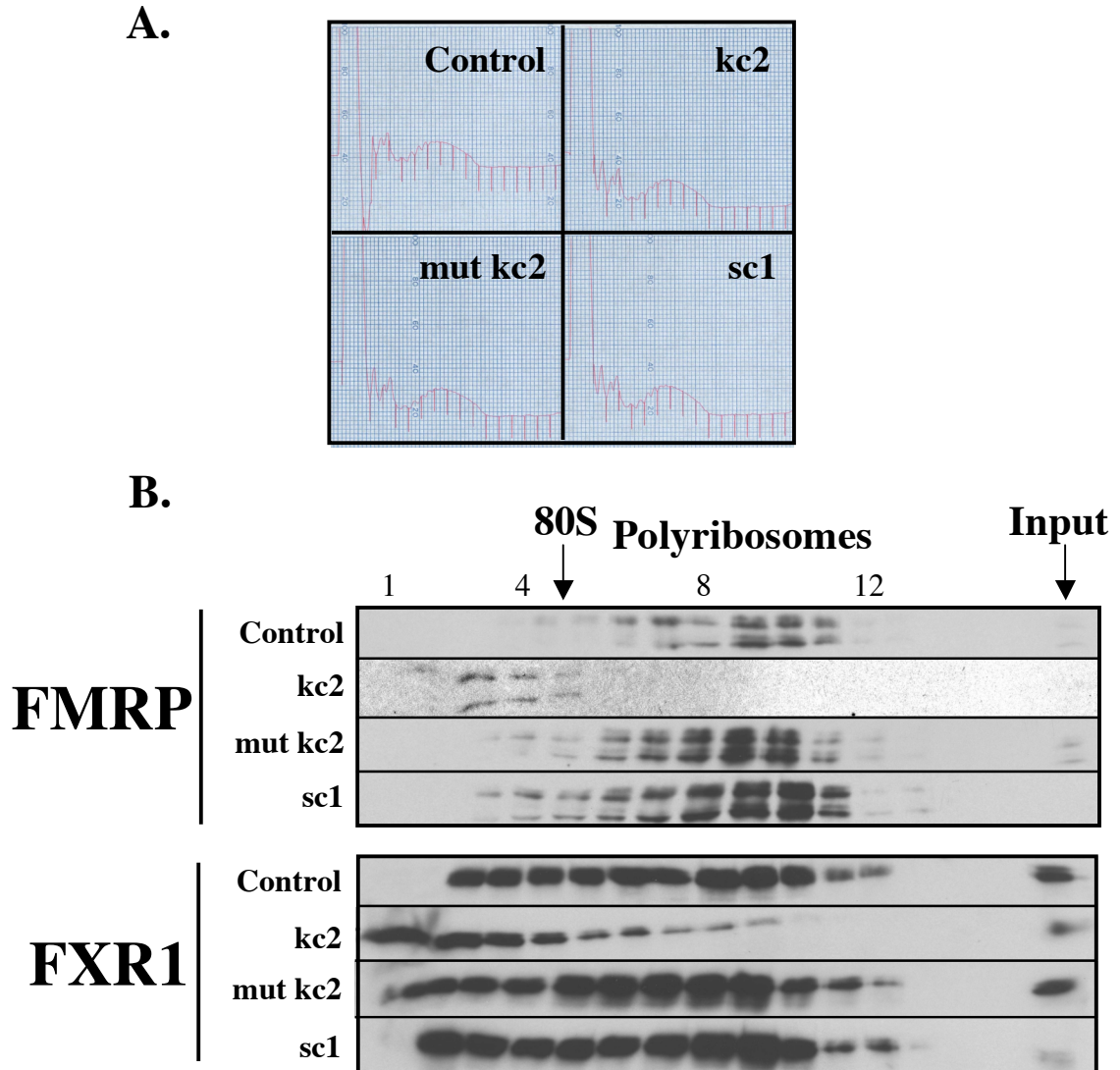


Figure 6.4 FMRP and FXR1 are competed off polyribosomes by kissing complex RNA in human neuroblastoma cells. UV absorbance profiles (**A**) and Western blots (**B**) against FMRP and FXR1 for polyribosome gradient fractionations of IMR32 cell cytoplasmic extracts. After cell lysis with hypotonic buffer containing 0.3% NP40, post-mitochondrial supernatants were incubated with 500nM kc2, mutant kc2, or sc1 RNAs, or an equal volume of RNA binding buffer (Control), before 20-50% sucrose gradient centrifugation.

FXR2 do not bind gqRNA, a result that is supported by the low conservation of these domains (Figure 6.2).

In order to differentiate between the possibilities that FXR1 and FXR2 might be sensitive to kcRNA by direct binding or through their heterodimerization with FMRP, kcRNA incubation experiments were performed using cerebral cortices from FMRP knockout mice. Polyribosomal profiles are intact in FMRP knockout mice, with or without incubation with 500nM RNA (Figure 6.5A, and ribosomal protein L7 in Figure 6.5B). FXR1 and FXR2 cosediment with polyribosomes in the absence of FMRP (Figure 6.5B). Incubation of FMRP KO brain lysate with 500nM kc2 RNA results in a shift of both FXR1 and FXR2 from bimodal peaks in fractions 3-6 and 9-11 (of 16) to a single peak in fractions 1-7. Interestingly, nine percent each of FXR1 and FXR2 may indeed interact with polyribosomes via FMRP, since there is a reproducible shift of these proteins when the distributions are compared between samples from FMRP WT and KO mice. However, it is evident in Figure 6.5 that both FXR1 and FXR2 are able to associate with polyribosomes by binding kissing complex RNA, irrespective of whether FMRP is present.

Unrelated polyribosome-bound proteins do not dissociate with kcRNA

To test whether the ability of excess kcRNA to disrupt interaction with polyribosomes is specific for the FXR family of RNA-binding proteins, gradient fractions from Figure 6.5 were probed for a several neuronal non-FXR RNA-binding proteins. Elongation factor 1alpha (EF1 α , Figure 6.6A), the neuronal ELAV-related proteins HuB, HuC, and HuD (Hu, Figure 6.6B), and eukaryotic translation initiation factor 4E (eIF4E,

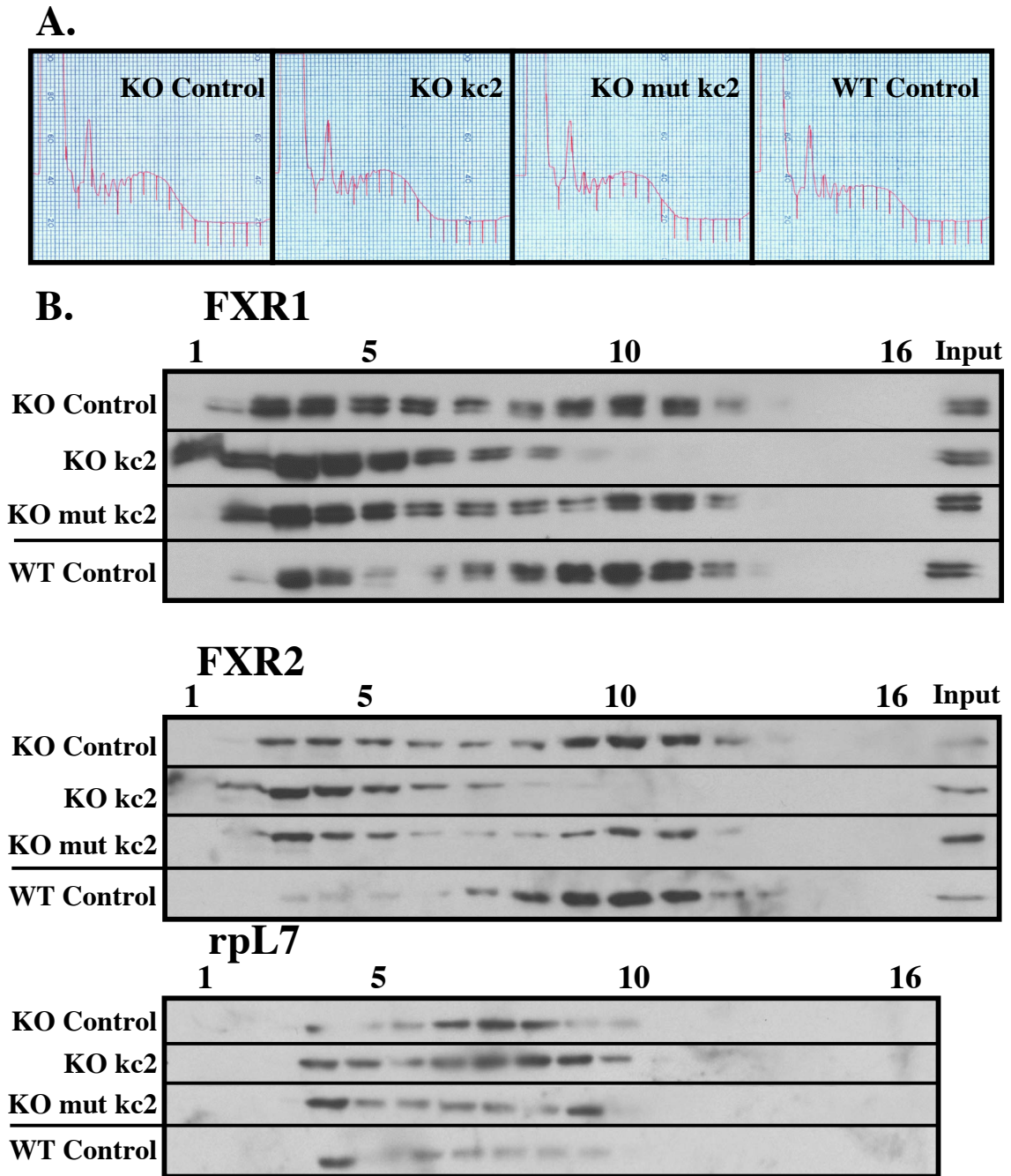


Figure 6.5 FXR1 and FXR2 are shifted off polyribosomes by kissing complex RNA independently of FMRP. A254 traces (A) and Western blots (B) from polyribosome separations of wild type and FMRP knockout mouse cerebral cortices. Post-mitochondrial supernatants were incubated with 500nM kc2 (kc2) or mutant kc2 (mut kc2) RNAs or RNA binding buffer (Control) before sucrose gradient centrifugation.

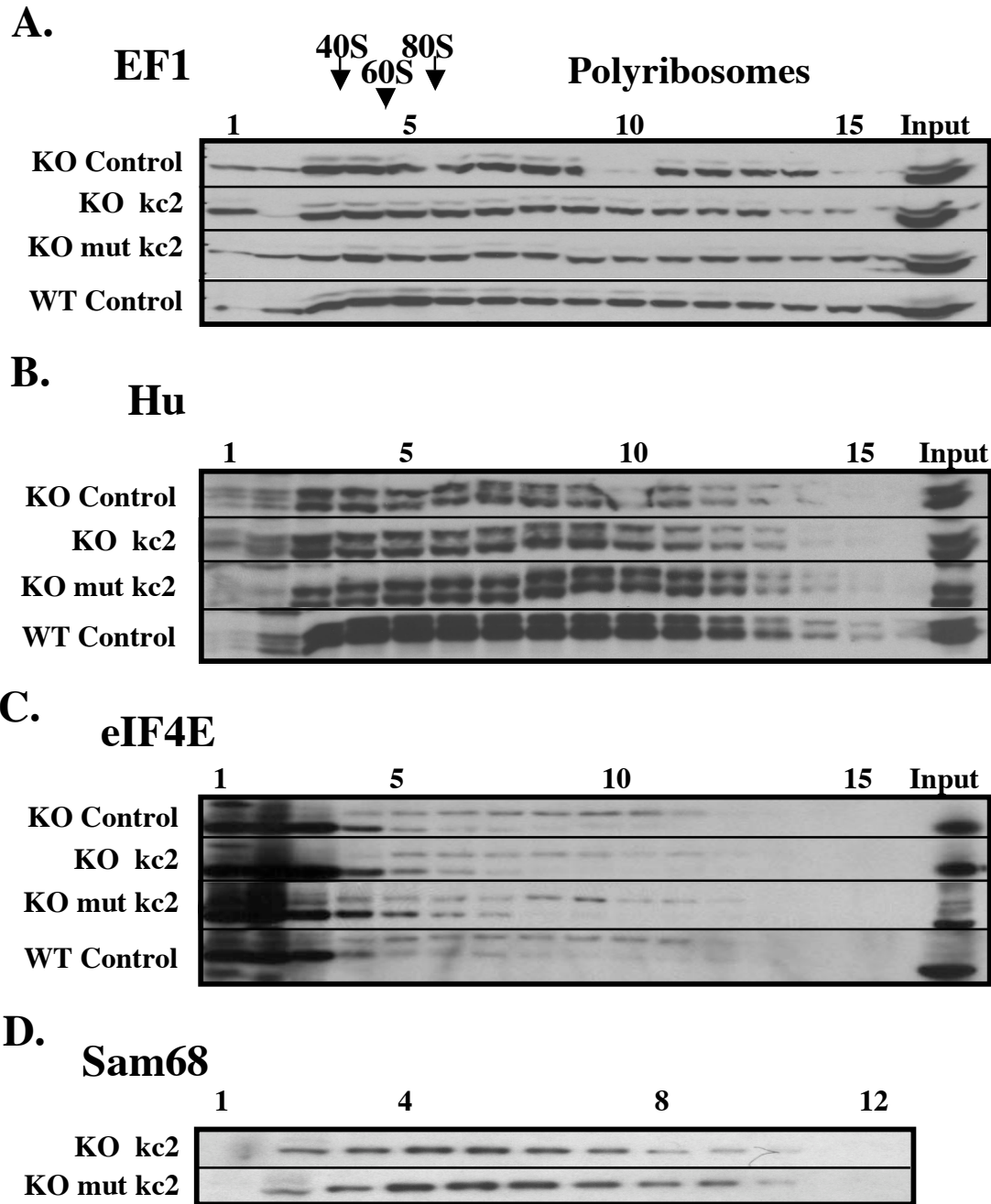


Figure 6.6 Control polyribosome-associated proteins do not shift with kissing complex RNA. Western blots of TCA-precipitated gradient fractions shown in Figure 6.5 (A-C) or a replicate experiment (D) were probed for polyribosome-associated proteins that are not known to interact with FMRP: elongation factor 1alpha (A), the neuronal Hu proteins HuB, HuC and HuD (B), translation initiation factor 4E (C), and Sam68 (Src-associated in mitosis, 68kDa, D).

