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Hayley McCausland April 3, 2014

Effect of FMRP deficiency on the expression and mRNA translation of the potassium channel Kv4.2 in human cells

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Abstract

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Fragile X syndrome (FXS) is the most common monogenic cause of intellectual disability. FXS is marked by hyperactivity, and approximately 25% of patients have epilepsy, but the underlying molecular causes are unknown. FXS is caused by transcriptional silencing of the fragile X mental retardation gene 1 (*FMR1*), resulting in the loss of the fragile X mental retardation protein (FMRP). FMRP is an mRNA binding protein involved in the regulation of mRNA translation. Recent findings show that FMRP regulates the expression of the A-type potassium channel Kv4.2, which plays an essential role to regulate hippocampal excitability, suggesting that dysregulated expression of Kv4 channels may cause epilepsy and hyperexcitability in patients with FXS. To date, it is unclear whether FMRP acts as a translational activator or inhibitor of Kv4.2 mRNA. We hypothesize that mRNA translation and levels of the A-type potassium channel Kv4.2 are dysregulated in a human cell model of FXS. In order to test this hypothesis, we have analyzed (1) Kv4.2 expression levels and (2) Kv4.2 mRNA translation in a human *in vitro* cell model for FXS. We tested how the manipulation of FMRP levels with short interfering RNA (siRNA) sequences, or by overexpression of FMRP in a human cell line affects Kv4.2 expression and mRNA translation. Our initial analyses of human Kv4.2 protein expression *in vitro* using a luciferase reporter system showed decreased expression of Kv4.2 protein after knockdown of *FMR1*. However, polysome association studies suggested that Kv4.2 mRNA translation was unchanged after knockdown of *FMR1*. FMRP overexpression led to a decrease in Kv4.2 expression and mRNA translation in both assays. Based on these and previous studies, we propose a new model for the regulation of Kv4.2 mRNA by FMRP via two separate binding sites in its 3'UTR. We hypothesize that FMRP acts as a translational activator at the proximal binding site, and a translational inhibitor at the distal site. Preliminary experiments support this hypothesis. Taken together, this study not only provides evidence that FMRP is an important regulator of Kv4.2 expression in human cells, but has also led to the development of a novel, testable model of how FMRP regulates the mRNA translation of the specific target mRNA Kv4.2.

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Introduction

Fragile X Syndrome

Fragile X syndrome (FXS) is the most common inherited form of intellectual disability and is frequently associated with epilepsy and autism (Bassell and Warren, 2008). About 1 in 4000 males and 1 in 7000 females are affected (Maurin et al., 2014). Males with the full mutation have severe cognitive deficits, including problems with short-term memory, working memory, and executive functions (Garber et al., 2008). Symptoms include hypersensitivity to sensory stimuli, hyperexcitability, social anxiety, self-injurious behavior, and aggressiveness towards others (Tsiouris and Brown, 2004, Hagerman et al., 2009). Because the disorder is X-linked, females are generally less affected than males (Garber et al., 2008).

Current treatments for FXS are wholly symptom based, though clinical trials for disease-mechanism targeted treatments are ongoing. Hyperactivity and impulsivity are most commonly treated with stimulants, though their effectiveness varies between individuals (Berry-Kravis and Potanos, 2004). α2-adrenergic agonists are used to treat hypersensitivity to sensory stimuli (Berry-Kravis and Potanos, 2004). Pharmacological treatments are usually combined with behavioral interventions like occupational therapy, individualized education plans, and parenting techniques, though the extent of their effectiveness is unknown (Glaser et al., 2003).

FXS is caused by transcriptional silencing of the fragile X mental retardation gene 1 (*FMR1*) on the X chromosome. The mutation consists of a trinucleotide repeat expansion in the 5' untranslated region (UTR). Normally, there are ~30 CGG repeats in the 5'UTR of *FMR1*. The full mutation consists of >200 CGG repeats, which usually leads to methylation (Hagerman et al., 2009). A premutation with 55-200 CGG repeats in the 5'UTR has been found in families of fragile X patients. The premutation can increase in length in subsequent generations and lead to

the full mutation, resulting in FXS (Bassell and Warren, 2008). Those with the premutation may show symptoms not seen with the full mutation, like ovarian insufficiency in females, and fragile X-associated tremor/ataxia syndrome (FXTAS) (Hagerman and Stafstrom, 2009).

Fragile X Mental Retardation Protein (FMRP)

The full mutation on *FMR1* leads to silencing of fragile X mental retardation protein (FMRP). FMRP is an mRNA binding protein with a large number of mRNA targets, up to 4% of all mRNAs present in the brain (Bassell and Warren, 2008, Gross et al., 2012). FMRP is ubiquitously expressed but is most abundant in the brain and testes. In neurons FMRP is highly expressed in the cytoplasm, which includes cell bodies, dendrites, and axons (Braat and Kooy, 2014). FMRP has at least three mRNA binding domains: the two hnRNP-K-homology (KH) domains, KH1 and KH2, and an arginine-glycine-glycine (RGG) box (Fig.1) (Bassell and Warren, 2008). Previous studies have shown that the RGG box recognizes and binds to a G-quartet in target mRNAs, and binding is made more specific by the preceding sequence (Darnell et al., 2001). The KH2 domain is necessary for interaction with the "kissing complex" structure in the RNA, a complex tertiary RNA structure, which is involved in binding of FMRP to polyribosomes (Darnell et al., 2005). FMRP has multiple roles in the cell, but is most often recognized as an inhibitor of mRNA translation at the synapse (Ronesi and Huber, 2008). Most recently, it has been proposed that FMRP represses mRNA translation by ribosome stalling, which would aid in delayed translation of the mRNA at the synapse (Darnell et al., 2011). While Darnell et al. find that FMRP associates with elements in the coding region of the target mRNAs, this observed ribosome stalling may also be explained by the binding of FMRP to G-quartets in the 3'UTR (untranslated region) of many mRNA sequences, thus interfering with polyribosome activity (Melko and Bardoni, 2010). Noncoding microRNAs (miRNA) may also be involved in

mediating the effect of FMRP on translation (Jin et al., 2004a, Edbauer et al., 2010, Muddashetty et al., 2011). Dephosphorylation of FMRP has been shown to lead to upregulated translation, suggesting that post-translational modifications are key to the function of FMRP (Sidorov et al., 2013). A few recent studies suggest that FMRP can also act as a translational activator. For example, FMRP has been shown to be a positive regulator of nitric oxide synthase 1 (NOS1) mRNA when FMRP binds to G-quartet regions in NOS1 (Kwan et al., 2012).

The localization of FMRP in dendrites and the synapse, along with its translation activity at the synapse, make FMRP an important player in the regulation of synaptic plasticity (Sidorov et al., 2013). *Fmr1* KO mice, like Fragile X patients, lack FMRP and display hyperactivity, impaired spatial memory, and audiogenic seizures, making them a strong model system for FXS (Bakker et al., 1994). Both FXS patients and *Fmr1* KO mice display increased density of immature dendritic spines, suggesting a critical role for FMRP in dendritic spine maintenance (Irwin et al., 2001, Irwin et al., 2002). FMRP is also essential for synapse formation and axonal pruning during development in drosophila (Gatto and Broadie, 2008). Dysregulated long-term potentiation (LTP) in cortical areas and hippocampi of *Fmr1* KO mice further demonstrate the role of FMRP in synaptic plasticity (Li et al., 2002, Lauterborn et al., 2007).

mGluR Theory of FXS

The mGluR (metabotropic glutamate receptor) theory is one possible explanation for the impaired synaptic plasticity in FXS (Bear et al., 2004). This theory is based on the observation that signaling through mGluRs is exaggerated and stimulus-independent in FXS animal models (Huber et al., 2002). It posits that dysregulated mGluR signaling may be a cause of neuronal defects in FXS, and thus represents a promising therapeutic target. Strong activation of mGluRs reverses LTP and causes long-term depression (LTD) (Palmer et al., 1997), and previous studies

have shown that mGluR-dependent LTD is exaggerated in FXS (Huber et al., 2002). Exaggerated LTD may slow synapse maturation, leading to epilepsy, cognitive impairment, and increased density of dendritic spines (Bear et al., 2004). LTD triggered by mGluR activation requires local mRNA translation, unlike NMDA receptor-induced LTD (Huber et al., 2000), making FMRP a candidate for involvement in LTD. Activation of group 1 mGluRs also stimulates synthesis of FMRP at the synapse (Weiler et al., 1997) and leads to translocation of FMRP and *Fmr1* mRNA into synaptic spines (Antar et al., 2004). Importantly, it has been demonstrated that mGluR LTD is enhanced and protein synthesis-independent in *Fmr1* KO mice (Huber et al., 2002). This has led to the exploration of FXS treatment with mGluR antagonists. The mGluR5 antagonist MPEP was shown to ameliorate audiogenic seizures in *Fmr1* KO mice (Yan et al., 2005), and clinical trials with mGlu5 antagonists are currently ongoing.

FXS and Epilepsy

About 25% of all FXS patients develop epilepsy (Bassell and Warren, 2008). Electroencephalogram (EEG) data from FXS patients with seizures indicates benign focal epilepsy and shows centrotemporal spikes (Hagerman and Stafstrom, 2009). Many individuals have abnormal EEG readings, without exhibiting epileptic seizures (Hagerman et al., 2009). In most cases, the seizures are treated with anticonvulsants and disappear in adolescence (Hagerman and Stafstrom, 2009). Similarly, *Fmr1* knockout mice have an unusually high susceptibility to audiogenic seizures, display epileptiform discharges, and have hyperexcitable neurons (Musumeci et al., 2000, Chuang et al., 2005, Yan et al., 2005). However, the precise molecular mechanisms that cause increased excitability and impaired synaptic plasticity in FMRP-deficient neurons in the central and peripheral nervous system have not yet been

addressed in a human model. There is hope that interventions correcting this imbalance might be promising therapeutic strategies to treat FXS and other brain illnesses.

A-type Potassium Channel Kv4.2

One important regulator of excitability in the brain is the potassium channel Kv4.2. Kv4.2 potassium channels are highly expressed in the central nervous system and are concentrated in hippocampal CA1 pyramidal cells (Serôdio and Rudy, 1998). Kv4.2 is also found in the CA2 and CA3 regions of the hippocampus, the caudate putamen, pontine nucleus, and medulla (Birnbaum et al., 2004). In dendrites of neurons, Kv4.2 channels are increasingly more concentrated further from the soma. The channels mediate transient A-type outward currents, which rapidly hyperpolarize cells in response to depolarization, thereby diminishing the back-propagation of action potentials into dendrites (Brager and Johnston, 2014). This controls the excitability of a neuron and regulates its capability to undergo long-lasting changes in signal transmission. As such, Kv4.2 is critically involved in the regulation of dendritic excitability and plasticity in the hippocampus. Current models show that Kv4.2 channels can aid in regulation of LTP at synapses lacking AMPA receptors, termed “silent synapses.” At these synapses, lower densities of Kv4.2 channels allow for further back-propagation of depolarization in the dendrite, thus activating NMDA receptors and allowing for long-term potentiation (LTP) (Birnbaum et al., 2004).

Kv4.2 protein expression or function was shown to be altered in several neurological diseases, suggesting that accurate regulation of Kv4.2 is essential for normal brain function (Birnbaum et al., 2004). Kv4.2 is clustered in the CA1 region of the hippocampus, especially around postsynaptic membranes (Alonso and Widmer, 1997) and has been shown to decrease following kainic acid-induced seizures in the rat hippocampus (Francis et al., 1997, Lugo et al., 2008). In addition, Kv4.2 knockout mice are more susceptible to audiogenic seizures (Barnwell

et al., 2009). A mutation in Kv4.2 has been linked to temporal lobe epilepsy in humans (Singh et al., 2006). Importantly, it was recently discovered that a mutation in KCND2, the gene coding for Kv4.2, was found in identical twins with autism and seizures (Lee et al., 2014).

FMRP Regulates Kv4.2

Two studies (Gross et al., 2011, Lee et al., 2011) have recently shown that FMRP regulates Kv4.2 expression. Gross et al. (2011) have shown that Kv4.2 surface expression is reduced in *Fmr1* knockout mice, suggesting that FMRP positively regulates Kv4.2 mRNA translation and cell surface expression. However, Lee et al. (2011) have shown that Kv4.2 is upregulated in a mouse model of FXS. The reasons for this apparent discrepancy are not yet understood. The use of *Fmr1* KO mice with different genetic backgrounds in each study may have contributed to the conflicting results. Gross et al. studied *Fmr1* KO mice with a C57BL/6J background, while Lee et al. used *Fmr1* KO mice with a FVB background. While observation of audiogenic seizures is reliably observed across different FXS mouse models, the behavioral phenotype depends largely on genetic background, age, and experimental setting (Gross et al., 2012). Another contributing factor to the discrepancy in results could be due to different Kv4.2 constructs used in each study. Gross et al. studied the rat Kv4.2 3'UTR, while Lee et al. studied the mouse Kv4.2 3'UTR, which is more similar in length to the human sequence.