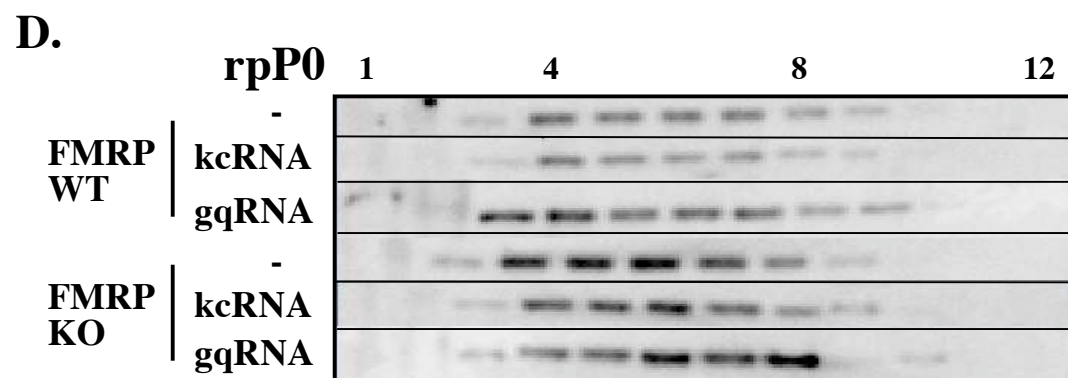
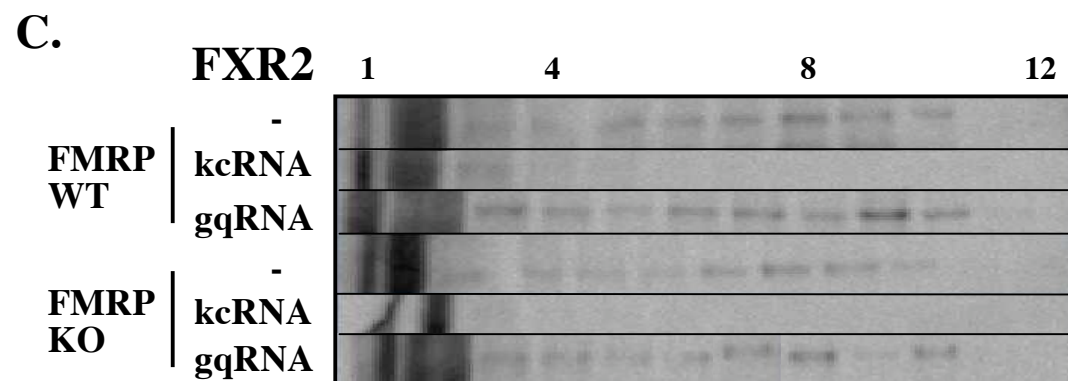
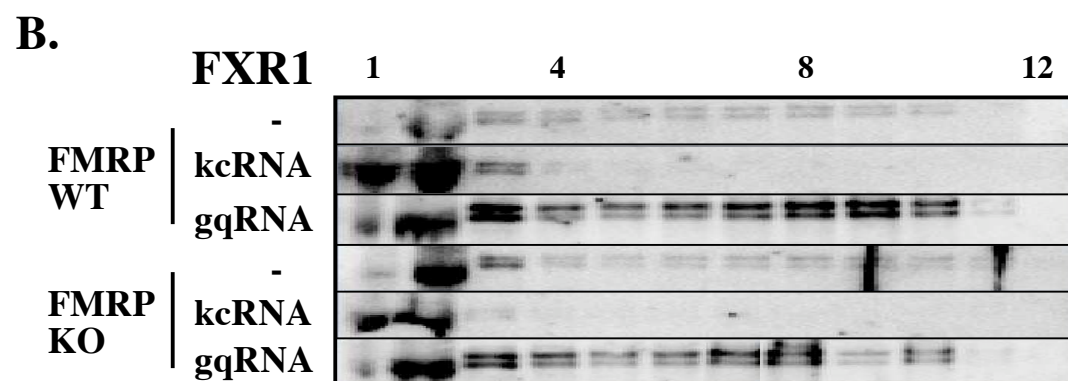
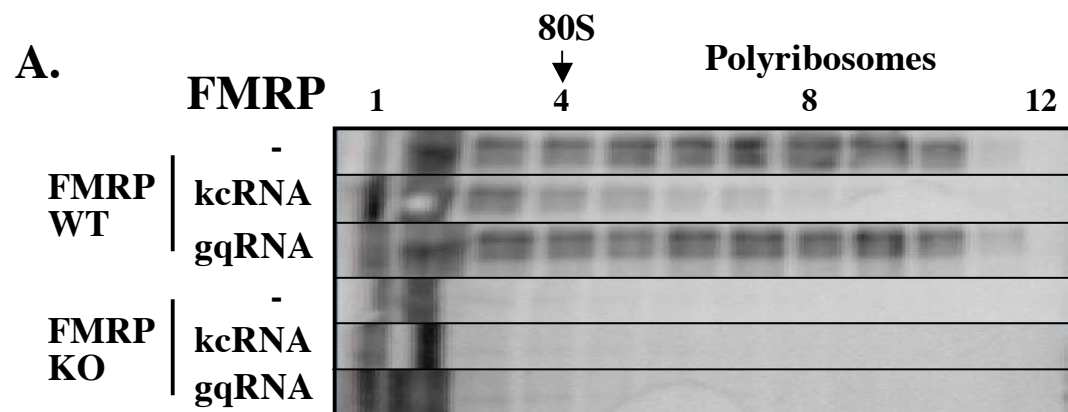
Figure 6.6C) are all unaffected by the absence of FMRP and incubation with kcRNA. Fractions from a similar experiment were probed for the RNA splicing and export factor Sam68 (Figure 6.6D), which remains bound to polyribosomes in the presence of kcRNA.

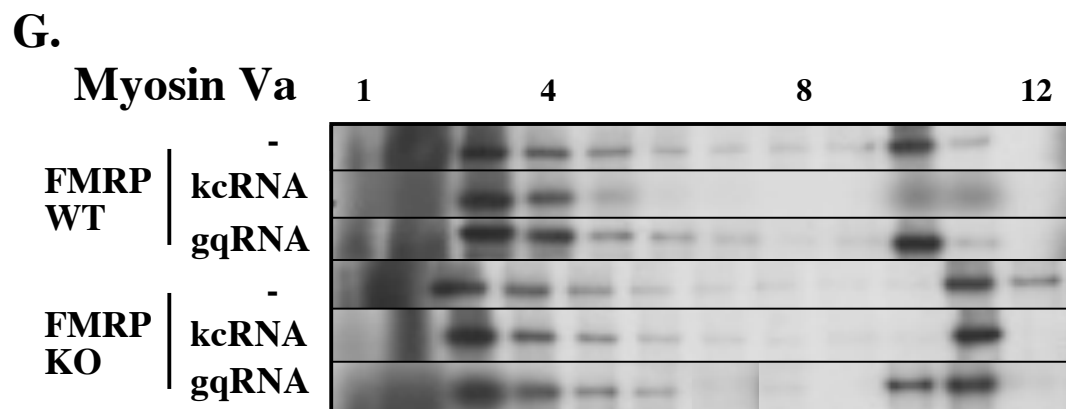
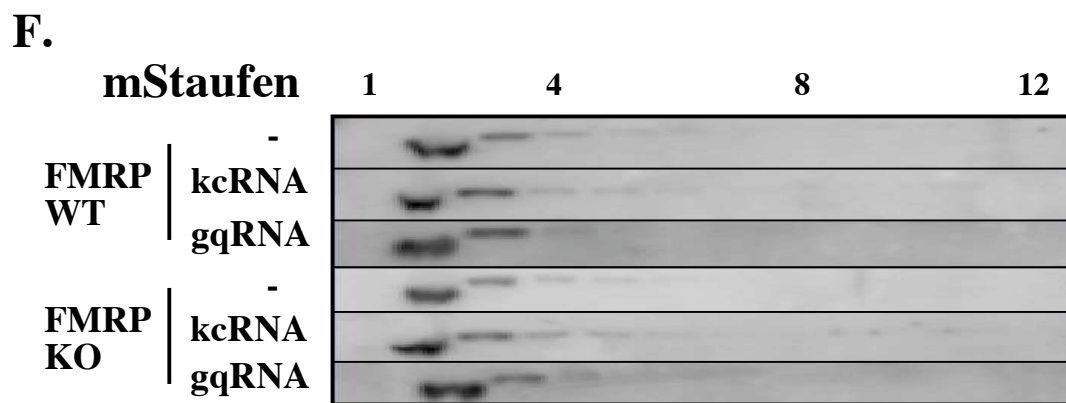
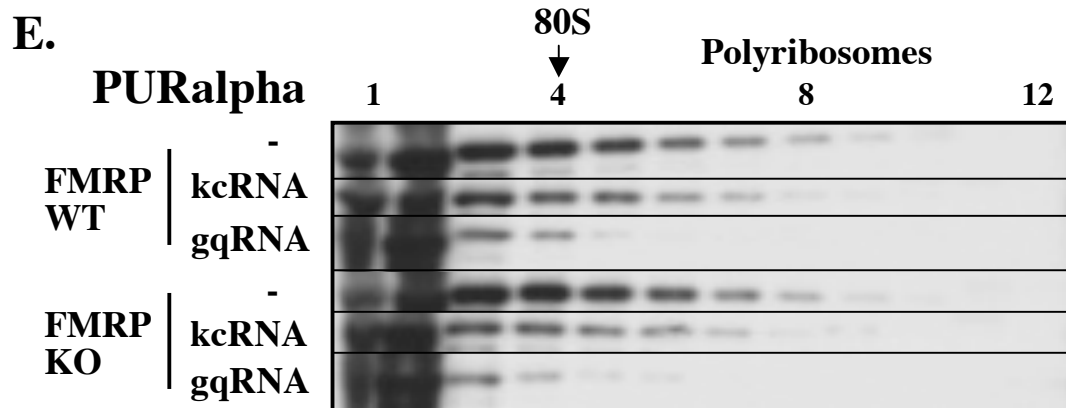
Polyribosomal distributions of FMRP-interacting proteins