A recent functional study investigating the electrophysiological consequences of the loss of FMRP supports the hypothesis that FMRP is a positive regulator of Kv4.2. The study found that in *Fmr1* KO mice A-type K^+ current (I_{KA}) is reduced in CA1 pyramidal neurons, especially in the dendrite (Routh et al., 2013). Moreover, *Fmr1* KO neurons were activated at more negative potentials, were less voltage dependent, and had increased back-propagation of action potentials

when compared to wild-type neurons (Routh et al., 2013). These results suggest that FMRP knockout leads to functional downregulation of Kv4.2.

In order to further elucidate the effect of *FMR1* silencing on Kv4.2, and to evaluate Kv4.2 as a potential therapeutic target, it is important to examine loss of FMRP in human cells. Notably, recent unpublished experiments from the Bassell lab may suggest decreased levels of Kv4.2 in post-mortem hippocampal brain tissue from FXS patients (Mishra A., 2012) (Fig.2). The results were inconclusive due to a small sample size and patient comorbidities. However, the results do provide further support for the hypothesis that Kv4.2 expression is decreased in fragile X syndrome. In order to further test the hypothesis, it is necessary to utilize a more stable human model system. Human embryonic kidney 293 (HEK293) cells in culture allow for this approach.

Based on the previous studies of the Bassell lab and others, we hypothesize that altered expression of Kv4.2 potassium channels might cause impaired synaptic plasticity and hyperexcitability in FXS. We speculate that, normally, FMRP activates translation. FMRP may limit the downregulation of Kv4.2, which is induced by synaptic activity, and thus prevent hyperexcitation.

Previous data of the Bassell lab has demonstrated that Kv4.2 mRNA associates with FMRP in cortical brain lysates from mice, and is translationally dysregulated in *Fmr1* KO mice (Gross et al., 2011). Reporter assays using the rat Kv4.2 3'UTR showed that FMRP is a positive regulator of the translation of rat Kv4.2 mRNA. However, while the rat and the human Kv4.2 gene are very homologous in the 5'UTR, the coding sequence, and parts of the 3'UTR, the most notable difference is that the human Kv4.2 3'UTR is 907 base pairs longer than the rat Kv4.2 3'UTR and contains a second FMRP binding site (Lee et al., 2011) (Fig.3). The homologous section of the rat and human Kv4.2 3'UTRs is ~1.5kb. Differential effects of the two FMRP

binding sites on Kv4.2 3'UTR mRNA have not been explored.

Here, we examine if and how protein levels and mRNA translation of human Kv4.2 are regulated by FMRP in a human cell culture model. We found that, in human cell models of FXS, protein expression of the human A-type potassium channel Kv4.2, but not mRNA translation, is reduced after knockdown of FMRP. In addition, we found that overexpression of FMRP decreases expression and mRNA translation of Kv4.2 in human cells. Importantly, we also began to examine the differences between the FMRP-mediated translational regulation of Kv4.2 3'UTR at its two FMRP binding sites. Our preliminary results indicate that the more proximal FMRP binding site in the Kv4.2 3'UTR is positively regulated by FMRP, while the distal FMRP binding site is negatively regulated.

The current research provides crucial insight into species-specific regulation of Kv4.2 mRNA by FMRP, and will provide rationale to pursue Kv4.2 as a therapeutic target for FXS, with the hope that correcting dysregulated A-type currents will lead to effective treatments for hyperactivity, seizures, and sensory hypersensitivity in patients with FXS. This may also guide the development of novel treatment strategies for patients with other forms of epilepsy.

Experimental Methods

Cloning of Reporter Constructs

The EGFP-human Kv4.2 3'UTR plasmid was cloned by first amplifying human Kv4.2 3'UTR using Kv4.2 primers (forward: gatctcgaggagaattcgagccctggc; reverse: gatggatccgtcctgttcaagcatgcacatg) using a Kv4.2 cDNA clone template (*Origene*). The human Kv4.2 3'UTR and pEGFP-C1 (plasmid, enhanced green fluorescent protein-C1) (*Clontech*) were cut with Fast Digest (FD) Xho1 and BamH1 (*Fermentas*) and ligated with T4 DNA ligase (*Fermentas*) according to the manufacturer's instructions. Clones were transformed into DH5α chemo-competent cells. Colonies were inoculated in LB media containing appropriate antibiotics. After ~16 hours DNA was isolated by alkaline hydrolysis using *QIAGEN* buffers, followed by isopropanol precipitation. DNA was analyzed by restriction digest to identify pEGFP plasmids containing the Kv4.2 insert. Positive clones were retransformed and DNA was purified with the *QIAGEN* Maxi-prep kit. (Fig.4A). Constructs were verified by sequencing.

In order to clone human Kv4.2 3'UTR into a pGL3 firefly luciferase plasmid, pEGFP-Kv4.2 3'UTR was linearized with FD Xho1, and ends were filled using Klenow (*Fermentas*). Then the Kv4.2-3'UTR was cut out using BamH1. The pGL3 plasmid was cut with FD Xba1 and its ends were filled using Klenow, followed by restriction digest with fast digest BamH1. Then, Kv4.2-3'UTR and pGL3 were ligated with T4 DNA ligase (*Fermentas*) according to manufacturer's instructions. Clones were transformed into DH5α chemo-competent cells. Colonies were inoculated in LB media containing appropriate antibiotics. After ~16 hours DNA was isolated by alkaline hydrolysis using *QIAGEN* buffers, followed by isopropanol precipitation. DNA was analyzed by restriction digest to identify pGL3 plasmids containing the Kv4.2 insert. Positive clones were retransformed and DNA was purified with the *QIAGEN* Maxi-

prep kit. (Fig.5). Constructs were verified by sequencing.

The 5' (Δ3) and 3' (Δ5) parts of human Kv4.2 3'UTR were amplified by PCR, and cloned into pEGFP-C1 using BamH1 and Xho1 (*Fermentas*) (Fig.4B,C). To clone the rat Kv4.2-human Kv4.2 Δ5 hybrid construct, the rat Kv4.2 3'UTR was cut out of the FFL-rat Kv4.2 3'UTR plasmid using Fast Digest Xho1 and Sma1 and ligated into pEGFP-C1 as described above. Next, the Δ5 part of human Kv4.2 3'UTR (distal part) was amplified using primers including a Kpn1 sequence (forward: gat**ggtacc**catgctgcacatgacag; reverse: gat**ggtacc**gtccaagcatgcacatg) and ligated into the pEGFP-rat Kv4.2 3'UTR plasmid using Kpn1. Clones were transformed into DH5α chemo-competent cells. Colonies were inoculated in LB media containing appropriate antibiotics. After ~16 hours DNA was isolated by alkaline hydrolysis using *QIAGEN* buffers, followed by isopropanol precipitation. DNA was analyzed by restriction digest to identify EGFP plasmids containing the Kv4.2 insert. Positive clones were retransformed and DNA was purified with the *QIAGEN* Maxi-prep kit. (Fig.4B-D). Constructs were verified by sequencing.

Cell Culture

Human embryonic kidney 293 (HEK293) cells were cultured on 10cm tissue culture dishes in Dulbecco's Modified Eagle Medium (DMEM; 25mM glucose, 1mM pyruvate) (*Invitrogen*) with 10% fetal bovine serum (FBS) and 1x penicillin streptomycin (P/S) (*Invitrogen*) added. Cells were plated at ~30% confluency and passaged every 2-3 days once cells reached ~100% confluency.

FMR1 Knockdown

For *FMR1* knockdown, the cells were transfected, using Lipofectamine 2000 (*Invitrogen*) in Opti-MEM (*Invitrogen*) according to the manufacturer's protocol, with either *FMR1*-specific

siRNA (uggcgcuuucuacaaggcauuugua) or scrambled siRNA control (ugguuuccaucggaauuacugcgua) (STEALTH RNAi^{TM}, *Invitrogen*). For luciferase assays, cells in 12-well plates at ~30% confluency were transfected with 40pmol siRNA. For polysome gradients, cells in 10cm plates at ~30% confluency were transfected with 160pmol siRNA.

Time Course of FMR1 Knockdown

Cells were plated at ~20% density in 8 wells of a 12-well plate. Two wells were transfected in each of four conditions: (1) si*FMR1* (20pmol) (2) siCtr (20pmol) (3) si*FMR1* (40pmol) (4) siCtr (40pmol). Cells from each condition were lysed at either 48 hours or 72 hours after transfection with the lysis buffer (20mM Tris-HCl 7.5, 100mM KCl, 5mM $MgCl_2$, NP40, protease inhibitor, 100X cyclohexamide, SuperAse-In 20U/μL) and spun down at 12,000rcf for 10 min.

FMRP Overexpression

For FMRP overexpression cells were transfected, using Lipofectamine 2000 (*Invitrogen*) in Opti-MEM, with either a construct containing EGFP fused in frame to the human FMRP open reading frame (pEGFP-FMRP) or with pEGFP-C1 according to the manufacturer's protocol. For luciferase assays, cells in 12-well plates at ~30% confluency were transfected with 1μg DNA. For polysome gradients, cells plated in 10 cm dishes at ~30% confluency were transfected with 4μg DNA.

Protein expression detection with luciferase assays

First, FMRP was either knocked down or overexpressed, as described above. Cells were

incubated overnight at 37°C. Then, cells were transfected with 0.5µg firefly luciferase-βactin, 0.5µg firefly luciferase-rat Kv4.2 3'UTR, or 11µg firefly luciferase-human Kv4.2 3'UTR construct, in addition to 0.08µg renilla luciferase. Cells were again incubated overnight at 37°C. The luciferase assay was conducted using the Dual-Glo Luciferase Assay System (*Promega*) and protocol. The system allows for sequential measurement of firefly luciferase activity and renilla luciferase activity, which acts as an internal control. Emission of light (proportional to luciferase activity) was measured using the *Veritas* luminometer. Results were normalized to renilla luciferase as a control for transfection efficiency.

Polysome analysis and RNA purification from sucrose gradients

HEK293 cells after *FMR1* knockdown or FMRP overexpression, respectively, were transfected with 8µg human GFP-Kv4.2 3'UTR reporter construct. Cells were incubated overnight at 37°C. In a separate experiment, cells were transfected only with the GFP-human Kv4.2 3'UTR construct and treated with 1mM puromycin for 1 hr. Cells were lysed using a lysis buffer (20mM Tris-HCl 7.5, 100mM KCl, 5mM $MgCl_2$, NP40, protease inhibitor, 100X cyclohexamide, SuperAse-In 20U/µL) and spun down at 12,000rcf for 10 min. Resulting supernatant was loaded on a 15-45% linear sucrose gradient (prepared in 20 mM Tris-HCl 7.5, 100 mM KCl, and 5 mM MgCl) and spun at 38,000rcf for 2 hours in the *Beckman Coulter* ultracentrifuge SW41 rotor. A small fraction of the supernatant (~30µl) was kept for western blot analyses. A 60% sucrose solution was used to push each sample out of the centrifuge tube without disturbing the gradient. The gradient was separated into 10 fractions using the Foxy R1 Fractionator and UV absorption at 245nm recorded with the UA-6 UV Detector (example readings shown in Fig.6). RNA was isolated from each fraction with Trizol (*Invitrogen*) and

mRNAs of interest were analyzed by qRT-PCR.

RNA Purification

After collection of sucrose fractions, 300µl of each sample was suspended in 900ul Trizol LS Reagent (*Invitrogen).* RNA was purified according to the manufacturer's instructions. In order to visualize the RNA pellet, 2µl Pellet Paint Co-Precipitant (*Millipore*) were added. The purified RNA pellet was resuspended in 20µl water.

cDNA Synthesis and qRT-PCR

7.5µl of each RNA sample was used to generate cDNA by reverse transcription in a 10µl volume (*iScript system, Biorad*) and 2µl of the reaction were used for quantitative real-time PCR (*Roche 480 Lightcycler Master Mix and System*). GFP-specific primers were used to analyze siRNA knockdown experiments (forward: aaggacgacggcaactacaag; reverse: atgccgtttcttctgcttgtcg). Human Kv4.2 3'UTR-specific primers (forward: agcatgctttctacgccatt; reverse: ttgcaatacacaggaacctttc) were used to analyze FMRP overexpression experiments.

Protein Detection by Western Blotting

Analysis of *FMR1* knockdown and overexpression: Equal amounts of HEK293 cell lysate were resolved on 8% SDS polyacrylamide gels and transferred to PVDF membranes. Blots were probed with antibodies against FMRP (rabbit anti-FMRP antibody, 1:2000), followed by horseradish peroxidase-conjugated secondary antibody (anti-rabbit IgG, 1:2000) and α-tubulin (mouse anti-tubulin, 1:5000), followed by horseradish peroxidase-conjugated secondary antibodies (anti-mouse IgG, 1:4000). Tubulin was used as a loading control. Bands were

visualized using autoradiography. The blots were quantified using *Image J* software. Levels of FMRP were normalized to tubulin.

Analysis of *FMR1* knockdown time course: Equal amounts of HEK293 cell lysate were resolved on 8% SDS polyacrylamide gels and transferred to PVDF membranes. Blots were probed with antibodies against FMRP (rabbit anti-FMRP, 1:2000) and α-Tubulin as a loading control (mouse anti-Tubulin, 1:5000), followed by fluorescently labeled secondary antibodies (goat anti-rabbit 800CW, 1:5000 and goat anti-mouse 680RD, 1:10,000, *Li-Cor*). Bands were visualized using the *Odyssey* system for fluorescence detection. Blots were quantified using the *Li-Cor Image Studio.* Levels of FMRP were normalized to Tubulin.