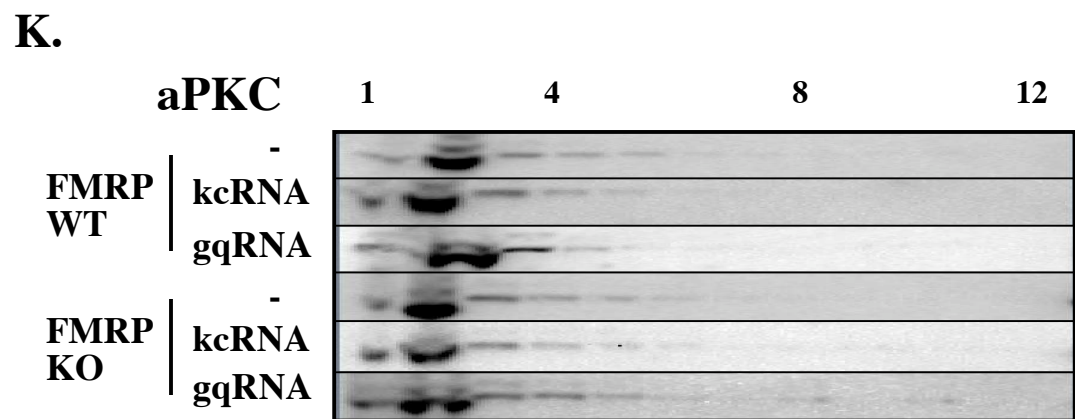
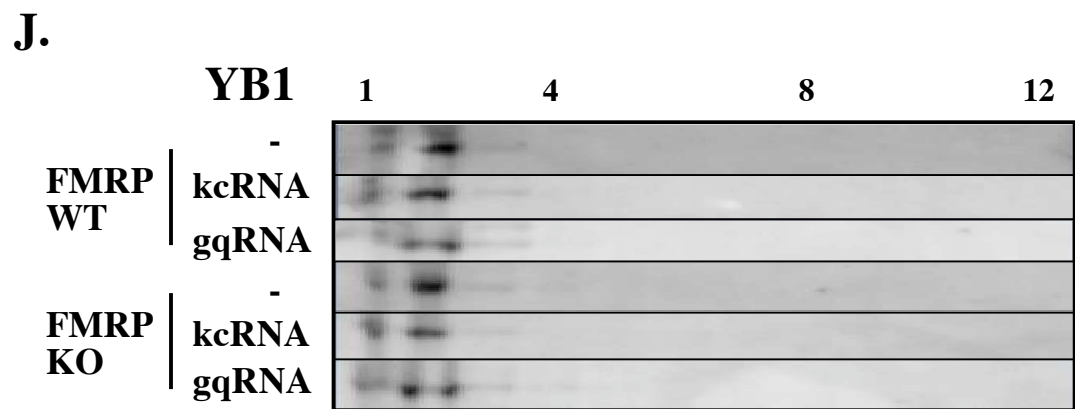
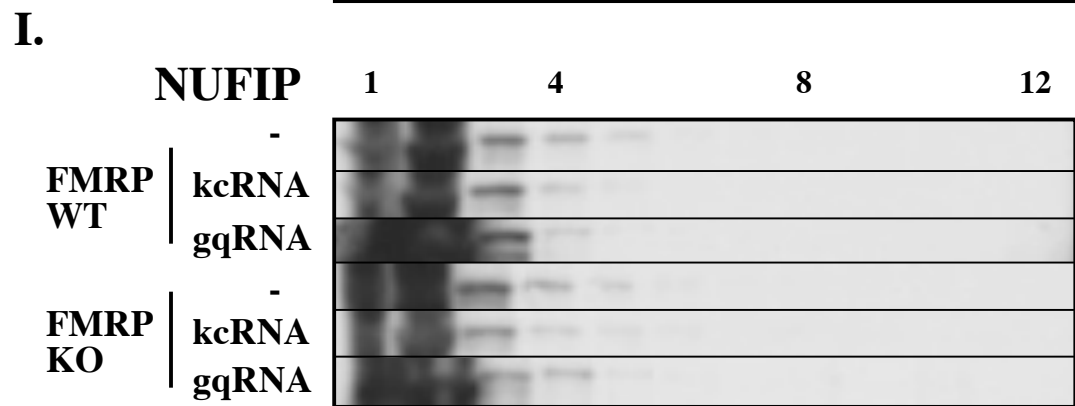
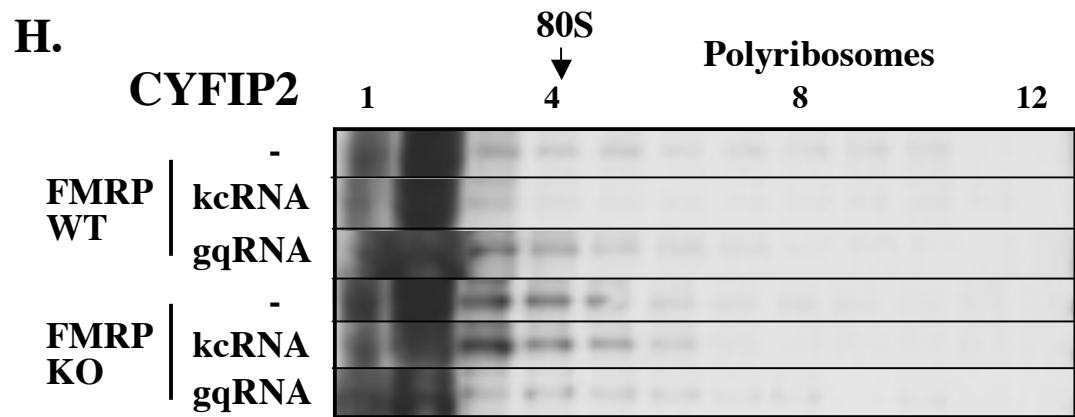
FMRP has been shown to co-precipitate with a large number of neuronal proteins, which include nuclear FMRP-interacting protein 1 (NUFIP1) and 82-FIP (Bardoni et al., 1999; Bardoni et al., 2003b); cytoplasmic FMRP-interacting proteins 1 and 2 (CYFIP1 and CYFIP2) (Schenck et al., 2001); Nucleolin (Ceman et al., 1999); Y box-binding protein 1 (YB1) (Ceman et al., 2000); Pur α , mStaufen and Myosin Va (Ohashi et al., 2002); the mouse homolog of *Drosophila lethal giant larvae* (mLgl) and atypical Protein Kinase C zeta (aPKC ζ) (Zarnescu et al., 2005); ribosomal proteins L5 and L11; Dmp68 (Ishizuka et al., 2002); poly(ADP-ribose) glycohydrolase (PARG) (Gagne et al., 2005); the Argonaute protein Ago2 (eIF2C2) (Caudy et al., 2002; Ishizuka et al., 2002; Jin et al., 2004); Ran-binding protein 9 (RanBP9/RanBPM) (Menon et al., 2004); and nuclear export receptor NXF2 (Lai et al., 2006). Using antibodies against some of these proteins, we have screened Western blots from polyribosome gradients of fractionated wild type or FMRP knockout brain lysate treated with kcRNA or gqRNA (Figure 6.7). Figure 6.7A demonstrates that FMRP is shifted off polyribosomes when treated with kcRNA, compared to control (-) and gqRNA-treated samples. (Signal evident in lanes one and two of the FMRP knockout samples is non-specific background chemiluminescence due to the large amount of protein loaded in these lanes. These samples are from FMRP knockout brain lysate.) Confirming results shown in Figures 6.3, 6.4 and 6.5, FXR1

Figure 6.7 Polyribosome distributions of FMRP-interacting proteins in mouse brain.

Western blots of TCA-precipitated sucrose gradient fractions from wild type or FMRP knockout mouse cerebral cortex polyribosomes. Post-mitochondrial cortex supernatants were treated with kc7 RNA (kcRNA), sc1 RNA (gqRNA) or RNA binding buffer (-). The gradient positions of the 80S single ribosome and polyribosomes are indicated. Blots were probed with antibodies to FMRP (**A**, 2F5), FXR1 (**B**, 830), FXR2 (**C**, 1G2), ribosomal protein P0 (**D**, Biodesign), Pur alpha (**E**, Abnova), mouse Staufen (**F**, Chemicon), the neuronal myosin Va (**G**, Sigma), cytoplasmic FMRP-interacting protein 2 (**H**, courtesy of B. Bardoni), nuclear FMRP-interacting protein (**I**, courtesy of B. Bardoni), Y-box binding factor 1 (**J**, Abcam), and the atypical Protein Kinase C zeta (**K**, Sigma).







(Figure 6.7B) and FXR2 (Figure 6.7C) cosediment with polyribosomes in the presence and absence of FMRP. This interaction is disrupted by kcRNA, but not gqRNA, in both wild type and FMRP knockout cerebral cortex. The distribution of ribosomal protein P0, a component of the 60S subunit, is not altered by a lack of FMRP or incubation with kcRNA or gqRNA (Figure 6.7D).

Ohashi and colleagues identified an mRNP containing FMRP, Pur α , mStaufen and myosin Va (Ohashi et al., 2002). This complex is released from polyribosomes by EDTA, and is abolished by treatment with RNase. Pur α binds both single-stranded DNA and RNA, and is reported to control the expression of BC1 RNA, as well as that of a number of other genes. (Kobayashi et al., 2000; Ohashi et al., 2000; Zhang et al., 2005) It also cosediments with polyribosomes, and Pur α -null mice exhibit impaired neural development. (Gallia et al., 2000; Khalili et al., 2003) Therefore, it has been proposed that Pur α may assist mRNP assembly in an RNA-dependent manner and be involved in targeting mRNPs to polyribosomes in cooperation with other RNA-binding proteins (Ohashi et al., 2002). We find that the majority of cytoplasmic Pur α does not cosediment with polyribosomes, but is in fact found in the upper region of the gradient, likely in a non-polyribosomal mRNP (Figure 6.7E). However, there is a portion of Pur α that is found in fractions 4-9 that may be bound to polyribosomes. This interaction is not affected by the loss of FMRP (FMRP KO -), but does exhibit a slight shift in the presence of kcRNA, and a more significant shift in the gqRNA-treated gradients. These shifts do not require FMRP, since the kcRNA and gqRNA shifts are comparable between wild type and knockout gradients. This finding clearly demonstrates that cytoplasmic Pur α interacts with large complexes via RNA, but that this interaction may not be sequence-

specific. Further study of the literature reveals that Puro α binds G-rich sequences in both RNA and single-stranded DNA. (Gallia et al., 2000) Since both kcRNA and gqRNA contain stretches of multiple G residues (gqRNA more so than kcRNA), it is likely that these spiked-in RNAs compete with endogenous targets for binding by Puro α . This may be related to FMRP biology in that Puro α could be bound to mRNA cargoes that also carry FMRP. It is likely that specific interaction of Puro α with an mRNA does not depend on the RNA binding abilities of FXR KH domains or the FMRP RGG box.

mStaufen, the mouse homolog of *Drosophila staufen*, is an RNA-binding protein thought to be involved in mRNA transport and localization. In addition to the coimmunoprecipitation of mStaufen with Puro α and FMRP (Ohashi et al., 2002), the Ortin laboratory reported that FMRP copurifies with granules containing hStaufen, ribosomes, cytoskeletal control proteins, and motor proteins (Villace et al., 2004). We find that the majority of mStaufen is found in fraction 2 (of 12), a region of the 20-50% sucrose gradient that contains mRNPs, and possibly 40S ribosomal subunits (Figure 6.7F). Faint bands can be seen in fractions 3-8, indicating that mStaufen may be present on polyribosomes, as has been previously reported (Ohashi et al., 2002). Incubation with kcRNA or gqRNA does not appear to alter the distribution of mStaufen in either wild type or FMRP knockout cerebral cortex.

A fourth component of the mRNP containing FMRP, Puro α and mStaufen is myosin Va, a non-muscle myosin that is expressed in brain and involved in the transport of vesicles and mRNAs (Evans et al., 1998). When centrifuged through a 20-50% sucrose gradient, myosin Va exhibits an unusual distribution. There are two peaks present: the first in fraction three, corresponding to mRNPs and the 40S ribosomal

subunit, and the second in fractions 10-12, possibly containing large granules and/or cytoskeletal networks (Figure 6.7G). Of note, while others have described an association of FMRP with cytoplasmic granules (De Diego Otero et al., 2002; Krichevsky and Kosik, 2001; Mazroui et al., 2002; Villace et al., 2004), in our hands, this is the first evidence of a complex of that size, as assayed by gradient centrifugation. We do not see a granule “peak” in polyribosome profiles from brain and tissue culture cells. FMRP, FXR1 and FXR2 are present in fractions 10-11, but do not display the same peak of protein concentration that is seen for myosin Va. Myosin Va is present in the intervening fractions 4-9. The distribution in fractions 3-9 does not change with the absence of FMRP or treatment with kcRNA or gqRNA. The myosin Va peak in fractions 10-11 does seem to shift from fraction ten in wild type brain, to fraction eleven in FMRP knockout brain. However, this change in distribution is only one fraction, which could be due to technical artifact in gradient fraction collection. Further analysis requires better resolution of these fractions (e.g. a 35-60% sucrose gradient divided into more fractions of smaller volume.) From these data, myosin Va does not display a gross change in polyribosome distribution when treated with kcRNA or gqRNA.

CYFIP1 and CYFIP2 share 88% sequence identity, and contain no known functional motifs. They were first identified as FMRP-interacting proteins through a yeast two-hybrid screen (Schenck et al., 2001). In addition, the *Drosophila* proteome contains a single homolog, CYFIP/Sra-1, that interacts with dFmr1 (Schenck et al., 2003). Interestingly, CYFIP1 interacts only with FMRP, while CYFIP2 interacts with all three of the FXR family members via the same N-terminal domain that mediates heterodimerization between FXR proteins. CYFIP1 interacts with Rac1 (Kobayashi et

al., 1998), a Rho GTPase, and both family members are thought to regulate actin polymerization to shape synapse morphology as part of the WAVE/SCAR complex (Kim et al., 2006; Pilpel and Segal, 2005; Schenck et al., 2004). We find that a small amount of CYFIP2 cosediments with polyribosomes in an FMRP-independent manner (Figure 6.7H). Incubation with kcRNA or gqRNA does not alter this distribution.