Statistics

Statistics were performed using SPSS 21. Data was tested for normality using the Shapiro-Wilk test, and appropriate parametric or non-parametric tests were used. Two-tailed student's t-tests were performed on qRT-PCR quantification of Kv4.2 mRNA and Western blot quantifications. Significance was defined at $p \leq 0.05$. Friedman's two-way analysis of variance with Games-Howell post hoc tests was performed on results of the luciferase assay. Error bars indicate standard error of the mean (SEM).

Results

Previous animal studies examining the effect of FMRP on the potassium channel Kv4.2 have shown conflicting results. One study showed that Kv4.2 is downregulated in *Fmr1* KO mice (Gross et al., 2011). The other showed that Kv4.2 is upregulated in *Fmr1* KO mice (Lee et al., 2011). In this thesis, we sought to determine the effect of FMRP on the potassium channel Kv4.2 in human cells. We approached this through two principle aims. The first was to examine the effect of *FMR1* knockdown on Kv4.2 expression and mRNA translation. The second was to analyze the effect of FMRP overexpression on Kv4.2 expression and mRNA translation. In order to investigate protein expression, we conducted luciferase assays, and to measure mRNA translation, we utilized polysome gradients.

Based on the results of our first two aims, we developed a novel hypothesis that FMRP differentially regulates Kv4.2 expression via two separate binding sites in the human Kv4.2 3'UTR. We postulate that FMRP mediates translational activation at the more proximal binding site and mediates inhibition of translational activation at the more distal site. In this thesis, we cloned, and initially tested the effect of FMRP on Kv4.2 deletion constructs that will serve as tools to test this hypothesis in the future.

FMR1 knockdown decreases expression of a human Kv4.2 reporter

We first examined the effect of *FMR1* knockdown (KD) on the enzymatic activity of a luciferase reporter construct containing the human Kv4.2 3'UTR. *FMR1* KD in HEK293 cells was used as an *in vitro* model of FXS in human cells. As a read-out for the effect of the Kv4.2 3'UTR on protein expression, we conducted luciferase assays. The luciferase assay is an indirect measure of protein expression that measures levels of light emitted by firefly luciferase

following application of its substrate. Here, we generated a reporter construct containing the coding sequence for firefly luciferase immediately followed by the 3'UTR of human Kv4.2 (FFL-huKv4.2) (Fig.5), as previous studies have shown that FMRP binds to and regulates Kv4.2 mRNA translation via the 3'UTR of mouse and rat Kv4.2 (Gross et al., 2011, Lee et al., 2011). For these experiments, cells were first transfected with an *FMR1*-specific siRNA (si*FMR1*) or a scrambled control siRNA (siCtr). The following day, cells were transfected with the firefly luciferase-human Kv4.2 3'UTR construct (FFL-huKv4.2, Fig.4), FFL-ratKv4.2 3'UTR (Gross et al., 2011) as a positive control, or FFL-βActin as a negative control. All cells were also transfected with a renilla luciferase control that does not contain the Kv4.2 3'UTR. Renilla luciferase activity is a measure of basal levels of luciferase expression, thus acting as an internal control for transfection efficiency. The next day, the cells were lysed and light levels recorded after subsequent exposure to firefly and renilla luciferase-specific substrates. The luciferase assay showed that after siRNA-mediated knockdown of FMRP, there was a significant decrease in activity of the reporter containing human Kv4.2, as compared to the control (Fig.7) (Friedman's Two-Way Analysis of Variance, Games-Howell post hoc, $p = 0.001$, $N=9$). The expression of the βActin reporter did not change significantly. In contrast to the Bassell lab's previous studies (Gross et al., 2011), there was no significant change in the expression of the rat Kv4.2 3'UTR (Fig.7). Western blot analyses showed that FMRP expression was reduced by *FMR1*-specific siRNA (Fig.9A). We cannot fully explain the apparent lack of an effect of *FMR1* KD on rat Kv4.2 expression, but speculate it may be caused by lower efficiency of the *FMR1* knockdown, and/or by the use of a different cell system (HEK293 cells versus Neuro2A cells) than was used in the previous study (Gross et al., 2011). The results, taken with the high concentration at which the human Kv4.2 3'UTR was transfected for the luciferase assays (11μg),

relative to the rat Kv4.2 3'UTR (0.5μg), suggest that the human Kv4.2 3'UTR might experience a stronger inhibitory effect on protein expression compared to the rat sequence, providing support for the idea that FMRP has an inhibitory binding site on human Kv4.2 that the rat sequence lacks.

Identification of actively translating polysomes by puromycin treatment

In order to establish which polysome fractions contained mRNA that was being actively translated, we utilized cells treated with puromycin. Puromycin is an antibiotic that aborts translation by mimicking a tRNA molecule (Nathans, 1964), leading to polysome "run-off" of the transcripts. Cells were transfected with a reporter construct containing the coding sequence for GFP fused to the human Kv4.2 3'UTR and allowed to express overnight. Then, cells were treated with puromycin for 1 hour before being lysed. Cyclohexamide (10μg/mL) in the lysis buffer and sucrose gradient acted to stall translation and create stability of ribosomal subunits on mRNA. Cell lysate was added to the sucrose gradient and centrifuged. This allowed for distribution of mRNA by its sedimentation coefficient, which is proportional to the number of polysomes attached to the mRNA. Polysomes are chains of ribosomes on an mRNA (Mašek et al., 2011), and multiple polysomes attached to an mRNA indicate active translation. Gradients were fractionated and the UV absorption along the gradient was measured. The UV trace indicates where the 40s, 60s, and 80s ribosomal subunits, and polysomes are within the sucrose gradient (Fig.6A). The UV trace of the puromycin-treated cells shows a clear lack of polysomes (Fig.6B), indicating the successful disassembly of actively translating polysomes.

After the sucrose fractions were collected, we purified RNA from each sample and synthesized cDNA. Then, we performed qRT-PCR using GFP-specific primers in order to determine which fractions contained actively translating human Kv4.2 mRNA. When compared

to untreated cells, puromycin-treated cells showed a decrease in Kv4.2 mRNA levels in fractions 6-10 (Fig.8A), indicating the presence of actively translating polysomes. Normalization of GFP mRNA levels in fractions 6-10 to the sum of mRNA in all fractions shows a significant reduction in mRNA levels in the heavy, polysome-containing fractions (6-10) in puromycin-treated cells (Fig.8B) (N=5, paired samples t-test, $p = 0.008$). These results justified our assumption that fractions 6-10 contain actively translating Kv4.2 mRNA and provided the basis for the analysis of the *FMR1* knockdown and FMRP overexpression polysome gradients described below.

FMR1 knockdown has no significant effect on association of human Kv4.2 mRNA with actively translating polysomes

To measure mRNA translation after *FMR1* knockdown, cells were first transfected with either si*FMR1*, or siCtr. The following day, cells were transfected with the GFP-human Kv4.2 3'UTR construct. Cells were lysed on the third day, allowing for two days of siRNA-mediated knockdown. Fractions were collected and analyzed as described for the puromycin-treated cells. The qRT-PCR analyses of seven independent experiments indicate that there was no significant change in Kv4.2 mRNA translation after knockdown (Fig.9B-C). Western blot quantification indicated that knockdown occurred in all experiments when normalized to tubulin (Fig.9A) (N=5, paired t-test, $p = 0.13$).

FMR1 knockdown is more effective after 72 hours

We expected a decrease in human Kv4.2 mRNA translation after siRNA-mediated knockdown of *FMR1*. This was expected due to the results of the luciferase assay (Fig.7) and previous data showing a decrease in rat Kv4.2 3'UTR translation after knockdown in a murine

Neuro2A cell line (Gross et al., 2011). As such, we tested the effectiveness of the knockdown, and whether an additional day of knockdown, or an increased concentration of siRNA, would be more effective. In order to examine this, cells were transfected with either si*FMR1* or siCtr at either the concentration used for the polysome gradients, or twice the concentration. Half of the cells were allowed to express the siRNA for 48 hours (as used in the polysome assays), and the other half for 72 hours before lysing. Western blot analysis showed a stronger decrease (though not a complete loss) of FMRP levels after 72 hours compared to 48 hours of knockdown when normalized to tubulin (Fig.10). These results indicate that a 72 hour knockdown could be used in future experiments to increase knockdown efficiency.

No significant difference in puromycin-sensitivity of Kv4.2 mRNA following FMR1 knockdown

Previous studies have shown that the number of ribosomes on a given mRNA does not necessarily demonstrate active translation. In these studies, significant inhibition of the synthesis of specific proteins did not result in the expected decrease in the number of ribosomes on the associated mRNA (Olsen and Ambros, 1999, Clark et al., 2000). A more recent study demonstrated that, in the presence of FMRP (cells from wild-type mice), more ribosomes remained on FMRP mRNA targets after treatment with puromycin than in cells lacking FMRP (from *Fmr1* KO mice) (Darnell et al., 2011). This method allows for the separation of actively translating and stalled ribosomes on FMRP mRNA targets. Our initial experiments using puromycin in cells with normal FMRP levels demonstrated that the polysome gradient method indeed quantified actively translating Kv4.2 mRNA. However, to ensure that a potential effect of FMRP levels on Kv4.2 mRNA association with actively translating polysomes was not masked

by a large proportion of Kv4.2 mRNA in translationally inactive ("stalled") ribosomes, we tested if puromycin-sensitivity of Kv4.2 mRNA changes depending of FMRP levels. To do this, we knocked down FMRP and then treated the cells with puromycin. Preliminary data indicates no differences in the effects of puromycin on Kv4.2 mRNA levels in heavy fractions of *FMR1* knockdown cells compared to control cells (Fig.11). In summary, these experiments indicate that, at least with the polysome gradient method, no significant difference in human Kv4.2 mRNA translation following *FMR1* knockdown can be detected.

FMRP overexpression decreases expression of a human Kv4.2 reporter

In order to further test our hypothesis that FMRP regulates Kv4.2, we also examined the effect of FMRP overexpression on Kv4.2 expression and mRNA translation. To do this, we first cloned a construct containing GFP fused to the open reading frame of human FMRP (GFP-FMRP). For the luciferase assay, cells were first transfected with the GFP-FMRP construct. The following day, cells were transfected with the FFL-huKv4.2 3'UTR, FFL-ratKv4.2 3'UTR (Gross et al., 2011) as a positive control, or FFL-βActin as a negative control. All cells were also transfected with renilla luciferase control that does not contain the Kv4.2 3'UTR. The next day, the cells were lysed and light levels recorded. Results from the luciferase assays show a significant decrease in activity of the reporter containing human Kv4.2, as compared to the control, after overexpression of FMRP (Fig.12) (Friedman's Two-Way Analysis of Variance with Games-Howell post hoc, $p = 0.008$, $N=7$). Interestingly, after overexpression of FMRP, expression of rat Kv4.2 showed a significant increase in activity of the reporter containing human Kv4.2, as compared to the control (Fig.12) (Friedman's Two-Way Analysis of Variance with Games-Howell post hoc, $p = 0.001$, $N=7$), which is in line with the previously published

study of the Bassell lab (Gross et al., 2011).

FMRP overexpression decreases association of human Kv4.2 mRNA with actively translating polysomes

We also examined the effect of FMRP overexpression on Kv4.2 mRNA translation. We transfected HEK293 cells with the GFP-FMRP construct. The next day, cells were transfected with a GFP-human Kv4.2 3'UTR construct. Cells were lysed on the third day. Fractions were collected and analyzed as described for the puromycin-treated cells, except a human Kv4.2-specific primer was used during qRT-PCR. After FMRP overexpression, HEK293 cells exhibited reduced levels of Kv4.2 mRNA in actively translating polysome fractions compared to cells transfected with only pEGFP-C1 (Fig.13B,C) (paired samples t-test, $p = 0.012$, N=4). Western blot analysis indicated the overexpression was successful in each experiment (Fig.13A). These results were in line with the observed reduction in activity of a luciferase reporter containing the human Kv4.2 3'UTR following FMRP overexpression.

Increased levels of Kv4.2Δ3 mRNA translation after overexpression of FMRP

A previous study showed two FMRP binding sites in the mouse Kv4.2 3'UTR (Lee et al., 2011), which are also present in the human Kv4.2 3'UTR (Fig.3). Interestingly, the rat Kv4.2 3'UTR is significantly shorter than the human sequence, and contains only the proximal FMRP binding site (Fig.3). We speculate that both binding sites are regulated differentially by FMRP (see Fig.16 and Discussion), which may explain the differences observed in the luciferase assay between human and rat Kv4.2 reporters. To further test this hypothesis, we cloned deletion constructs containing either one of the two binding sites (Fig.14), as well as a "humanized" rat

construct that contains the rat Kv4.2 3'UTR followed by the 3' portion of the human Kv4.2 3'UTR, which is not present in the rat Kv4.2 3'UTR. A preliminary polysome gradient was carried out with the 5' portion of the human Kv4.2 3'UTR (GFP-Kv4.2Δ3) construct and overexpression of FMRP. The GFP-Kv4.2Δ3 is similar to the rat sequence, as it only contains the proximal FMRP binding site and is missing the distal binding site (Figs.3,14). Initial results indicate that overexpression of FMRP led to an increase in the translation of Kv4.2Δ3 mRNA (N=1), similar to what is observed using the rat reporter, and opposite to the effect on the full length human Kv4.2 3'UTR.