Nuclear FMRP-interacting protein 1 (NUFIP1) is a nucleocytoplasmic shuttling protein predominantly found in the nucleus. However, it has been co-localized with subsynaptic polyribosomes by electron microscopy (Bardoni et al., 2003b). NUFIP1 interacts with the N-terminal region of FMRP involved in heterodimerization and binds ribohomopolymers *in vitro*, specifically poly(G) and poly(U) (Bardoni et al., 1999). In contrast to the co-localization of NUFIP1 with polyribosomes, we find that NUFIP1 does not cosediment with polyribosomes in brain lysate from either wild type or FMRP knockout mice (Figure 6.7I). These findings are not mutually exclusive, as NUFIP1 may be incorporated in mRNPs that are translationally repressed and in a reserve pool at the synapse. Furthermore, unlike Pur α , NUFIP1 distribution does not change when incubated with kcRNA or gqRNA, even though NUFIP1 also binds G-rich RNA (Bardoni et al., 1999). Perhaps NUFIP1 is part of a translationally silent mRNP that includes FMRP, and that is important for correct transport and localization of a given mRNA. Interestingly, NUFIP1 does not interact with FXR1 or FXR2 in a pull-down assay (Bardoni et al., 1999), and may compete for binding to FMRP.

The coimmunoprecipitation of Y box-binding protein 1 (YB1) with FMRP is RNA-independent (Ceman et al., 2000). YB1 is both a transcription factor and a core element of mRNP complexes, specifically a component of both translationally repressed

and actively translating mRNPs. (Kohno et al., 2003) In the polyribosome gradient fractionations shown in Figure 6.7J, cytoplasmic YB1 cosediments with small mRNPs; it does not seem to be associated with polyribosomes.

dFmr1 interacts both genetically and biochemically with PAR complex members PAR6, dLgl, and aPKC ζ (Zarnescu et al., 2005). This relationship is conserved for the mouse homologs of these proteins. *dlgl* is an oncogene and encodes a cytoskeletal protein involved in cellular polarity and cytoplasmic transport that is phosphorylated by aPKC ζ . (Vasioukhin, 2006) Atypical-PKC ζ cosediments primarily with smaller complexes irrespective of FMRP genotype, although faint bands are visible in fractions 4-7 (Figure 6.7K). Incubation with kcRNA or gqRNA does not alter this distribution. Thus, mRNAs associated with both FMRP and the PAR/Lgl/aPKC ζ complex are likely to be translationally repressed.

Several groups have identified dAgo2 and the mammalian homolog of dAgo1, eIF2C2/hAgo2, in complexes that co-purify respectively with dFmr1 and FMRP. (Caudy et al., 2002; Ishizuka et al., 2002; Jin et al., 2004) The Argonaute proteins are components of the RISC complex and bind miRNAs. They are thought to mediate one mechanism of translational repression through miRNA targeting of mRNAs. (Chen and Meister, 2005) Mammalian Ago2 (eIF2C2) has been shown to cosediment with polyribosomes from cells in culture (Mourelatos et al., 2002). Due to specificity of anti-mammalian Argonaute antibodies (a kind gift of T. Tuschl) for the human homologs of these proteins, we could not include mAgo1 and mAgo2 in Figure 6.7. Instead, human neuroblastoma cells (IMR-32) in culture were lysed and treated with 500nM RNA (kcRNA, mutant kcRNA or gqRNA) or 30mM EDTA, then analyzed by gradient

centrifugation and Western blotting. hAgo1, a closely related family member of hAgo2, is partially associated with polyribosomes, but is not affected by incubation with RNA ligands for FMRP (Figure 6.8A). hAgo2 exhibits a slightly different distribution, with a peak in fractions 6-11 which shifts to lighter fractions following treatment with EDTA (Figure 6.8B). However, like hAgo1, hAgo2 does not shift when incubated with kcRNA or gqRNA.

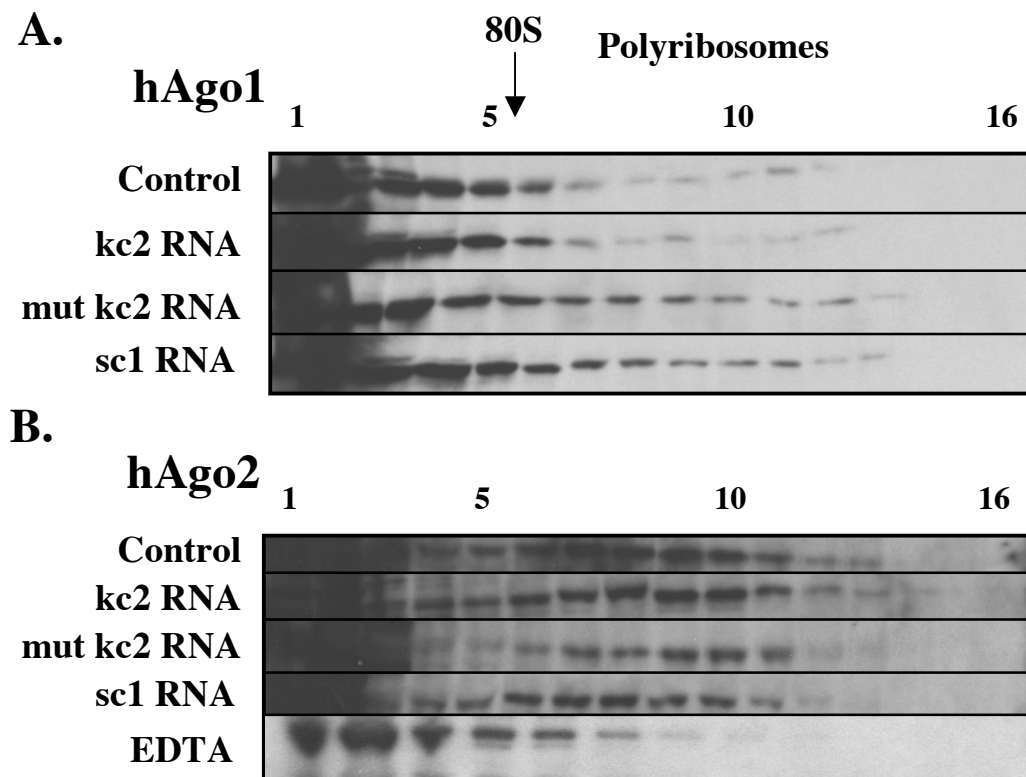


Figure 6.8 Mammalian Argonaute proteins 1 and 2 are not shifted by FMRP RNA ligands in human IMR32 cells. Western blots from polyribosome gradient fractionations in Figure 6.4 were probed for Ago1 (**A**) and Ago2 (**B**) (antibodies courtesy of T. Tuschl). 20-50% sucrose gradient distributions of hAgo1 and hAgo2 do not shift when incubated with 500nM FMRP RNA ligands. hAgo2 is associated with polyribosomes, since the peak in fractions 6-13 (Control, top panel) shifts to the top of the gradient when treated with 30mM EDTA.

Protein-protein interactions are not disrupted by kcRNA

While the KH2 domains of FXR1 and FXR2 bind kcRNA directly, and both FXR1 and FXR2 are competed off polyribosomes by kcRNA in the absence of FMRP, it is possible that incubation with kcRNA can also disrupt the heterodimerization of these proteins. In fact, both KH1 and KH2 have been implicated in heterodimerization (Siomi et al., 1994). To test this hypothesis, immunoprecipitations from wild type mouse cerebral cortex lysate for FMRP and an irrelevant antibody to an RNA-binding protein (anti-Nova) were performed in the presence of 500nM kc2 RNA or mutant kc2 RNA. FXR2 specifically coprecipitates with FMRP when incubated with either RNA (Figure 6.9). Thus, the KH2:kcRNA interaction does not disrupt heterodimerization of FMRP and FXR2. To further test this hypothesis, coimmunoprecipitations were carried out in a mild isotonic buffer under several different conditions, as shown in Figure 6.10. To control for non-specific interactions, FMRP knockout brain lysate was used with and without 500nM kcRNA, and FMRP wild type brain lysate was incubated with a control antibody. Experimental conditions included incubation with 500nM kcRNA, 500nM gqRNA, 500nM tRNA, 0.1% deoxycholate (DOC), 30mM EDTA, or RNase A. FXR1 coprecipitates with FMRP in all conditions, but shows some background non-specific precipitation in the immunoprecipitations from FMRP knockout brain and with a control antibody. FXR2 also coprecipitates with FMRP, but seems to require release from polyribosomes for efficient capture. It is interesting that FXR1 exhibits an increased interaction with FMRP in the presence of gqRNA versus kcRNA, while FXR2 coprecipitation with FMRP is greatly reduced by gqRNA compared to kcRNA. MyoVa is precipitated in all conditions, including the three controls. The only decrease in

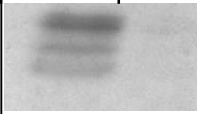


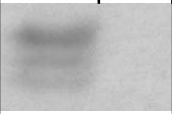
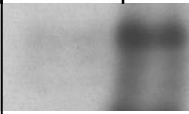
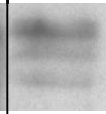






kcRNA	kc2					mutant kc2				
IP matrix	control Ab		FMRP 7G1-1			control Ab		FMRP 7G1-1		
	Sup	IP	Sup	IP	Tot	Sup	IP	Sup	IP	Tot
FMRP										
FXR2										

Figure 6.9 Kissing complex RNA does not abrogate heterodimerization of FMRP with FXR2. Post-mitochondrial supernatant from mouse cerebral cortex (lysed in 1X brain polyribosome buffer, see Chapter 2) was treated with 500nM kc2 or mutant kc2 and incubated with protein A-sepharose pre-bound to 7G1-1 or control (rabbit anti-Nova) antibody for 2 hours at 4°C. IP matrix was washed twice with lysis buffer and extracted by boiling in 1X Laemmli sample buffer (Bio-Rad). 10% of IP eluate (IP), 1% of pre-IP lysate (Tot), and 1% of post-IP supernatant (Sup) were analyzed by SDS and Western blotting for FMRP (1C3) and FXR2 (1G2).

Figure 6.10 Co-immunoprecipitations of FMRP with interacting proteins under a variety of treatments. Mouse cerebral cortices from wild type and FMRP knockout mice were lysed and post-mitochondrial supernatants were prepared as in Figure 6.9. Lysates were incubated as noted for “IP condition” with 500nM of the indicated RNA, 0.1% sodium deoxycholate (DOC), 30mM EDTA, 2ug/ml RNase A (RNase), or buffer (blank). After pre-clearing with protein A-sepharose for 30 min at 4°C, aliquots were taken for pre-IP total input samples (T). Lysates were immunoprecipitated with mixture of two monoclonal antibodies to FMRP (7G1-1 and 2F5) pre-bound to bridging anti-mouse Fcgamma antibody fragment (Jackson Labs):protein A-sepharose. Control antibody IP eliminates both monoclonal antibodies to IP with bridging antibody alone. IP matrices were incubated for 2 hours at 4°C and washed twice with lysis buffer before boiling in 1X Laemmli sample buffer. 1% of Input (T), 10% of IP eluate (IP), and 1% post-IP supernatant (S) are analyzed by Western blotting with antibodies to FMRP (2F5), FXR1 (830, courtesy of B. Bardoni), FXR2 (1G2), myosin Va (Sigma), Pur alpha (Abnova), mStaufen (Chemicon), atypical Protein Kinase C zeta (Sigma), and ribosomal protein P0 (Biodesign). (Of note, signal for FMRP saturated in some IP conditions, producing low signal “circles” inside FMRP bands.)

MyoVa capture occurs when treated with deoxycholate, indicating that stringent washes may yield a more specific result. Puro α shows maximal coprecipitation with FMRP when both proteins are released from polyribosomal complexes, as seen in the EDTA- and RNase-treated conditions. Since the Puro α -FMRP interaction has been previously reported to be RNA-dependent (Ohashi et al., 2002), perhaps this interaction is an artifact of incomplete RNase digestion. Under these conditions, mStaufen, aPKC ζ and ribosomal protein P0 do not coimmunoprecipitate with FMRP. Thus, KH2:kcRNA interaction does not disrupt heterodimerization of FXR proteins. Other FMRP-interacting proteins are more difficult to capture using this method. The effects of kcRNA and gqRNA on these interactions remain unclear.