Discussion

Hyperactivity, hypersensitivity to stimuli, and epileptic seizures are recognized as symptoms of fragile X syndrome (FXS), but their molecular mechanisms are poorly understood. Previous studies have examined the cause of neuronal hyperexcitability in mouse models and found that FMRP regulates Kv4.2, a potassium channel critical for regulating neuronal excitability. However, the conclusions reached by each study were in opposition (Gross et al., 2011, Lee et al., 2011). A recent functional study supported the hypothesis that FMRP is a positive regulator of Kv4.2 by demonstrating that *Fmr1* KO mice have reduced A-type K^+ current (I_{KA}) in the hippocampus (Routh et al., 2013). The current study provides further insight into the effects of FMRP on Kv4.2 in a human cell model of fragile X syndrome.

Here, we show that decreased levels of FMRP lead to reduced expression of a human Kv4.2 3'UTR reporter (Fig.7). This is in line with the model suggested by the Gross (2011) and Routh (2013) studies, which suggest FMRP is a positive regulator of Kv4.2 expression. However, in two different experimental approaches, we show that overexpression of FMRP decreases the mRNA translation and expression of the human Kv4.2 3'UTR (Figs.12-13). These findings suggest that FMRP inhibits Kv4.2 expression, as proposed by Lee et al. (2011). Based on these seemingly contradictory results, and results of previous studies, we postulate that the Kv4.2 3'UTR contains two binding sites for FMRP, which are regulated in an opposing manner: the more proximal site is translationally activated by FMRP, and the more distal site is translationally inhibited by FMRP (Fig.16). Under normal conditions, FMRP is preferentially bound to the activating site. When FMRP is in excess, binding to the inhibitory site occurs and overrides the activating effects (Fig.16). We provide initial experimental support for this hypothesis by demonstrating increased Kv4.2 3'UTRΔ3 mRNA translation after overexpression

of FMRP (Fig.15). Taken together, the results of this study led to the development of a testable hypothesis for a novel mechanism of FMRP-mediated translational control of its target mRNA Kv4.2, which may explain the controversial results of previous studies.

Our results indicate that when FMRP is knocked down using *FMR1*-specific siRNA, there is a decrease in protein expression of human Kv4.2 3'UTR in human cells (Fig.7). These findings confirm the previous results from the Bassell lab showing that Kv4.2 3'UTR mRNA translation and expression are reduced in the brains of *Fmr1* KO mice (Gross et al., 2011). However, the luciferase assay provides an indirect measure of protein expression. The polysome gradient after siRNA-mediated knockdown of FMRP measures mRNA translation, thus providing a more direct measurement for the interaction of FMRP with Kv4.2 mRNA.

The results of the polysome gradient after *FMR1* KD indicate no significant change in the association of FMRP with Kv4.2 mRNA (Fig.9). These results do not support either theory concerning the effect of *Fmr1* KO on Kv4.2 in mice. Western blot analyses revealed that the *FMR1* knockdown was effective, and that 48 hours was a sufficient amount of time to allow for FMRP knockdown (Fig.9A). However, in all knockdown experiments, FMRP was still expressed. This is in contrast to the complete loss of FMRP in *Fmr1* KO mice and most cases of FXS in human patients. Small differences in FMRP expression may contribute to the inconsistent results observed. We went on to show preliminary data that indicates 72 hours of *FMR1* knockdown further decreases levels of FMRP, thus providing a more accurate model (Fig.10). In future experiments, cells will be allowed to express *FMR1* siRNA for 72 hours.

We also considered the possibility that the polysome gradient was not solely measuring actively translating Kv4.2 mRNA, but also Kv4.2 mRNA bound to stalled ribosomes. In order to examine this possibility, we adapted methods previously used to examine the effect of FMRP on

mRNA translation of various targets in FMRP KO and wild type mice (Darnell et al., 2011) through use of the translational inhibitor puromycin. Puromycin causes actively translating ribosomes, but not stalled (translationally inactive) ribosomes, to dissociate from mRNA by mimicking a tRNA molecule. Treating cells with puromycin in addition to *FMR1* KD did not reveal a significantly different association of Kv4.2 mRNA with polysomes compared to control cells (Fig.11). This evidence indicates that siRNA-mediated knockdown of *FMR1* does not result in a large difference in active translation. It is possible that our model system does not provide an accurate representation of the effect of FMRP loss in human cells. A complete knockout may be required to examine the role of FMRP in human cells. The availability of induced pluripotent stem cells from FXS patient cells, and neurons derived from these cells, will allow for future experiments analyzing Kv4.2 expression in human cell models that better reflect the disease status.

After overexpression of FMRP in HEK293 cells using a GFP-FMRP construct, there was a decrease in both protein expression and mRNA translation of the human Kv4.2 3'UTR (Fig.12-13). These results were contrary to our hypothesis. A previous study has shown that FMRP may non-specifically repress translation when overexpressed (Darnell et al., 2011), though these experiments do not examine Kv4.2 specifically. Our results show an *activating* effect of FMRP on the expression of a luciferase reporter containing the rat Kv4.2 3'UTR, thus making a general inhibitory effect of FMRP overexpression unlikely. One explanation for the decrease in Kv4.2 translation and expression after FMRP overexpression is that FMRP's multiple binding sites on the human Kv4.2 3'UTR differentially regulate translation of Kv4.2. Lee et al. have previously shown that there are two separate FMRP binding sites on the mouse Kv4.2 3'UTR sequence (2011). We hypothesize that the proximal FMRP binding site may be a positive regulator of

Kv4.2 mRNA translation, and the distal FMRP binding site may be a negative regulator of Kv4.2 mRNA translation (Fig.16). We speculate that, under normal conditions, FMRP preferentially binds to the proximal (activating) binding site (Fig.16A). Moreover, we speculate that binding of FMRP to the inhibitory, distal site, overrides any activating effect of FMRP. The model proposes that in FXS, lack of FMRP-mediated activation decreases Kv4.2 expression (Fig.16B). Our inconclusive results under *FMR1* knockdown conditions may be caused by the small (and variable) amounts of remaining FMRP bound to the activating site (Fig.16C). However, if FMRP is overexpressed, both binding sites are occupied by FMRP. We speculate that the inhibitory effect overrides the activating effect, leading to inhibition of Kv4.2 expression (Fig.16D). Under physiological conditions, binding of FMRP to the two different sites may be regulated by posttranslational modifications, or trans-acting factors, such as other proteins or microRNAs.

Our model also explains why effects of FMRP overexpression on the rat Kv4.2 3'UTR, in contrast to the human Kv4.2 3'UTR, are consistent with FMRP as an activator of Kv4.2 mRNA translation. The rat sequence only contains the proximal, activating FMRP binding site, but not the more distal, inhibitory site (Fig.15E,F). Initial data on Kv4.2 Δ3 (the sequence homologous to the rat Kv4.2 3'UTR) mRNA translation after FMRP overexpression are in accordance with the idea of activation at the proximal site. Results show that after overexpression of FMRP, Kv4.2 Δ3 mRNA translation is increased compared to the control (Fig.15).

The association of FMRP with the RNA-induced silencing complex (RISC) (Jin et al., 2004b) suggests the involvement of microRNAs (miRNA) in the regulation of Kv4.2. The Bassell lab has previously demonstrated the involvement of microRNAs with another FMRP target, PSD-95 mRNA. PSD-95 mRNA is normally inhibited by an FMRP-activated complex of AGO2 and miR-125a, which is reduced in *Fmr1* KO mice (Muddashetty et al., 2011). Kv4.2

mRNA may also be regulated directly by miRNAs (unpublished results of the Bassell lab). It is hypothesized that microRNAs bind to Kv4.2 mRNA in the absence of FMRP, thus preventing translation of Kv4.2 mRNA. FMRP may compete with, and prevent the binding of, miRNAs to Kv4.2 mRNA, thus leading to translational activation. Interestingly, miRNAs that preferentially target Kv4.2 associate within or close to the proximal FMRP binding site on the 3'UTR, which is present in both the rat and human Kv4.2 3'UTRs. The current results thus fit the hypothesis suggesting a role for microRNAs in the translational regulation of Kv4.2 by FMRP.

The accessory subunits of Kv4.2 may also contribute to the surface level expression of Kv4.2. Kv channel-interacting proteins (KChIPs) and dipeptidyl-peptidase-like proteins (DPLs) associate with Kv4.2 simultaneously to effect its functional properties (Jerng et al., 2005). KChIPs bind to the cytoplasmic N-terminus of Kv4 channels, where they increase surface trafficking and remodel gating behavior (Bähring et al., 2001). The main function of KChIPs is to regulate A-type current, thus influencing long-term potentiation (Lilliehook et al., 2003). DPLs associate with Kv4 channels, where they facilitate surface expression and modify channel properties (Jerng et al., 2004). The altered or absent expression of these Kv4.2 accessory subunits may affect the expression of Kv4.2 differentially in HEK cells compared to neurons. Currently, it is not known if and how FMRP regulates these Kv4.2 auxiliary subunits.

Current treatments for FXS only address the symptoms presented. As such, there are no specific treatments for FXS associated epilepsy. However, understanding the molecular mechanisms of epilepsy in FXS may generate valuable information concerning hyperactivity in FXS and lead to more targeted treatments that avoid undesirable side effects (Hagerman et al., 2009) . Several clinical trials for possible treatments of FXS are currently being conducted. AMPA receptor positive modulators have been tested in FXS patients with limited results. Based

on the mGluR theory of FXS (see introduction) several clinical trials using mGlu5 antagonists have been conducted with some success. Interestingly, mGluR signaling may regulate Kv4.2 itself. Activation of group 1 mGluRs inhibits Kv4.2-mediated currents and increases neuronal excitability in dorsal horn neurons, highlighting the possibility that Kv4.2 is a downstream target of mGluRs (Hu et al., 2007). Additionally, the mGluR antagonist MPEP was shown to partially rescue reduced surface levels of Kv4.2 in *Fmr1* KO mice (Gross et al., 2011). However, further exploration is needed.

The current study will continue with further exploration of the two FMRP binding sites on human Kv4.2 3'UTR. Polysome gradients and luciferase assays will be performed using the three constructs described in the methods section (Fig.14): 5' end of the human Kv4.2 3'UTR, 3' end of the human Kv4.2 3'UTR, and full length rat Kv4.2 3'UTR fused with 3' end of the human Kv4.2 3'UTR. To move into a more physiological relevant system, we plan to continue the experiments using human neurons grown from induced pluripotent stem cells (iPSCs). The results of this study may lead to a more complete understanding of how FMRP regulates Kv4.2 expression and function. In the future, this could help to develop mechanism-based therapies for hyperexcitability and epilepsy in FXS using Kv4.2 agonists. These therapies may also extend to possible treatment for epilepsy, and other diseases involving dysregulated neuronal excitability, associated with Kv4.2 dysfunction, like other autism spectrum disorders and Alzheimer's disease.

Figures

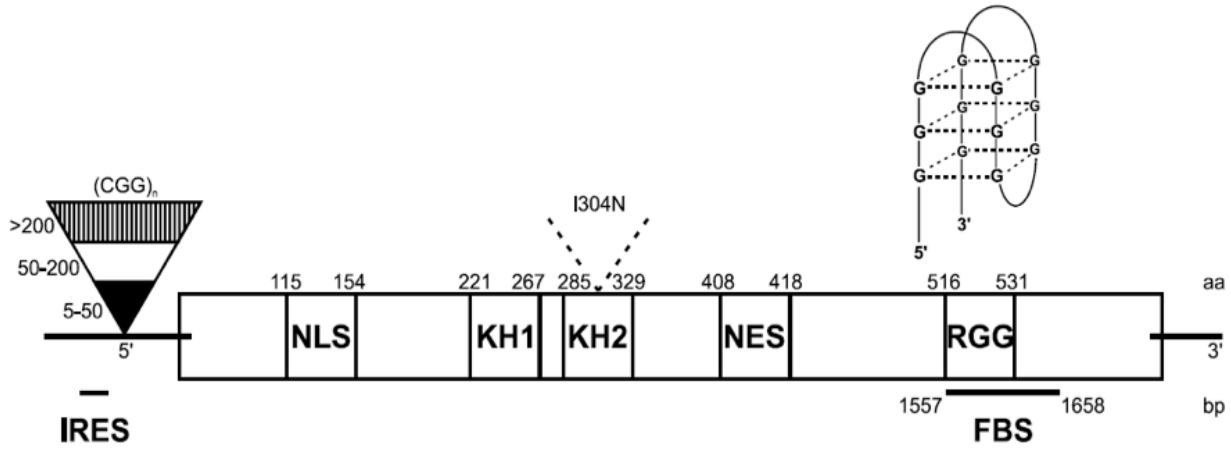


Figure 1. Domain structure for the RNA binding protein FMRP (Wells, 2006), including the three RNA binding motifs, KH1, KH2 and RGG box. *NLS* (Nuclear Localization Sequence), *KH* (hnRNP-K-homology domain), *NES* (Nuclear Export Sequence), *FBS* (FMRP binding site), *RGG* (arginine-glycine-glycine). A specific secondary structure on certain mRNAs (G-quartet, shown above the protein) was suggested to bind to the RGG region. The 5'UTR depicts the different classes of CGG repeats observed on *FMR1*: 5-50, normal; 50-200, premutation; >200, full mutation. I304N indicates the location of a point mutation observed in a FXS patient with particularly severe symptoms (De Boulle et al., 1993).

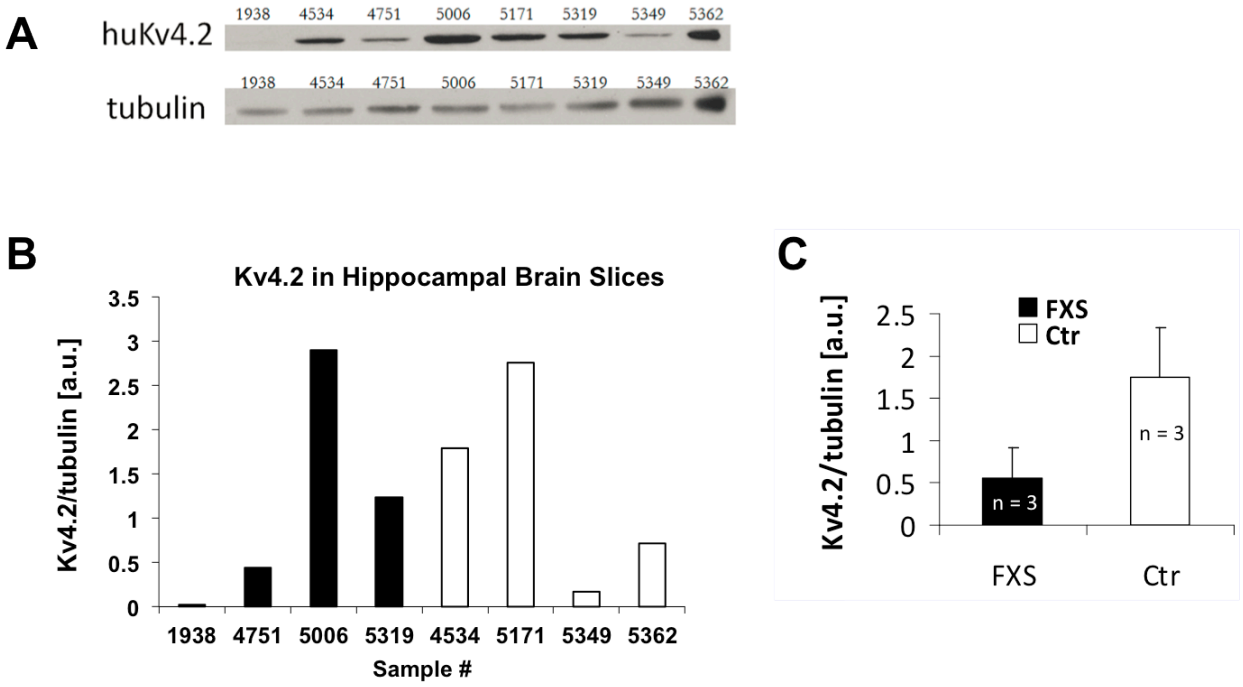


Figure 2. (A) Western blot analysis of human hippocampal tissue shows Kv4.2 and tubulin levels in both FXS (1938, 4751, 5006, 5319) and control (4534, 5171, 5349, 5362) samples. **(B)** All FXS patient samples showed low levels of Kv4.2, except 5006, who suffered from a neurodegenerative disorder that may have affected Kv4.2 levels. In general, controls showed higher levels of Kv4.2, except 5349, who died of drug intoxication and possibly suffered adverse neurological effects as a consequence. **(C)** The two outliers (5006, 5349) were removed and average Kv4.2 levels were shown to be lower in FXS patient samples than in control samples. N=3. (Mishra A., 2012, unpublished).

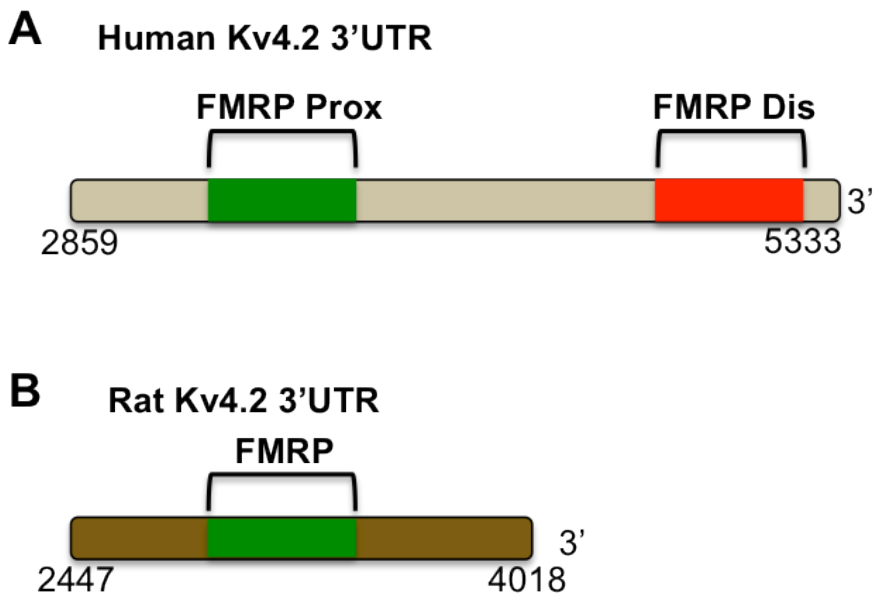


Figure 3. (A) Human (and mouse) Kv4.2 mRNA contain two FMRP binding sites in the 3'UTR: proximal and distal (*FMRP Prox, FMRP Dis*) (Lee et al., 2011). **(B)** The rat Kv4.2 3'UTR is ~900bp shorter than the human sequence and only contains the proximal binding site.

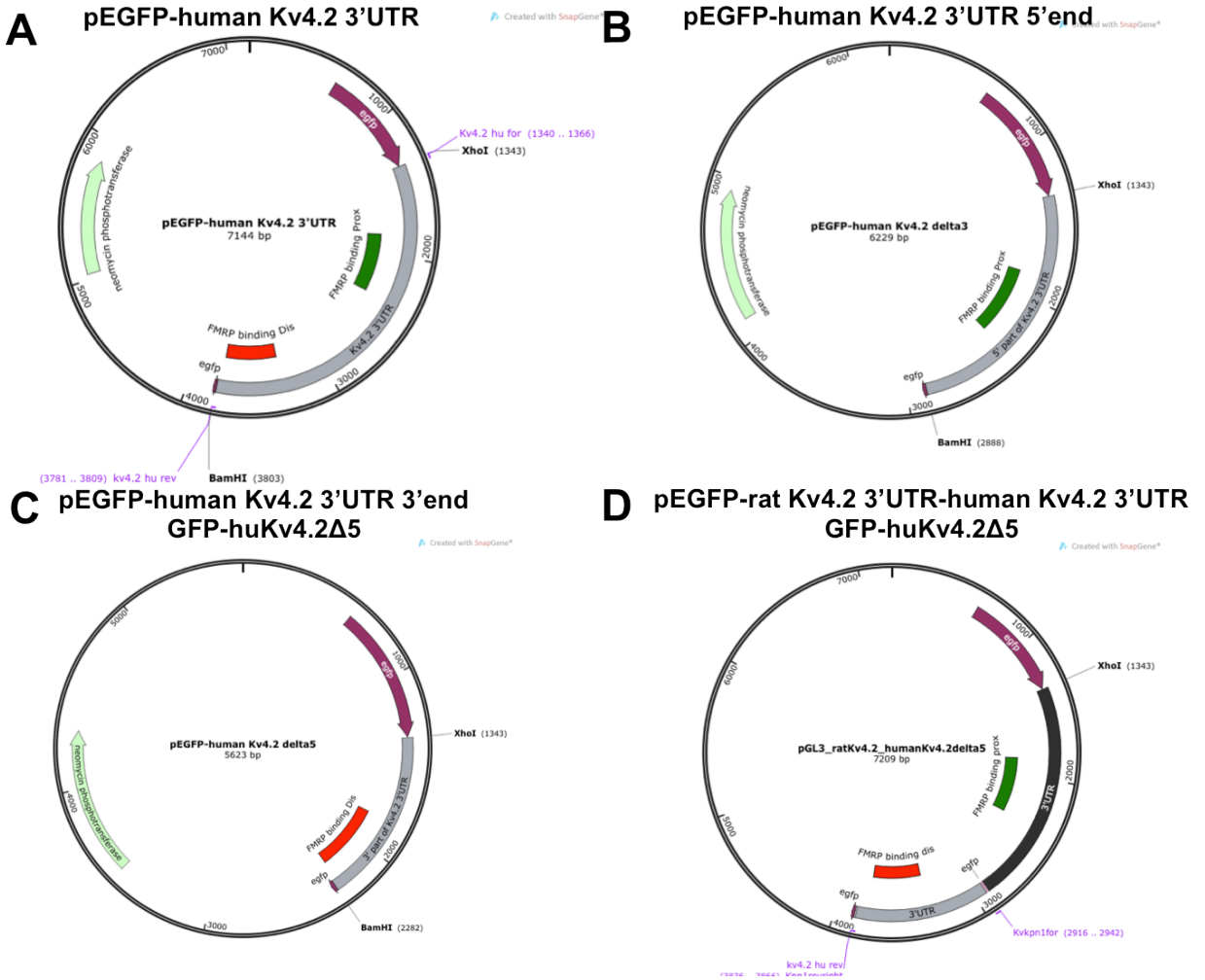


Figure 4. Plasmid maps built using *SnapGene* **(A)** Diagram of the plasmid containing the human Kv4.2 3'UTR cloned immediately following the EGFP (enhanced green fluorescent protein) coding sequence. Human Kv4.2 3'UTR was amplified from a cDNA clone (*Origene*) and cloned into *pEGFP-C1* (plasmid EGFP-C1) using Xho1 and BamH1. The construct contains the proximal (green) and distal (red) FMRP binding sites. **(B)** Diagram of the plasmid containing the 5' part of human Kv4.2 3'UTR (*Δ3*) cloned immediately following the EGFP coding sequence. Kv4.2 3'UTRΔ3 was PCR-amplified from the pEGFP-humanKv4.2 3'UTR plasmid and cloned into the pEGFP-C1 plasmid with Xho1 and BamH1. The construct contains only the proximal (green) FMRP binding site. **(C)** Diagram of the plasmid containing the 3' part of human Kv4.2 3'UTR (*Δ5*) cloned immediately following the EGFP coding sequence. Kv4.2 3'UTRΔ5 was PCR-amplified from the pEGFP-humanKv4.2 3'UTR plasmid and cloned into the pEGFP-C1 plasmid with Xho1 and BamH1. The construct contains only the distal (red) FMRP binding site. **(D)** Diagram of the plasmid containing the EGFP coding sequence followed by rat Kv4.2 3'UTR and human Kv4.2 3'UTR Δ5. Rat Kv4.2 3'UTR was PCR-amplified and subcloned into pEGFP-C1 using Sma1 and Xho1. PCR amplified human Kv4.2 3'UTR was subcloned via the Kpn1 site. The construct contains the proximal (green) FMRP binding site from the rat sequence, and the distal (red) FMRP binding site, which is only found in human Kv4.2 mRNA.

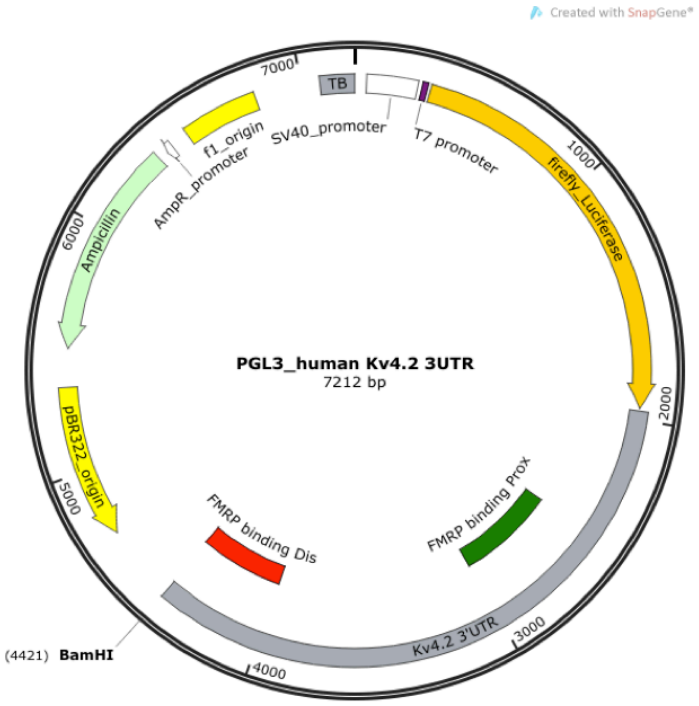


Figure 5. Diagram of the plasmid containing the coding sequence for firefly luciferase followed by human Kv4.2 3'UTR. The construct contains the proximal (green) and distal (red) FMRP binding sites. The Kv4.2 3'UTR was sub-cloned from pEGFP-Kv4.2 3'UTR into pGL3 using Xho1 and BamH1.