RNA decoys: a tool for domain-specific functional inhibition

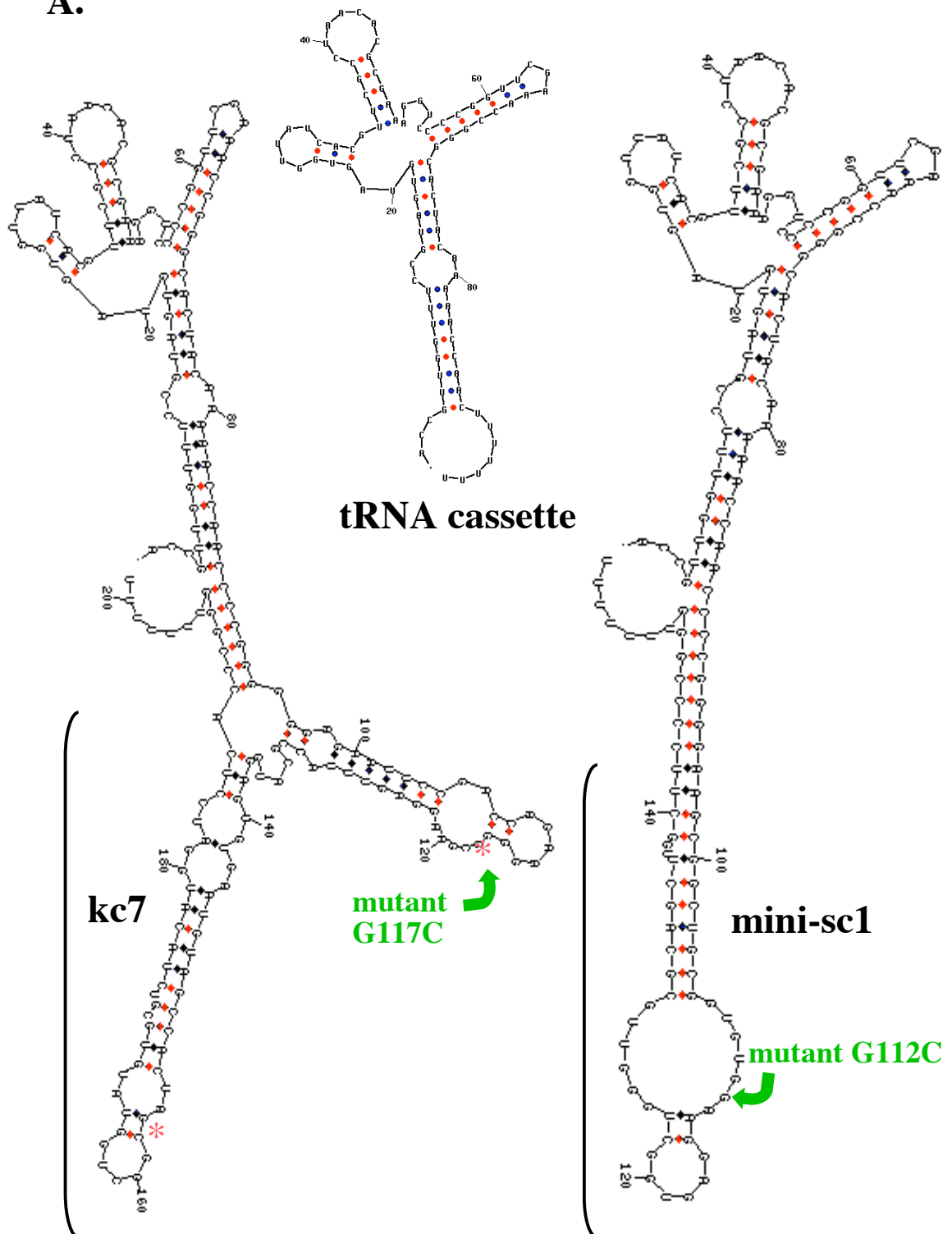
Since it is evident that the highly conserved KH2 domains of all three FXR family members bind kissing complex RNA with high affinity, and that only FMRP is able to bind g-quartet RNA motifs, these RNAs represent possible tools for domain-specific functional inhibition of these proteins *in vivo*. However, the *in vivo* expression of these RNAs is not trivial. Since they are short, non-translated RNAs with significant double-stranded regions, early attempts to express each high-affinity ligand as an individual 96nt species resulted in low expression levels and poor export from the nucleus (data not shown). A scheme for improved expression of these decoy RNAs is based on work by the Liebhaber and Taira laboratories. Their studies have shown that the human tRNA(Val) (Kuwabara et al., 2001) and the adenoviral VA1 RNA (Makeyev et al., 2002) are expressed at high levels and exported to the cytoplasm. Moreover, RNA sequences of

interest can be inserted at various locations within these RNAs without disrupting folding or nuclear export. Both of these RNAs have internal Pol III promoters that should increase expression by two to three orders of magnitude greater than that from Pol II promoters. We have constructed RNA decoys using either the tRNA or the VA1 cassettes, and containing kissing complex or g-quartet RNA motifs. Mfold-predicted structures for each species are shown in Figure 6.11 (Zuker, 2003). Mutations that disrupt binding to FMRP are indicated. *In vitro* transcribed RNA decoys using both cassettes bind purified FMRP with affinities close to those for FMRP SELEX target RNAs alone, as shown in Figure 6.12. This is the case when transcribed RNA is gel purified, denatured and refolded prior to incubation with recombinant FMRP (Figure 6.12A, top two panels). It is also true when *in vitro* transcribed RNA, after buffer exchange via G-25 sephacryl, is incubated directly with recombinant FMRP, bypassing any denaturation and refolding (Figure 6.12A, bottom panel, and B). In addition, FMRP does not bind tRNA or VA1 RNA alone, and does not bind mutated forms of the RNA decoys. While recombinant FMRP binds tRNA and VA1 cassettes that include kcRNA sequences, these RNAs may not function to disrupt FMRP:polyribosome complexes in mouse cerebral cortical lysate. Indeed, Figure 6.13 shows that while 500nM tRNA^{k7} completely disrupts FMRP association with polyribosomes (A), 1 μ M VA1^{k7} has no effect (B). For this reason, the VA1 cassette constructs were not used for further experiments.

To facilitate expression *in vivo*, tRNA expression constructs were cloned into the 3'LTR of a lentiviral vector developed by the Trono laboratory (Arrighi et al., 2004). This vector (pLVTHM, diagrammed in Figure 6.14) has the advantage of containing an

Figure 6.11 Predicted structures of RNA decoy molecules. Mfold-predicted structures of tRNA (**A**) and VA1 (**B**) expression cassettes and RNA decoys containing kc7 RNA or sc1 RNA sequences. A mutation that abrogates binding to FMRP is indicated for each. Pink asterisks denote kc7 nucleotides that form the base-pair “kiss” between loops.

A.



B.

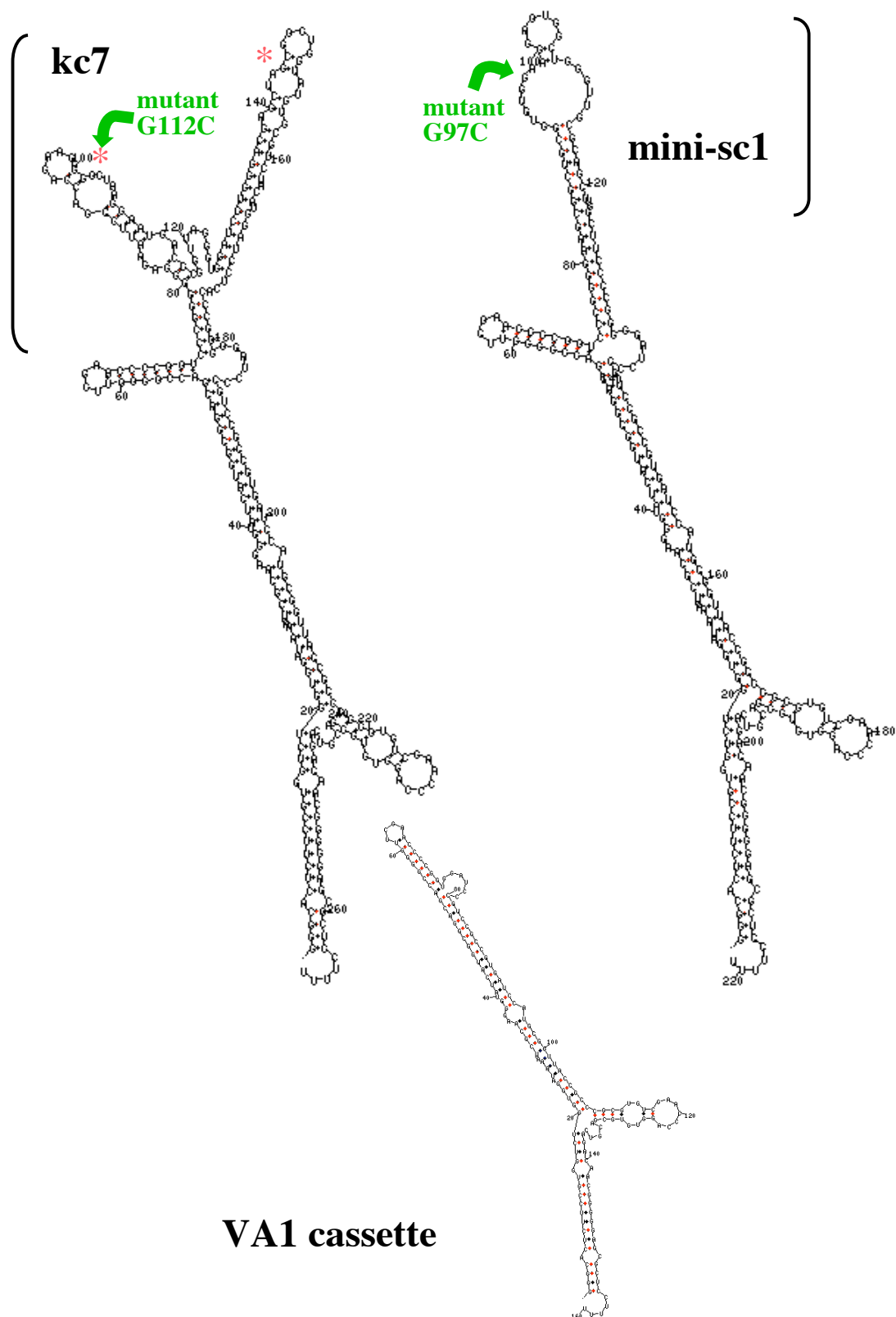
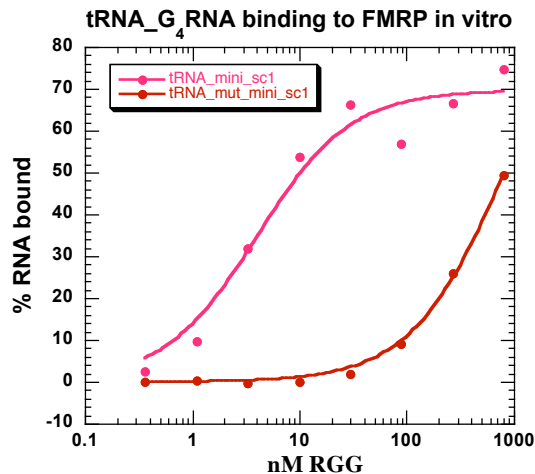
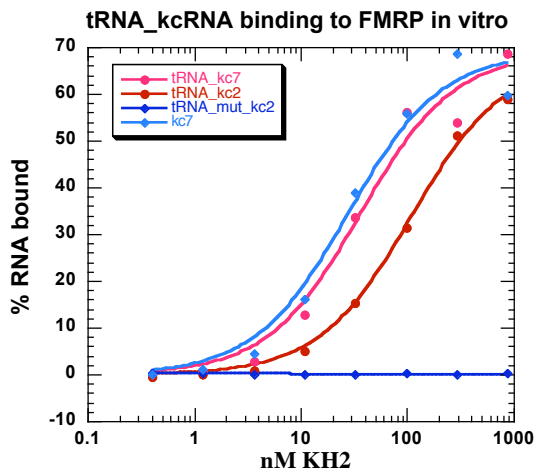
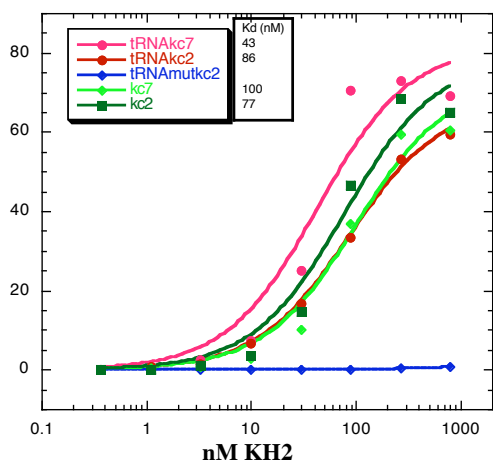


Figure 6.12 RNA decoys bind recombinant FMRP *in vitro*. (A) Filter binding assays for tRNA-based RNA decoys using recombinant FMRP KH2 (for kcRNA constructs, upper left panel) or C-terminus proteins (for sc1 RNA constructs, upper right panel). kc7 and kc2 are positive controls for protein activity. Upper panels test gel-purified, heat-denatured and refolded RNAs; binding affinities are listed at middle right panel. Middle left panel tests KH2 binding to co-transcriptionally folded RNA decoys (purified twice with G-50 sepharose columns, no heat denaturation); binding affinities are listed in figure legend. (B) Filter binding assays for co-transcriptionally folded VA1-based RNA decoys as in (A).