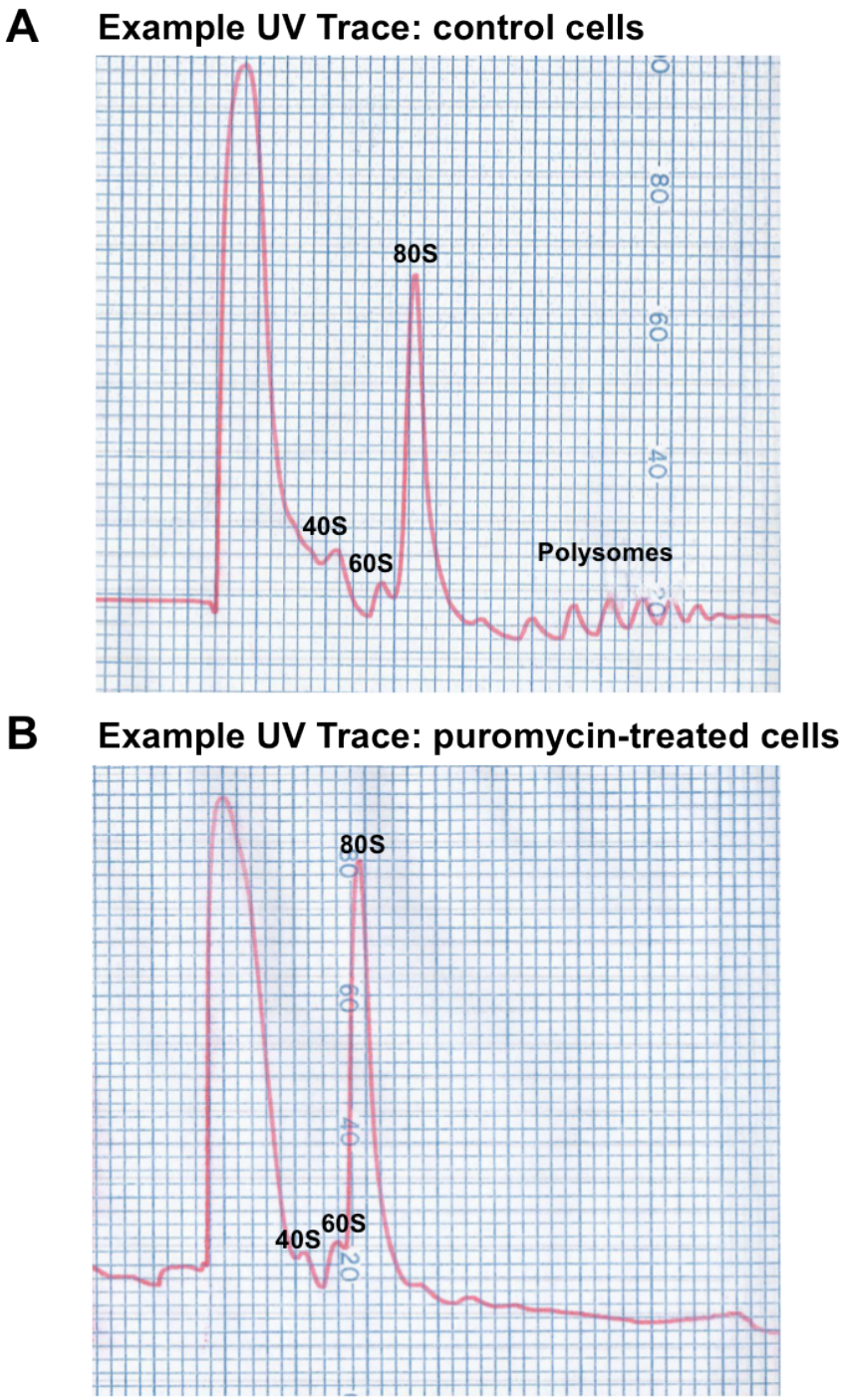


Figure 6. (A) Example absorption trace (245nm) from a polysome gradient of HEK293 cell lysates. 40s, 60s,and 80s ribosomal subunits, and polysomes are indicated. **(B)** Example absorption trace (245nm) from a polysome gradient of HEK293 cells treated with puromycin. 40s, 60s, and 80s ribosomal subunits are indicated. The typical peaks indicating polysomes are not detectable.

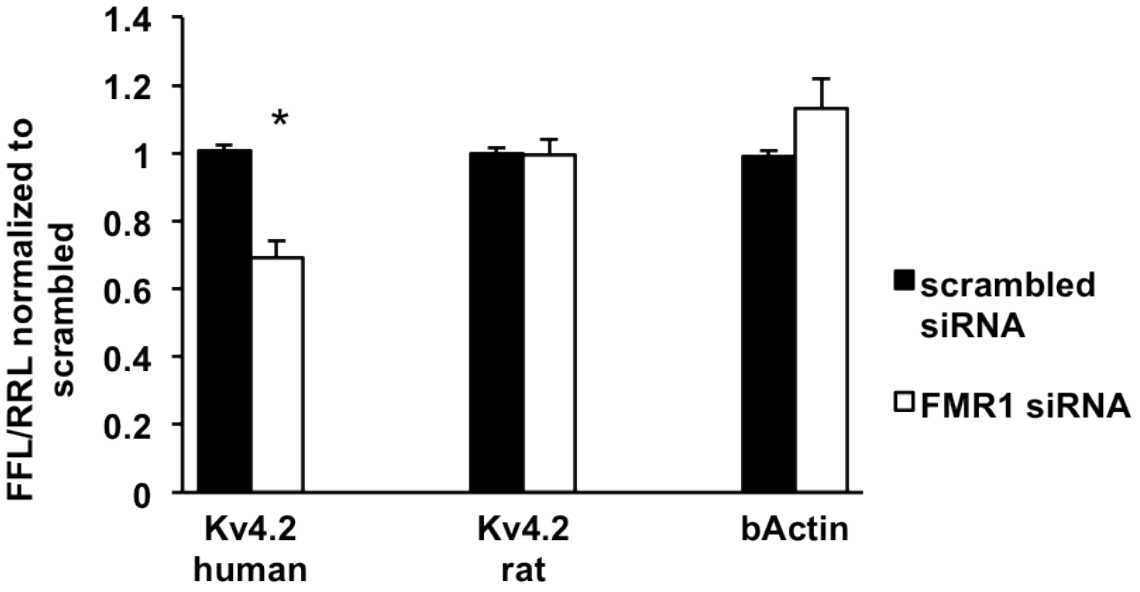


Figure 7. After knockdown of *FMR1* in HEK293 cells there was significantly **decreased** luciferase activity of a reporter containing the human Kv4.2 3'UTR ($p = 0.001$). *FMR1* knockdown did not significantly change expression of the luciferase reporters containing rat Kv4.2 3'UTR and βActin 3'UTR. N = 9, Friedman's Two-Way Analysis with Games-Howell post hoc tests. Luciferase activity was normalized to scrambled control for each reporter.

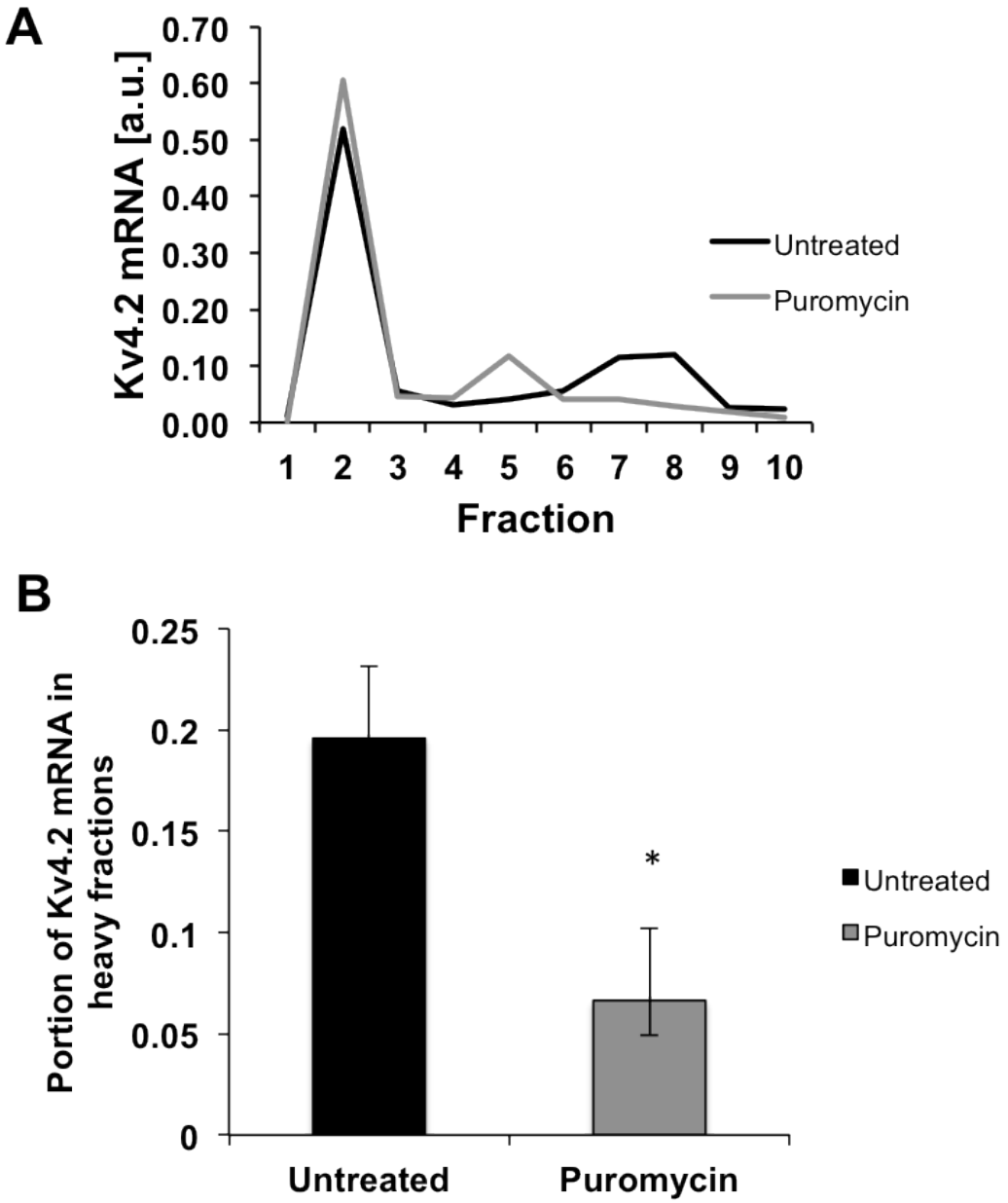


Figure 8. (A) Example qRT-PCR results showing puromycin-treated (gray) cells contain less Kv4.2 mRNA in the last five polysome containing fractions compared to untreated cells (black). **(B)** Quantification of 5 independent experiments showed cells treated with puromycin (gray) have significantly less mRNA in the last five fractions than untreated cells (black). N=5, Student's t-test, $p = 0.008$.