A.



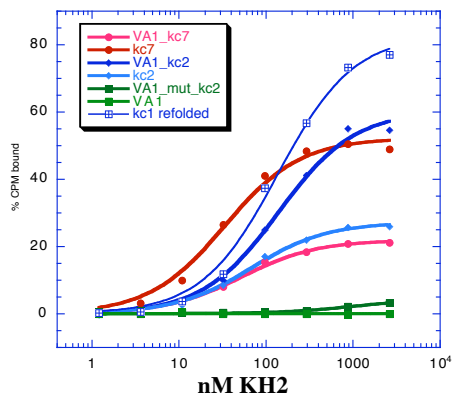
KH2 binds co-transcriptionally folded tRNAkcRNA



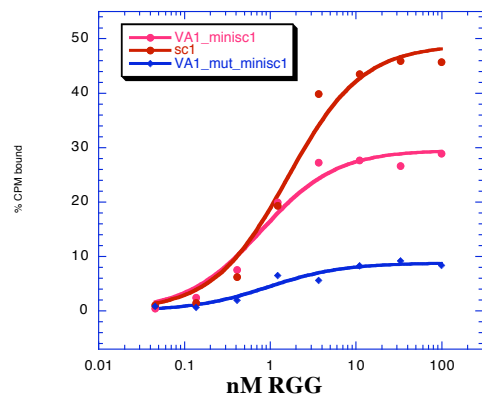
RNA	Kd (nM)
kc7	28
tRNA_kc7	36
tRNA_kc2	110
tRNA_mini_sc1	4
tRNA_mut_mini_sc1	808

B.

KH2 binds co-transcriptionally folded VA1_kcRNAs in vitro



FMRP binds co-transcriptionally folded VA1_gqRNA in vitro



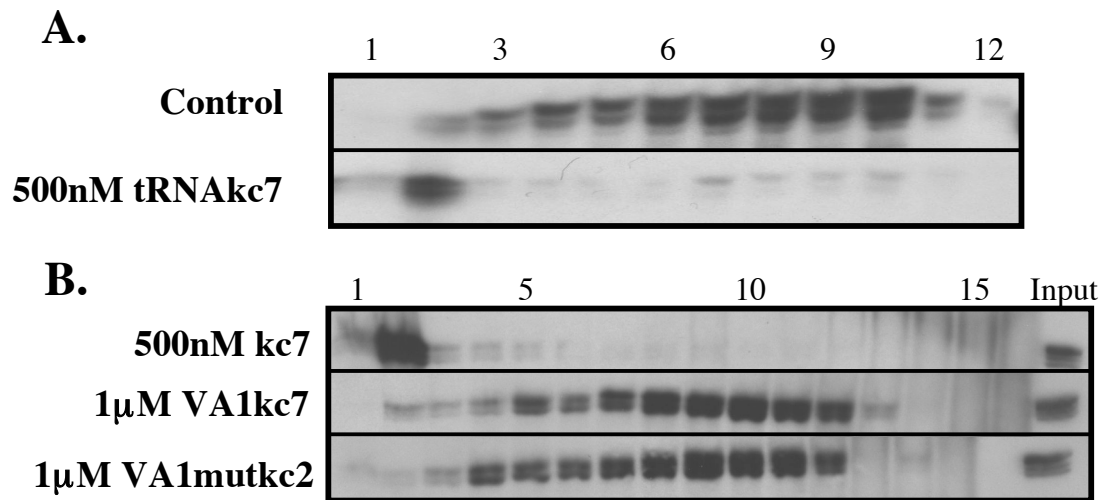


Figure 6.13 *In vitro* transcribed RNA decoys in polyribosome shift assay. Mouse cerebral cortex lysates were prepared for polyribosome fractionation and incubated with the indicated concentrations of *in vitro* transcribed RNAs before sucrose gradient centrifugation. Western blots of TCA-precipitated gradient fractions for FMRP indicate that tRNAkc7 RNA (**A**) competes FMRP off polyribosomes, whereas VA1kc7 RNA does not (**B**).

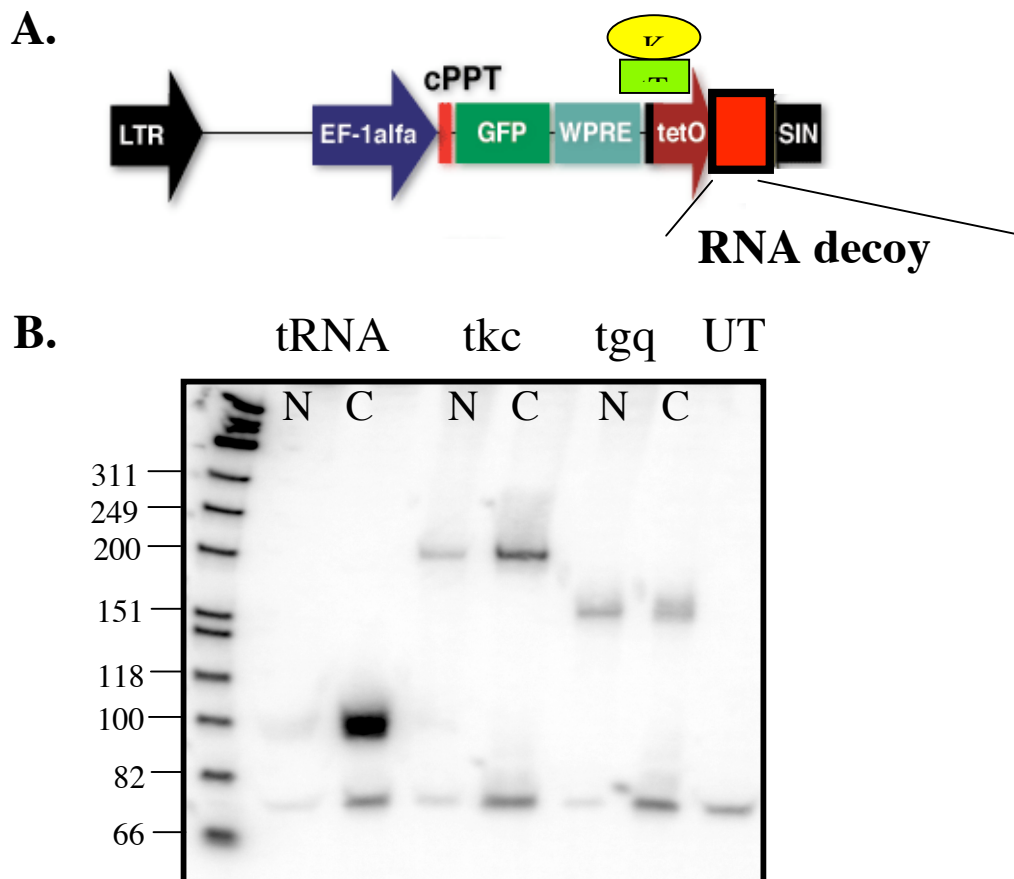


Figure 6.14 Lentiviral expression system for RNA decoys. (A) Schematic illustration of pLVTt adapted from D. Trono and colleagues. The H1 promoter (red box) of pLVTHM (D. Trono) in the 3'LTR has been replaced with the tRNA decoy expression cassette. (B) Northern blot for RNA decoy expression in 293T cells 40 hours post-lentiviral transduction with virus encoding empty tRNA cassette (tRNA), tRNA^{Akc7} (tkc), tRNA^{mini-sc1} (tgq), or untransduced (UT). 15ug of total nuclear (N) or cytoplasmic (C) RNA was electrophoresed on a denaturing 8% acrylamide/7M urea gel. After transfer to positively charged nylon membrane, blot was probed with P32-labeled anti-sense probe to the tRNA expression cassette. Ladder is single stranded DNA. Band at bottom of blot represents hybridization to a non-lentiviral sequence, possibly endogenous tRNA.

GFP reporter and a Tet-operator site adjacent to the RNA decoy cassette. When combined with an additional vector that contains a KRAB repressor fused to a Tet-repressor protein, expression of RNA decoys can be induced by doxycycline (Szulc et al., 2006). Figure 6.14B is a Northern blot from 293T cells transduced with lentivirus encoding tRNA^{Ac7} (tkc), tRNA^{Ala} (tgq) or the tRNA cassette alone. An antisense probe to the tRNA expression cassette reveals that all three RNAs are transcribed and exported to the cytoplasm, although export of tRNA^{Ala} is much less efficient than either tRNA^{Ac7} or the tRNA cassette.

In vivo activity of tRNA^{Ac7} and tRNA^{Ac2} was examined by polyribosome analysis of transduced cells. Briefly, high titer lentivirus was produced in packaging cells, concentrated, and used to transduce 15cm plates of 293T cells. This method produced approximately 90% EGFP-positive cells. Forty hours post-transduction, cells were lysed and layered on 20-50% sucrose gradients. Western blots from gradient fractions are shown in Figure 6.15A. FMRP in cells transduced with lentivirus encoding tRNA^{Ac7}, tRNA^{Ac2}, tRNA^{mutAc2}, or the tRNA cassette, remains associated with polyribosomes. Western blots from Figure 6.13A are reproduced here for comparison. In order to ensure that these RNAs were expressed, RNA was precipitated from aliquots of nuclear and cytoplasmic fractions of the cells used in Figure 6.15A. Northern blotting with an antisense tRNA probe (Figure 6.15B) reveals that the tRNA cassette, tRNA^{Ac7} and tRNA^{Ac2} RNAs were expressed and exported to the cytoplasm.

Since tRNA-based RNA decoys are expressed, exported to the cytoplasm, and can functionally interact with FMRP in a spike-in assay, it is not clear why these RNAs did not have the same effect *in vivo*. In order to more precisely quantify the *in vivo*

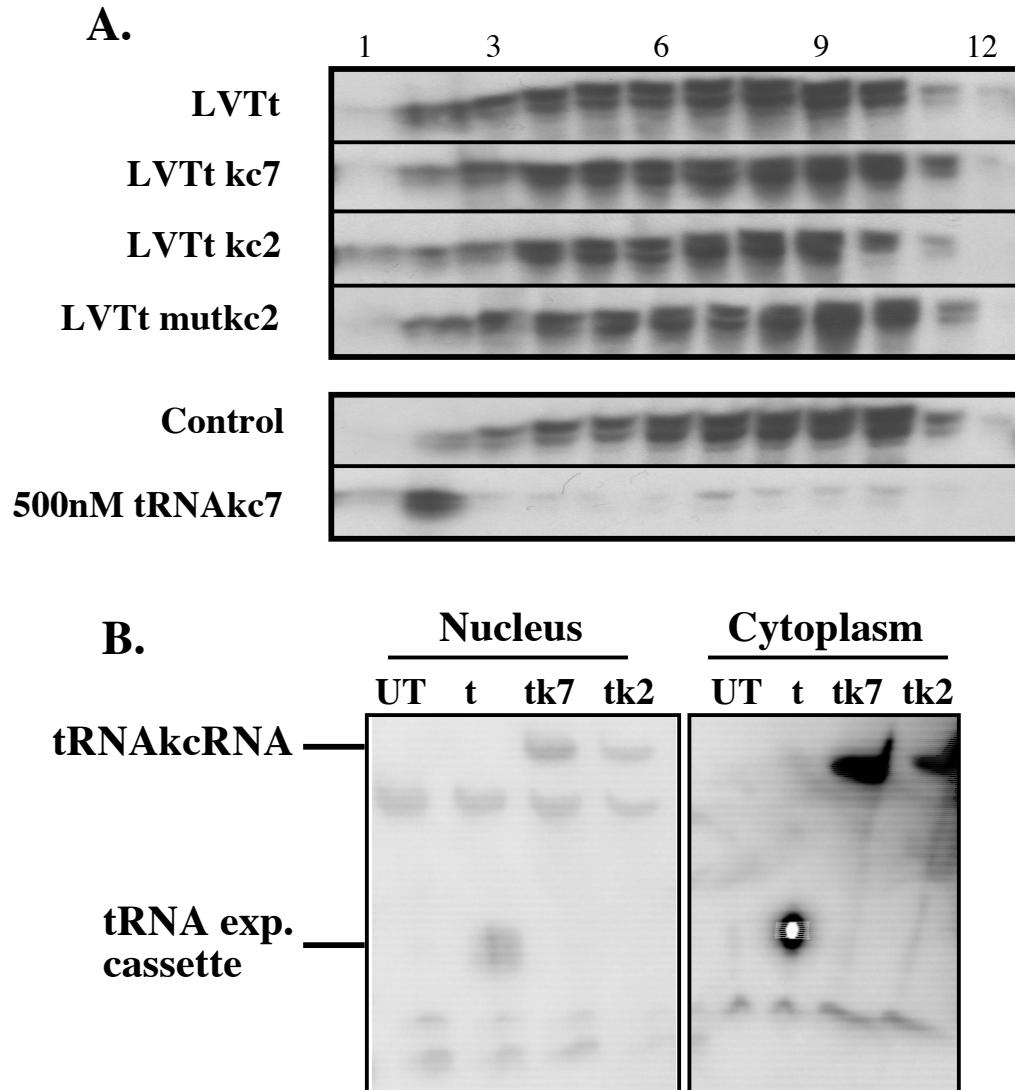


Figure 6.15 tRNAkcRNA does not compete FMRP off polyribosomes *in vivo*. (A) Western blots for FMRP of polyribosomal sucrose gradient centrifugations of 293T cells 40 hours post-lentiviral transduction with the indicated virus. Transduction efficiency was approximately 90% by GFP expression. Blots from Figure 6.13A are reproduced here for comparison. (B) RNA precipitated from nuclear and cytoplasmic fractions of cells in (A) is analyzed by Northern blotting with an antisense probe to the tRNA expression cassette (UT: untransduced, t: tRNA expression cassette, tk7: tRNAkc7, tk2: tRNA kc2.)