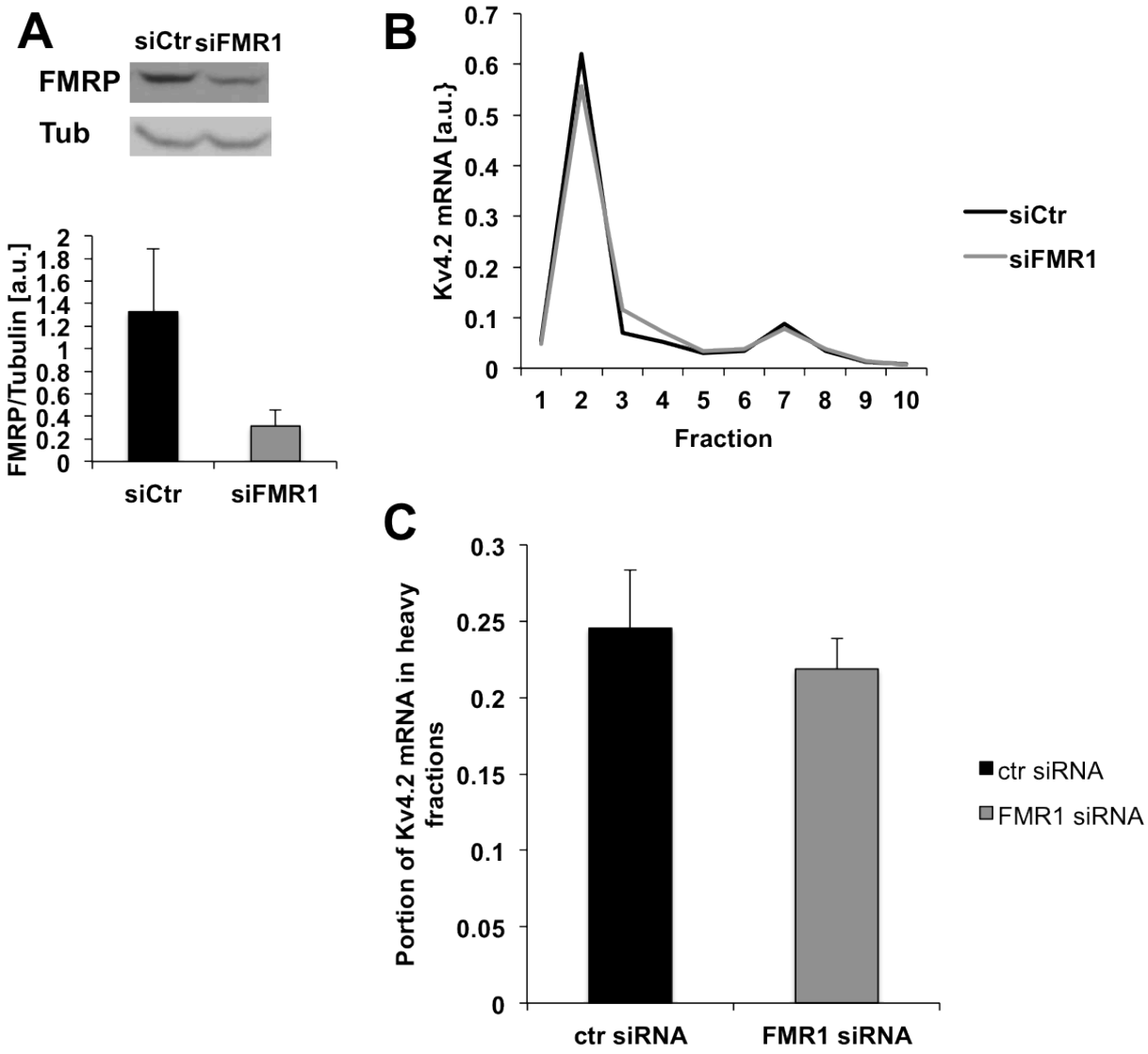


Figure 9. (A) Western blot analysis illustrates the *FMR1* knockdown was effective. While FMRP is clearly reduced in si*FMR1* cells compared to siCtr cells, it is still present in detectable levels, which is different from FXS patient cells, where FMRP is virtually absent. Tubulin (*Tub*) is present in all samples. Quantification of FMRP normalized to tubulin shows a decrease in FMRP after *FMR1* KD. N=5, paired samples t-test, p=0.13. **(B)** Example distribution of Kv4.2 mRNA on a polysome gradient showing the amount of Kv4.2 mRNA [a.u.] in each fraction in both si*FMR1* (gray) and siCtr (black) cells. **(C)** Quantification of 7 independent experiments showed cells transfected with *FMR1* siRNA (gray) show a slight decrease, but no significant change, in mRNA levels in the last five fractions compared to control cells (black). N=7.

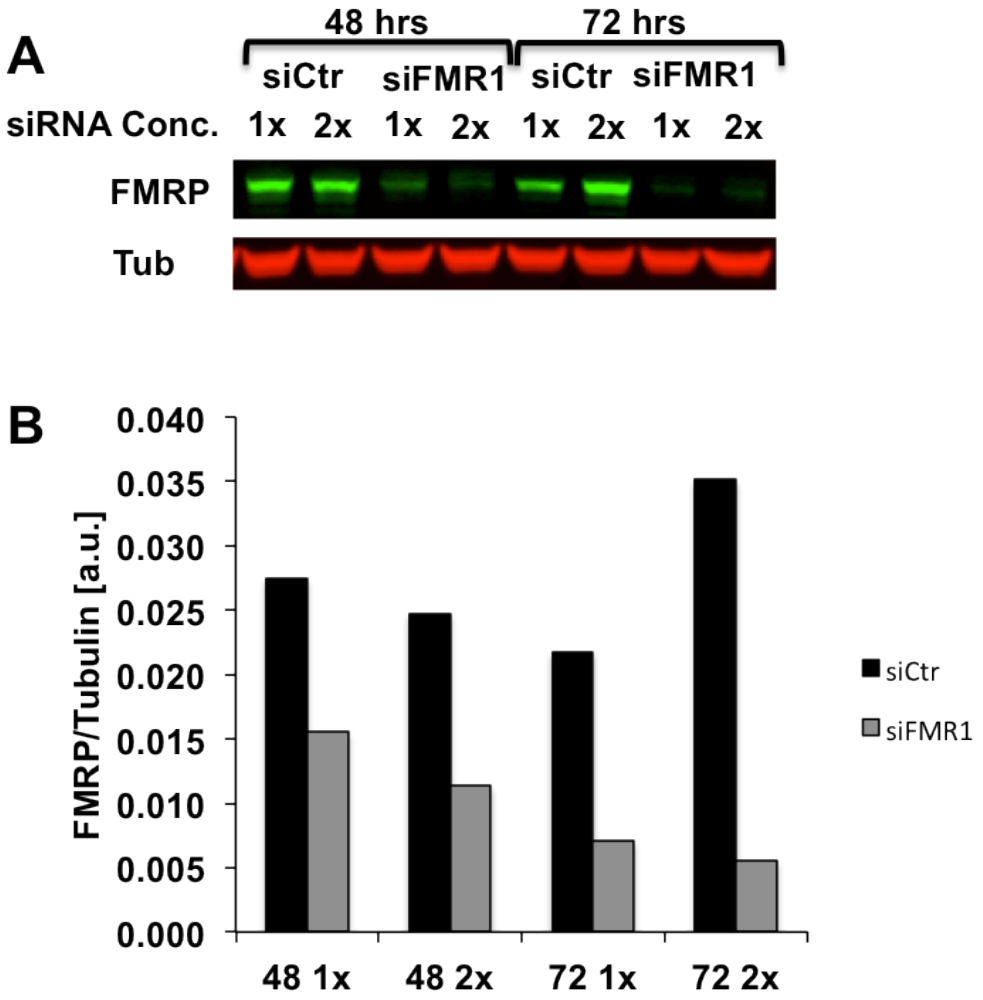


Figure 10. Time course of FMRP expression. **(A)** Western blot analysis of *FMR1* knockdown after transfection with either the standard concentration (1x) or twice that concentration (2x). Bands show presence of FMRP (green) and tubulin (*Tub*) (red). **(B)** Quantification of Western blots shows a stronger decrease in FMRP levels after 72 hours, though not a complete loss of FMRP, compared to 48 hours of knockdown when normalized to tubulin.

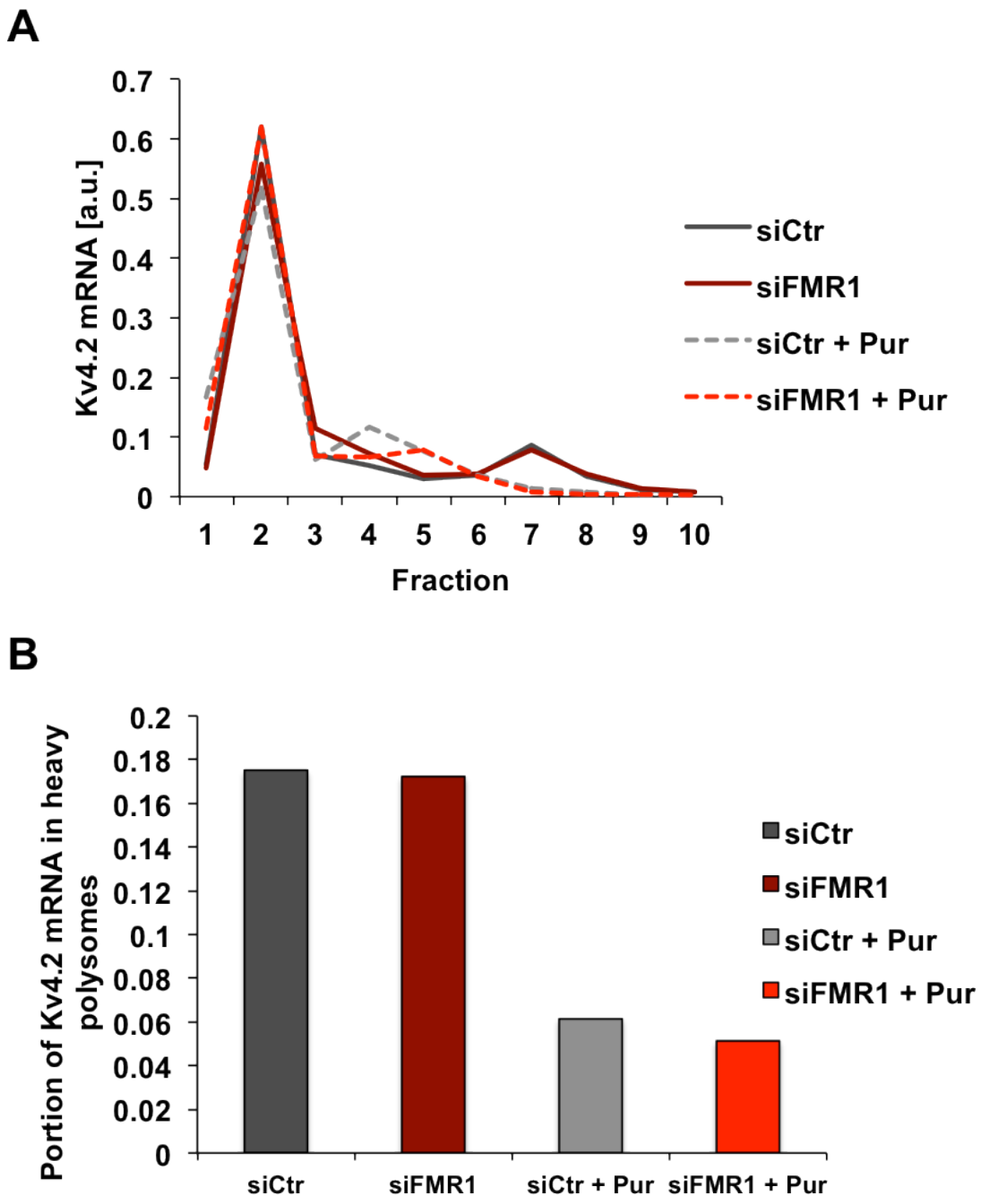


Figure 11. (A) Example distribution of Kv4.2 mRNA on a polysome gradient showing cells treated with puromycin (dotted lines) in addition to siRNA knockdown (grey for siCtr, red for si*FMR1*) contain less Kv4.2 mRNA [a.u.] in the last five polysome containing fractions compared to cells with siRNA knockdown only (solid lines). **(B)** Cells transfected with siRNA and treated with puromycin have a larger portion of puromycin-sensitive (e.g. actively translating) polysomes in heavy fractions compared to siRNA knockdown only (solids). N=1.

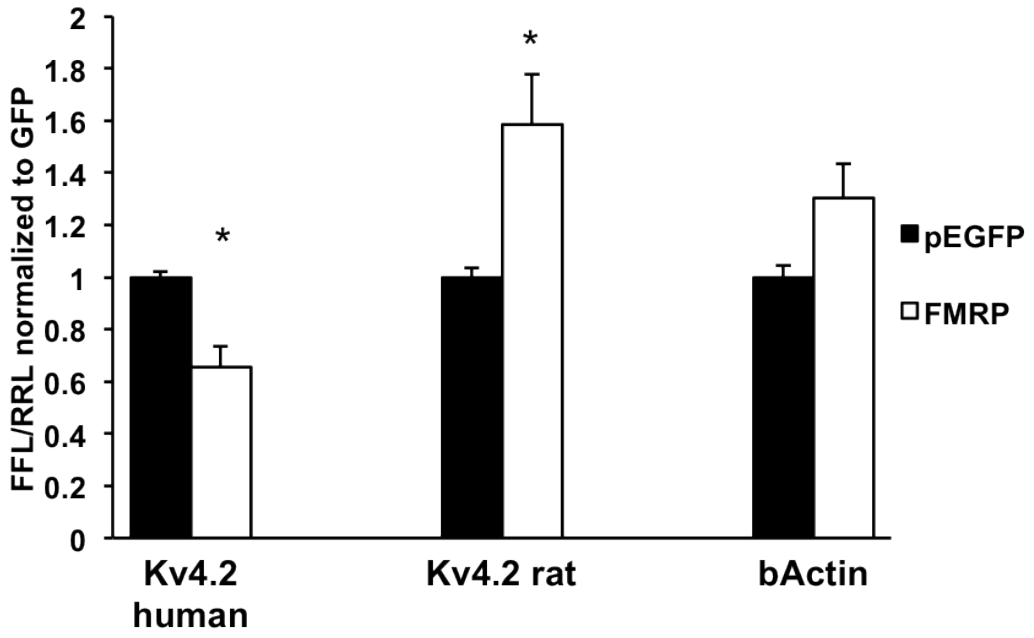


Figure 12. After overexpression of human GFP-FMRP in HEK293 cells there was **decreased** luciferase activity of a reporter containing the human Kv4.2 3'UTR ($p = 0.008$), but **increased** luciferase activity of a reporter containing the rat Kv4.2 3'UTR ($p = 0.001$). No significant change was detected for the βActin reporter. N=7, Friedman's Two-Way analysis with Games-Howell post hoc tests.