concentration of these RNA decoys, the antisense tRNA signal from 10 μ l pellets of transduced cells were compared to a known amount of *in vitro* transcribed tRNA^{Kc7} (Figure 6.16A). In sum, tRNA decoys are present at less than 1nM in transduced cells. In contrast, there are approximately six picomoles of FMRP in the post-mitochondrial supernatant from one juvenile (postnatal day 8) mouse cerebral cortex (Figure 6.16B). The 7G1-1 monoclonal antibody recognizes an epitope in the FMRP-specific variable loop of KH2 encoded by exon 11. 7G1-1 recognition of the indicated amounts of protein from wild type or FMRP knockout cerebral cortex were compared to known quantities of recombinant mouse FMRP KH2 (includes exon 11, but not exon 12.) A rough calculation based on the approximate volume of one mouse cerebral cortex (two hemispheres) yields an approximate concentration of 20nM FMRP in brain. No quantitation was done for FXR1 or FXR2, which also bind kcRNA with high affinity. Thus, it seems that the tRNA-based system of RNA decoy expression is not sufficient for effective functional inhibition of FXR KH2 domains. This may be due to a combination of factors, namely, insufficient decoy expression levels, sequestration of RNA decoys by unknown factors, and/or an increased susceptibility of FMRP to kcRNA binding when diluted approximately five-fold in brain lysis buffer. Further work is necessary to elucidate these requirements. Despite the difficulties in creating a viable system of *in vivo* RNA decoys for FXR family members, such an array of tool would be invaluable to the study of FMRP, FXR1 and FXR2 function in neurons, and could yield insights into the molecular mechanisms of Fragile-X mental retardation syndrome. Ultimately, a kissing complex RNA decoy could be used to create a conditional, KH2 domain-specific dominant negative simultaneously for all three FXR family members.

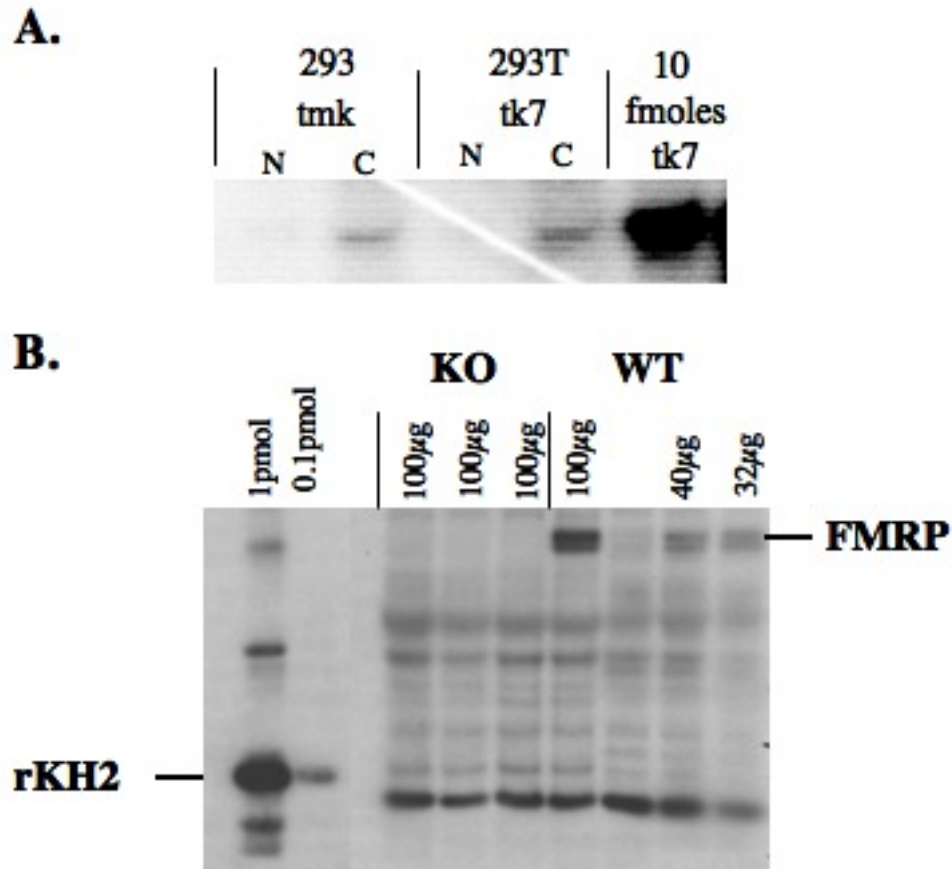


Figure 6.16 Quantitations of RNA decoy and endogenous FMRP expression. (A) Northern blot probed with labeled antisense tRNA expression cassette oligonucleotide of RNA from 10ul of packed 293 or 293T cells 40 hours post-lentiviral transduction with LVTt virus encoding tRNA^{mutkc2} (293) or tRNA^{kc7} (293T). 10 femtomoles of *in vitro* transcribed tRNA^{kc7} is included for comparison. (B) Western blot for FMRP (7G1-1) comparing indicated amounts of mouse brain lysate to recombinant mouse KH2 (includes exon 11 but lacks exon 12).

Discussion

We find that kissing complex RNA can disrupt the polyribosome association of FXR1 and FXR2, as well as FMRP. In their tandem KH domains, these family members share 90% sequence similarity. Our results indicate that these proteins bind to the same RNA motif, both *in vitro* and *in vivo*. Thus it is likely that they share a mechanism of action and pool of RNA targets in cells where they are co-expressed, i.e. neurons. Yet, from a common ancestral gene (*dfmr1* is representative), FXR family members have diverged to include C-terminal regions with only 40% sequence identity. In addition, these domains do not share a target RNA sequence. Therefore, while FXR1 and FXR2 are found in some cell types that do not express FMRP, they may also have differing roles in neurons. The potential for homodimerization and heterodimerization between these proteins adds another layer to the complexity of possibilities for functional roles and RNA targets. We can imagine that a common mechanism is mediated by KH2 binding to kissing complex RNA motifs that results in a polyribosome-associated complex. However, the identity of the FXR proteins bound to a given mRNA may determine a different translational fate. Alternatively, additional FMRP-binding sites, e.g. G-quartets, may specify a subgroup of mRNAs for a unique purpose at the synapse. Except for the negative data presented by our laboratory and the Mihailescu laboratory (Zanotti et al., 2006), there is as yet no data on whether the C-terminal domains FXR1 and FXR2 bind sequence specific RNA structural motifs. Changes in the protein composition of an mRNP are known to influence various steps in mRNA processing, transport, and regulation. These substitutions may accompany spatial changes, for example, when cytoplasmic PABC replaces nuclear PABPN1 on the poly(A) tail of an mRNA during export from the nucleus. (Reviewed in (Kuhn and Wahle, 2004)).

Changes in mRNP composition can also result from upstream signal transduction. For example, when nutrients are scarce, cells can restrict translational apparatus to only those transcripts necessary for survival in a quiescent state. This is accomplished using a general regulator of translation, Target of Rapamycin (mTOR). (Reviewed in (Hay and Sonenberg, 2004) Briefly, the presence of nutrients and/or growth factors activates mTOR, a kinase which phosphorylates eukaryotic translation initiation factor 4E (eIF4E)-binding proteins (4E-BPs), as well as other proteins involved in translational regulation. Phosphorylation of 4E-BP causes its dissociation from eIF4E and allows eIF4G binding that results in 40S ribosome subunit recruitment to the m7G-cap of an mRNA. Since eIF4E is required for cap-dependent translation, this is a general mechanism to regulate translation of most mRNAs. In neurons, cytoplasmic polyadenylation element (CPE)-containing mRNAs are bound by CPE-binding protein (CPEB) and maskin for transport into dendrites. Maskin also binds eIF4E, preventing interaction with eIF4G. When NMDA receptors are stimulated at synapses, Aurora A kinase is activated and phosphorylates CPEB, which induces mRNA polyadenylation and likely leads to dissociation of maskin from eIF4E, resulting translation of the mRNA. (Huang et al., 2002; Wu et al., 1998) FXR proteins may regulate translation of mRNA cargoes in a spatial-, temporal- and/or signal-transduction-dependent manner. In Fragile-X patients and FMRP knockout mice, the most striking morphological phenotypes in neurons are seen in the dendritic spines. (Comery et al., 1997; Hinton et al., 1991; Wisniewski et al., 1991) In addition, since there is a documented increase in group I metabotropic glutamate receptor (mGluR)-induced long-term depression (LTD) in hippocampal slices from FMRP knockout mice (Huber et al., 2002), FMRP activity is thought to be focused

at synaptic areas. It is possible that while FMRP, FXR1 and FXR2 all associate with polyribosomes via kissing complex RNA motifs, this association could be spatially or temporally specific for each protein. Indeed, a single kissing complex motif in a given mRNA could be sequentially bound by different FXR proteins during its lifetime. Finally, data showing that FMRP may be associated with changes in the quantity of polyribosomes in synaptoneurosomes in response to treatment with mGluR agonists, suggests that FMRP may be a downstream target of signal-transduction cascades induced by synaptic activity (Weiler et al., 2004).

The overlap in RNA sequence and structure specificity of the KH2 domains of FXR1, FXR2 and FMRP suggests that they may exhibit some functional redundancy in neurons. *Fmr1*-null mice have normal levels and subcellular distributions of FXR1 and FXR2 (Bakker et al., 2000). Yet, Fragile-X patients and *Fmr1*-null mice exhibit gross and molecular phenotypes that are apparently uncompensated by normal levels of FXR1 and FXR2. These abnormalities in dendritic spine development could be due to a hypomorphic effect of a loss of FMRP; i.e. increased levels of FXR1 and/or FXR2 could potentially reduce these effects. However, regulation of FXR family protein levels seems to be tightly regulated for a good reason. Overexpression of *dfmr* also results in neuronal structural abnormalities (Pan et al., 2004). An alternate hypothesis is that the abnormalities seen with decreased levels of FMRP are due to unique functions of FMRP, possibly due to loss of the RGG box and its ability to bind G-quartets. This suggests that any KH2-specific roles of FMRP are fulfilled by FXR1 and/or FXR2 in the absence of FMRP. The actual molecular differences in neurons that lack FMRP are likely due to both of the above possibilities, with the additional caveat that while FXR family members

share a means of association with polyribosomes, that association may result in a different fate for the mRNA, depending on which family member is bound.

While kcRNA is bound by the FXR protein family with high affinity and specificity, this does not preclude the possibility that additional proteins could bind kissing complex motifs. In fact, Pur α is shifted off polyribosomes by incubation with kcRNA. This effect is independent of FMRP. However, it is not specific to kissing complex RNA, since incubation with G-quartet RNA can produce a more pronounced shift in Pur α distribution. KH domains from several proteins (Nova1, Nova2, hnRNPE1, hnRNPE2, SF1 and ZBP) do not bind kcRNA *in vitro* (J. C. Darnell, manuscript in preparation.)