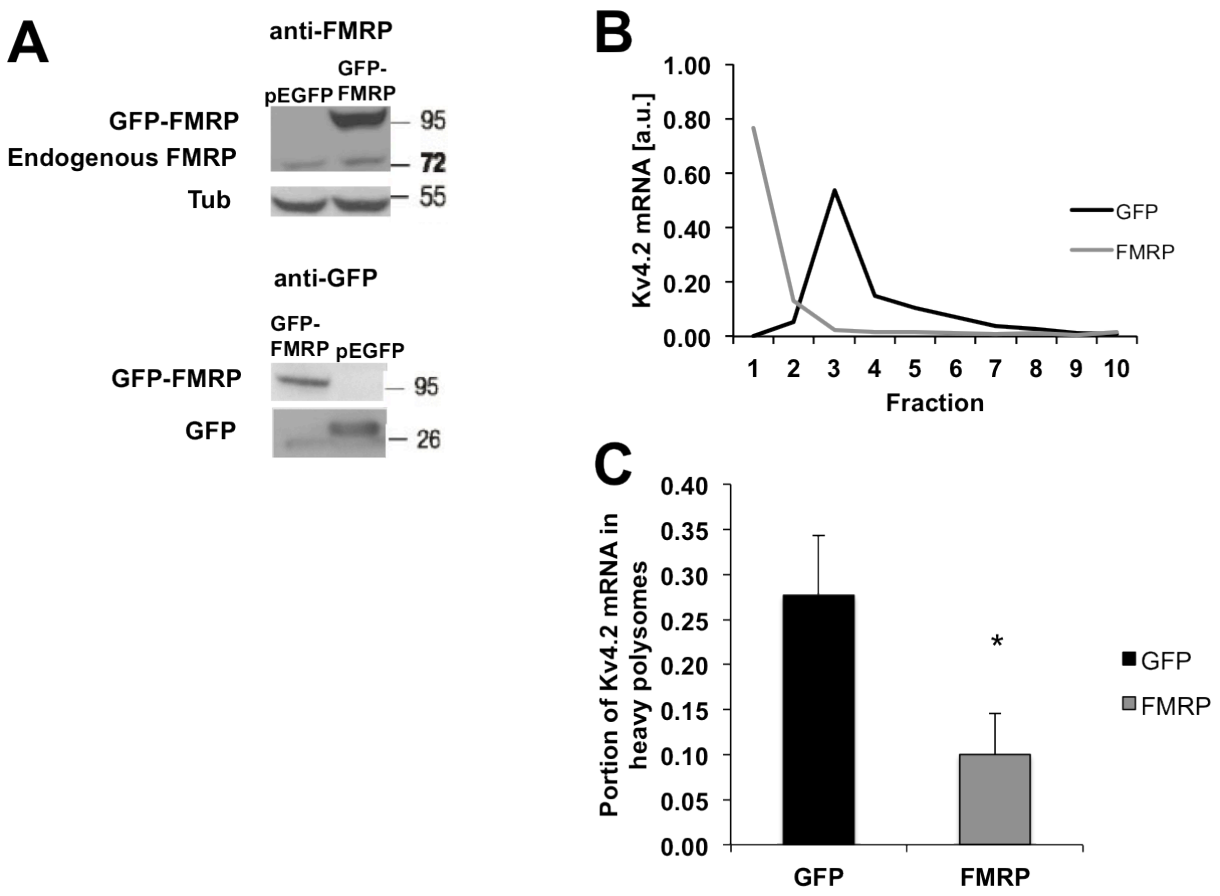


Figure 13. (A) Western blot analysis with an antibody against FMRP shows successful overexpression of the GFP-FMRP recombinant protein (~100kDa=75kDa FMRP, 26kDa GFP). Endogenous FMRP (75kDa) is present in both pEGFP and GFP-FMRP transfected cells. Tubulin (Tub), a loading control, is present in all samples. Western blot analysis with an antibody against GFP shows successful overexpression of the GFP-FMRP recombinant protein (~100kDa) and expression of endogenous GFP (~26kDa) in both GFP-FMRP and pEGFP samples. **(B)** Example distribution of Kv4.2 on a polysome gradient showing that cells overexpressing FMRP (gray) contain less Kv4.2 mRNA [a.u.] in the last five polysome containing fractions compared to GFP expressing cells (black). **(C)** Quantification of four independent experiments showed FMRP overexpression (gray) leads to a significant decrease in Kv4.2 3'UTR mRNA levels in the last five fractions compared to GFP control cells (black). N = 4. Paired samples t-test, p = 0.012.

Figure 14. Schematic of cloning constructs to test our hypothesis that FMRP regulates Kv4.2 translation differentially via two separate binding sites. The approximate locations of FMRP binding sites are shown on the full-length rat and human Kv4.2 sequences.

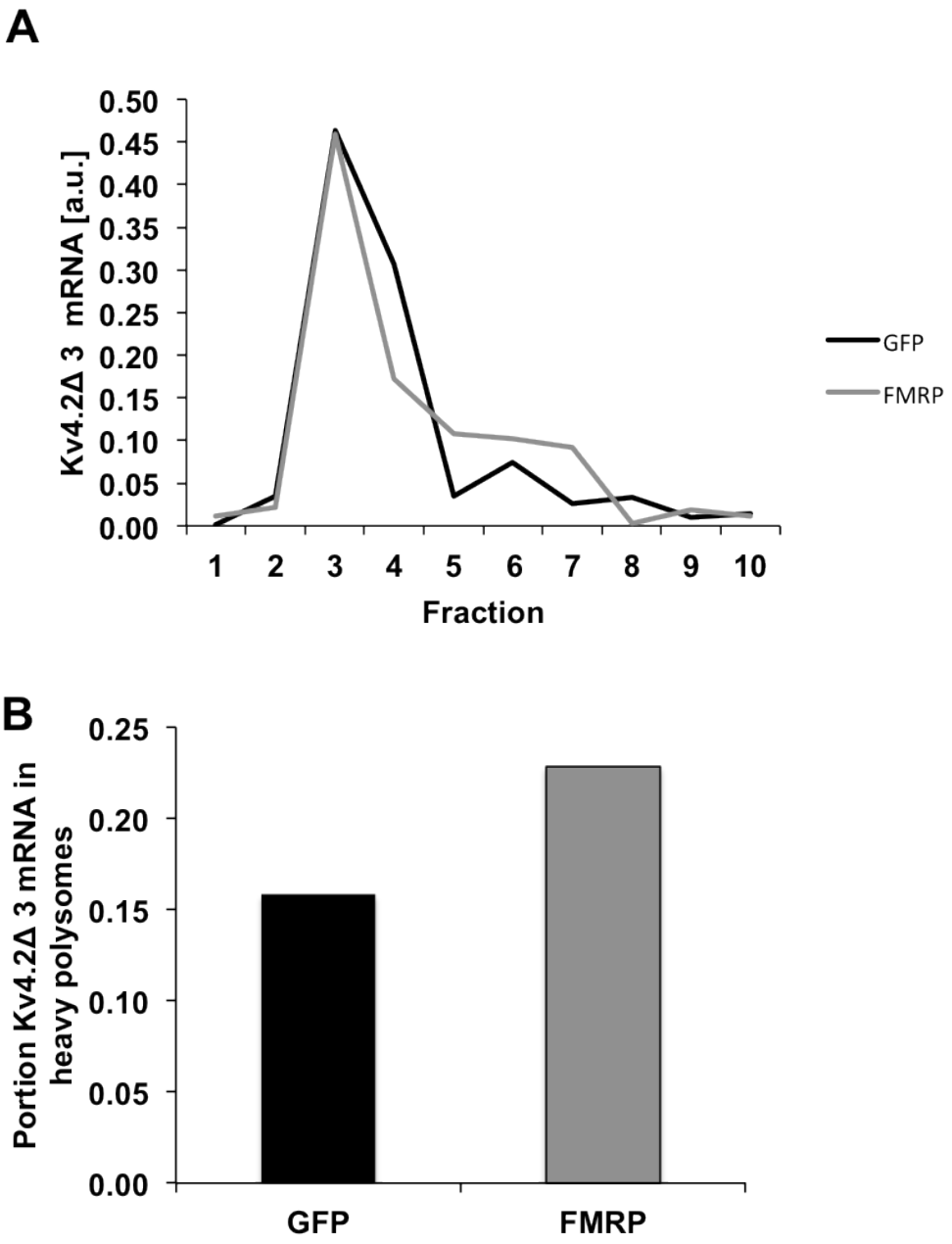
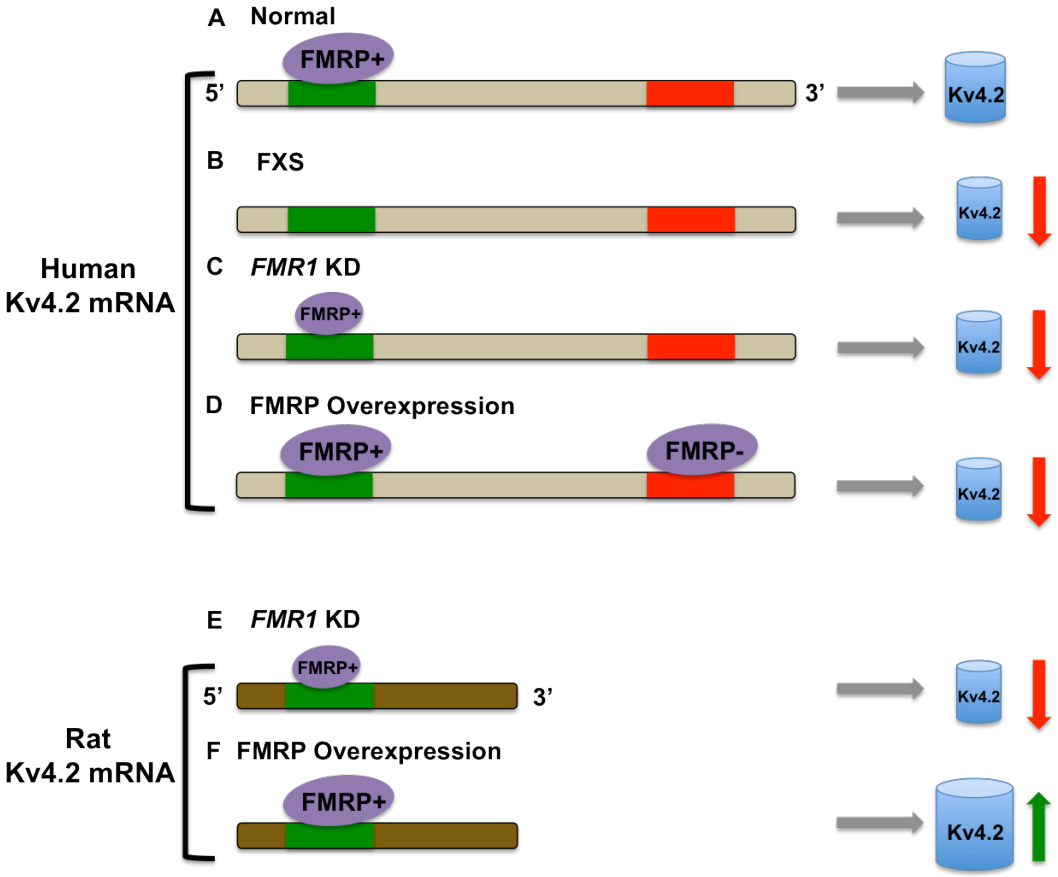


Figure 15. (A) Example distribution of Kv4.2Δ3 mRNA on a polysome gradient showing that cells overexpressing FMRP (gray) contain more Kv4.2Δ3 mRNA [a.u.] in the last five polysome containing fractions compared to GFP expressing cells (black). **(B)** After overexpression of FMRP, there is increased translation of Kv4.2 Δ3 mRNA (N=1). This corresponds to observations with the rat sequence, and is expected in the presence of only the proximal (activating) FMRP binding site.



G **Effects of FMRP on Kv4.2**

Condition	Luciferase Assay	Polysome Gradient
FMR1 KD + rat Kv4.2 3'UTR	Decrease	Decrease (Gross et al., 2011)
FMR1 KD + human Kv4.2 3'UTR	Decrease	No change
FMRP Overexpression + rat Kv4.2 3'UTR	Increase	N/A
FMRP Overexpression + human Kv4.2 3'UTR	Decrease	Decrease
FMRP Overexpression + human Kv4.2Δ3	N/A	Increase

Figure 16. Proposed model for the regulation of Kv4.2 mRNA by FMRP. **(A)** In normal (healthy) conditions, FMRP preferentially binds the proximal (activating) site leading to normal levels of Kv4.2. **(B)** In FXS (*Fmr1* KO) FMRP is absent. This leads to a decrease of Kv4.2 levels due to lack of FMRP-mediated activation. **(C)** When FMRP is reduced (experimental KD conditions) FMRP binds solely to the activating site in a limited fashion, thus leading to the observed decrease in Kv4.2. **(D)** When FMRP is overexpressed, both binding sites are occupied by FMRP, and inhibitory action at the distal site overrides the activating function at the proximal site, causing a decrease in Kv4.2. **(E)** Rat Kv4.2 is reduced in FMR1 KD conditions due to lack of FMRP-mediated translational activation. **(F)** Rat Kv4.2 is increased when FMRP is overexpressed. As there is no inhibitory (distal) site, FMRP's binding at the activating (proximal) site leads to an overall increase in Kv4.2 (Gross et al., 2011). **(G)** Summary table of the effect of FMRP on Kv4.2 expression and translation in the luciferase assays and polysome gradients.

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