Further permutations of FXR protein-containing mRNPs can result from the association of other proteins. A number of FMRP-interacting proteins have been identified. Some of these proteins specifically bind FMRP (e.g. CYFIP1), while others interact with all three family members (e.g. CYFIP2) (Schenck et al., 2001). Moreover, some of these interacting proteins (e.g. NUFIP1, CYFIP1, and CYFIP2) bind to the same N-terminal domain that mediates homodimerization and heterodimerization of FXR family members, potentially competing for this binding site (Bardoni et al., 1999; Schenck et al., 2001). We find that KH2 binding to kissing complex RNA does not disrupt heterodimerization between FMRP and FXR1 or FXR2. Thus, the variety of proteins that interact with FMRP through the N-terminal domain may also be associated with polyribosomes through FMRP. FXR1 and FXR2 show a nine percent shift from heavy polyribosomes to small complexes in wild type versus FMRP knockout cerebral cortex lysate. This suggests that, while FXR1 and FXR2 interact with polyribosomes

through direct binding of kissing complex motifs, they may also be able to bind FMRP:polyribosomes by means of protein-protein heterodimerization.

Studies are ongoing on *fmr1/fxr2* double knockout animals, and will provide more data on the functional redundancy of these proteins. However, a homozygous *fxr1*-null mutation is lethal at birth. Therefore, other methods are required to study what happens when all FXR proteins are functionally absent. Since kcRNA specifically binds to and is able to compete with endogenous targets of the KH2 domains of FMRP, FXR1 and FXR2, artificially introduced kcRNAs have the potential to abrogate function of these domains. On the other hand, gqRNA only binds FMRP. Thus the expression of these RNAs *in vivo* represents a system by which the specific roles of FXR proteins could be dissected with respect to the activities of their RNA binding domains, in the context of wild type proteins. The development of such a system is not trivial, due to difficulty in achieving sufficiently high levels of RNA decoy expression, since, to our knowledge, RNAs of this type are not normally produced by cells. However, if these difficulties can be overcome, an RNA decoy system could yield valuable insights into FXR function in translational regulation.

Acknowledgements

Jennifer Darnell provided the alignments shown in Figures 6.1 and 6.2, as well as the recombinant protein used in *in vitro* nitrocellulose binding assays. Antibodies to FXR1, NUFIP, CYFIP1, CYFIP2 and 83FIP were provided by Barbara Bardoni. Antibodies to hAgo1 and hAgo2 were provided by Tom Tuschl.

CHAPTER VII: GENERAL DISCUSSION

Nova proteins are partially bound to polyribosomes

In our studies of density gradient distributions of RNA-binding proteins in mouse brain, we found that approximately 10-25% of cytosolic Nova is shifted by EDTA treatment, supporting the hypothesis that this population of Nova proteins is associated with polyribosomes. Microarray comparisons between Nova-1 wild type and knockout spinal cord polyribosomal mRNAs have identified possible targets of Nova. However, the differences we observed were small, suggesting that translational regulation is likely to be a subtle process better studied through analysis of corresponding changes in the mRNA content of other polyribosome gradient regions such as mRNPs. Furthermore, none of our validated Nova alternative splicing targets (Ule et al., 2003; Ule et al., 2005b) show a greater than two-fold change between wild type and Nova-1 knockout spinal cord polyribosomes. These data represent the first biochemical assays showing a possible role for Nova proteins in translational control. As such these studies provide a foundation for future evaluation of this role in Nova biology; they provide a general platform of data from which to launch more specific experiments. While yielding some important results, these experiments were subject to some limitations. First, the lack of robust changes in polyribosomal mRNA populations in the absence of Nova-1 emphasizes the need for improved signal to noise ratios when using gradient density fractionation to isolate polyribosomes from the central nervous system. In essence, the presence of too many cell types and parts of cells (e.g. cell body vs. processes) obscures specific conclusions in this method. Clearer data for mRNA targeting and localization could be obtained by looking at specific neuronal layers using microdissection techniques. These experiments

did not eliminate redundant function as a confounding variable: comparing wild type to Nova-1/Nova-2 double knockout mice would yield maximum differences. Finally, further amplification of differences in the mRNA complement of polyribosomes can be achieved by measuring reciprocal changes in mRNA distribution over polyribosomal gradient fractionations. One can imagine that these methods could also be used to analyze activity-dependent changes in mRNA association with polyribosomes in a variety of paradigms such as seizure induction and glycine- or GABA- receptor blockade.

These proposed experiments could further refine our working model of Nova function in neurons. As such, we have evidence that Nova proteins bind nascent RNA transcripts in the nucleus and influence splice site selection for nearby exons (Dredge and Darnell, 2003; Jensen et al., 2000a; Ule et al., 2003; Ule et al., 2006; Ule et al., 2005b). It is most likely that cytoplasmic Nova proteins remain associated with previously alternatively-spliced mRNAs as a component of the exon-exon junction complex during nuclear export. At some point during cytoplasmic mRNA processing and transport, most Nova proteins disengage from mRNA complexes and return to the nucleus. However, we have not excluded a possible role for Nova in mRNA transport and/or localization. The 11-22% of cytoplasmic Nova proteins that are associated with brain polyribosomes may merely be a remnant of the exon-exon junction complex that has survived until an initial round of translation in the cytoplasm. Conversely, polyribosome-bound Nova may influence translational regulation of its target mRNA, perhaps by sequestering such mRNAs in a ribosome-loaded but inactive state, in readiness for a local signal that allows translation to proceed.

A conserved role for Fragile X Mental Retardation Protein and its family members FXR1 and FXR2

We found that FMRP is associated with actively translating ribosomal complexes in mouse cerebral cortex (Stefani et al., 2004). This is in agreement with previously published data showing that FMRP co-sediments with polyribosomes in tissue culture cell lines and rat synaptosomes (Corbin et al., 1997; Eberhart et al., 1996; Feng et al., 1997a; Feng et al., 1997b; Khandjian et al., 1996). However, our results directly refute the finding that FMRP does not associate with polyribosomes in mouse brain (Zalfa et al., 2003).

Our microarray screens of polyribosomal mRNA for differences between FMRP wild type and knockout mouse cerebral cortex show a remarkable lack of significant differences between the two genotypes in both mRNA populations (polyribosomal and total). This result contrasts with previous data identifying 251 mRNAs that show altered polyribosomal distribution in transformed white blood cells from individuals with fragile X syndrome compared to non-fragile X individuals, and 144 mRNAs differed in the total RNA population (31 mRNAs were present in both data sets) (Brown et al., 2001). Of the few genes that show subtle changes (between 1.27- and 1.42-fold) between wild type and FMRP knockout polyribosomes in our data, only one, proteolipid protein (Plp), has been previously identified as a target for FMRP (Wang et al., 2004). Our results imply that this method for evaluation of FMRP control of translational status for target mRNAs may be limited by an insensitivity to small differences in polyribosomal distribution, that may be visible only with measurement of reciprocal changes between mRNA populations (i.e. between lighter and heavier fractions of the sucrose gradient). In order to continue to use

this method for FMRP mRNA target identification, it is necessary to increase the signal to noise ratio by measuring these reciprocal changes or by using an animal model with a more severe phenotype (i.e. FMRP/FXR2 double knockout mice) to validate the subtle changes in mRNA-polyribosome association that we have identified in FMRP single knockout mice. Finally, activity-dependent changes in the functional interaction between FMRP and the translational apparatus have been proposed by a number of groups (Greenough et al., 2001; Hou et al., 2006; Huber et al., 2002; Weiler et al., 2004). A polyribosome-associated, transcriptome-wide analysis of the effect of FMRP combined with synaptic activity in the form of metabotropic glutamate receptor modulation or seizure induction could provide a more refined view of the activity-dependent actions of FMRP.

We have discovered that FMRP associates with polyribosomes via binding of KH2 to kissing complex (loop-loop pseudoknot) RNA motifs (Darnell et al., 2005a). Competition with exogenous kissing complex RNAs shifts FMRP off polyribosomes. This interaction is sequence- and structure-specific, as a single point mutation that disrupts one Watson-Crick base pair between the two loops fails to bind KH2, and fails to compete FMRP off brain polyribosomes. The binding of the RGG box to G-quartet RNA does not have an effect on polyribosomal association of FMRP in brain lysate. These findings support I304N data as a model for Fragile X mental retardation syndrome, and suggest that new mRNA targets of FMRP KH2 require identification. In addition, these data pose new questions of how, where and why the KH2 and RGG box RNA-binding domains interact with their unique target sequences. Do they identify separate classes of mRNAs, or populations that contain both types of motifs? Do they bind sequentially;

perhaps one domain facilitates helicase activity that unwinds one RNA structure, thereby allowing the binding of the other domain? Do they bring together RNA motifs in *trans* to effect translational regulation of via elongation or another as yet unidentified mechanism? These are all questions that can be addressed in further detail using G-quartet and kissing complex RNA sequences as tools to dissect the interactions of these domains.

Our experiments demonstrated that KH2:kissing complex RNA interaction is conserved in the FXR protein family. Both FXR1 and FXR2 bind kissing complex RNAs through KH2, and like FMRP, are effectively competed off polyribosomes by a similar concentration of exogenous RNA ligand. For FXR1 and FXR2 individually, approximately nine percent each appear to be associated with polyribosomes through FMRP, as they show a subtle shift in polyribosomal distribution in FMRP knockout mice. However, even in the absence of FMRP, FXR1 and FXR2 do bind polyribosomes via KH2 interaction with kissing complex RNA. We demonstrated that this relationship is specific to FXR family proteins, since the polyribosomal distributions of other RNA-binding proteins and FMRP-interacting proteins are unaffected by kissing complex RNA. RNA binding by KH2 does not abrogate heterodimerization of FMRP with FXR1 and FXR2. The high degree of sequence and functional conservation between the KH domains of the three Fragile X-related proteins is a clue to possible overlapping functions among these proteins and underscores the critical nature of this role.

An argument can be made that the cellular, behavioral and cognitive phenotypes of fragile X mental retardation syndrome are due to a loss of FMRP RGG domain RNA-binding activity alone, since FXR1 and FXR2 are present at normal levels in individuals

with fragile X syndrome. However, the existence of a severely affected fragile X patient with a point mutation in KH2 contradicts this hypothesis (De Boulle et al., 1993). I304N FMRP is able to bind G-quartet RNA through the RGG-box domain with wild type affinity (Darnell et al., 2001). Thus, even though the KH domains of FMRP, FXR1 and FXR2 may share similar roles in translational regulation, the loss-of-function of FMRP that occurs in fragile X syndrome likely includes roles for both the KH and RGG box domains. This warrants further investigation of the physiological roles of FXR family KH domains in translational regulation *in vivo*. In order to elucidate these roles, it will be important to develop domain-specific loss-of-function mouse models, such as an FMRP knock-in allele lacking the RGG box. Mice lacking both FMRP and FXR2 have been generated and show increased neurological phenotypes (Spencer et al., 2006). The I304N mutation, while representing a valuable model for fragile X syndrome, does not serve as a robust dominant negative for all three FMRP family members, since the majority of FXR1 and FXR2 proteins are associated with polyribosomes in the absence of protein-protein interactions with FMRP. Our attempts to create a KH2-specific triple functional knockdown system for FMRP, FXR1 and FXR2 by expression of kissing complex RNA decoys *in vivo* have been unsuccessful to date, due to difficulty expressing RNA decoys at a sufficient level. However, this type of methodology represents a unique strategy to achieve a more insightful analysis of what appears to be a highly conserved role for FXR-family proteins in translational control.

Our data suggest a new model for FMRP function in neurons. FMRP, as a nucleocytoplasmic shuttling protein, binds mRNAs either co-transcriptionally in the nucleus, during nuclear export, or once the mRNA has reached the cytoplasm. We find

that FMRP-bound mRNAs cosediment with the heaviest fractions of the polyribosomal distribution in a sucrose gradient. FMRP associates with polyribosomes via interaction between the KH2 domain and a kissing-complex RNA motif. The RGG domain of FMRP may add additional specificity to this protein-mRNA interaction through a G quartet motif in the same mRNA. Alternatively, it may bind a G quartet in a separate RNA moiety, be that microRNA, ribosomal RNA, structural non-coding RNA, or another mRNA. Since FXR1 and FXR2 also associate with polyribosomes via interaction with kcRNA motifs, we can envision a pool of mRNAs marked, and perhaps sequestered by their interactions with FMRP family members. These mRNAs are likely to be loaded with many ribosomes, in a state of translational repression that can be lifted on receipt of a local synaptic signal indicating a need for the protein products of these mRNAs. Such a signal could involve phosphorylation of FMRP family members and/or a conformational change that unwinds these mRNP complexes and allows translation to restart.

The Nova and Fragile X-related protein families share homology as RNA-binding proteins through their KH domains. Their diversity in displaying different polyribosomal associations, subcellular distributions, and likely different roles in post-synaptic translational regulation, demonstrate the important nature of RNA-binding proteins in human neurologic function and disease. Their pathophysiologic role is not necessarily limited to that in the diseases in which they were discovered. For example, FXR1 has been described as an autoantigen in a case of scleroderma (Bolivar et al., 1998). Of note, this is just one such example of how RNA-binding proteins are implicated in autoimmune neurological diseases. In regard to the biology of these protein families, persistent pursuit at the bench will not be lost in translation to the clinic.

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