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14. ABSTRACT

Project rationale and original aims: New evidence suggests that Fragile X Mental Retardation Protein (FMRP) may need to associate with ancillary factors to co-regulate the production of some of important synaptic proteins. Currently, there is a critical need to characterize these molecules. Some factors believed to associate with FMRP and co-regulate target mRNAs include a group of small regulatory molecules called microRNAs (miRNAs). There were **two goals** associated with this project. First, to use an established model system for FXS to identify and functionally characterize all FMRP-associated miRNAs and mRNAs involved in the control of synapse structure and function. Second, to investigate the molecular mechanisms involved in coordinated FMRP- and miRNA-mediated translational regulation. Progress towards both of these goals has shed light on the molecular pathogenesis of FXS. **Progress towards completion of aims:** First, after significant setbacks developing novel transgenic epitope-tagged FMRP protein that works efficiently *in vivo*, we have adapted and optimized *in vitro* approaches to efficiently enrich for and purify FMRP-associated miRNAs and mRNAs. These RNAs still need to be sequenced. Second, a serendipitous discovery as allowed us to make substantial progress towards understanding the molecular mechanisms involved in FMRP-mediated recruitment of miRNAs to target mRNAs. This will allow us to very rapidly and efficiently validate bona fide target mRNAs. Importantly, both findings provided strong preliminary data for a recent grant submission to NIH.

15. SUBJECT TERMS
Fragile X Syndrome (FXS), Fragile X Mental Retardation Protein (FMRP), FMRP- and microRNA-mediated mRNA repression

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INTRODUCTION:

Fragile X Syndrome (FXS) is the most common form of inherited mental retardation in humans and affects an estimated 1 in 4000 males and 1 in 8000 females in the general population. FXS is characterized by an array of intellectual and emotional problems including learning disabilities, developmental delay, and anxiety. In the brain, the neuroanatomical defects associated with FXS include an abnormal synaptic morphology. This alteration in synaptic architecture is thought to be central to the pathology of FXS. At the genetic level, FXS is caused by a mutation in the Fragile X Mental Retardation gene 1 (FMR1). In normal cells, FMR1 produces a protein called the Fragile X Mental Retardation Protein (FMRP). The normal function of FMRP is to bind to messenger ribonucleic acids (mRNAs) and turn off the production of important proteins required in the control of synapse structure and function. Current hypotheses regarding the pathology of FXS have focused on this conserved function for FMRP. The **idea** is that in FXS, key mRNA targets of FMRP are dysregulated leading to the development of synaptic defects. Unfortunately, little is known about the specific mRNAs that are controlled by FMRP or exactly how those mRNAs are regulated. Until all of these processes have been fully characterized, further progress towards developing a treatment for FXS will be hindered.

New evidence suggests that FMRP may need to associate with ancillary factors to co-regulate the production of some of important synaptic proteins. Currently, there is a **critical need** to identify and characterize all of these molecules. Some factors that are believed to associate with FMRP and co-regulate target mRNAs include a group of small regulatory molecules called microRNAs (miRNAs). With this in mind, there are two **long-term goals** associated with this project. First, to use an established model system for FXS to identify and functionally characterize all FMRP-associated miRNAs involved in the control of synapse structure and function. Second, to identify and functionally characterize all mRNA targets for these miRNAs. These long-term goals are obviously well beyond the scope of this proposal and will be examined in future experiments. As such, one primary outcome of this project is **expected** to be the accumulation of preliminary data sufficient to obtain funding for this project at the NIH Research Project Grant (R01) or equivalent level.

This work is believed to be **significant** because it represents the first logical step in a series of experiments expected to lead to the discovery of new potential therapeutic targets for the diagnosis, prevention, and/or treatment of FXS. The basic **idea** behind the work proposed here is simple. First, all miRNAs that associate with the FMRP protein in neurons will be identified using **innovative** techniques. Second, candidate mRNA targets for each of these miRNAs will be identified by bioinformatics. The emphasis will be on mRNAs with a characterized role in the control of synapse structure or function. Finally, interactions between miRNAs and potential mRNA targets will be confirmed using established approaches. This work is **expected** to rapidly identify novel candidates that associate with FMRP. This alone would represent a significant advance in the FXS field. However, it will be necessary to demonstrate a mechanistic link between FMRP, FMRP-associated miRNAs, and target mRNAs in the pathology of FXS in future experiments.

KEYWORDS:

Fragile X Syndrome (FXS)

Fragile X Mental Retardation Protein (FMRP)

microRNAs (miRNAs)

messenger RNAs (mRNAs)

translational regulation

synaptic plasticity

RNA sequencing (RNA-seq)

RNA immunoprecipitation (RIP)

OVERALL PROJECT SUMMARY:

The following description of research accomplishments will be organized as outlined in the approved statement of work (as **revised** in our **approved** request for a no-cost extension). Due to **unforeseeable complications**, we were forced to deviate from the original statement of work in several important ways (each will be indicated below). These deviations were primarily in methodology and in no way changed the overall Aims of the project. We had proposed a very tight 6-month timeline in our approved **revised** statement of work and had proposed significant back-end costs associated with RNA-sequencing. As indicated in our **revised** statement of work, items highlighted below in **RED** indicated a change in timeline; an asterisk (*) indicates a new subaim. These are presented as written in our **revised** statement of work.

Aim 1. Purification and deep sequencing of FMRP associated sRNAs (months 1-18).

*Aim 1a_1a. Optimization of FMRP immunoprecipitation – **unexpected complications** (months 1-16).

At the time the grant was submitted, we had developed but not tested transgenic fly lines that inducibly expressed a Protein A (PrA) tagged full length FMRP protein. The rationale for using PrA-tagged FMRP was primarily as a cost-saving measure [1]. These constructs would have allowed us to significantly scale-up our pull-down experiments with very little expense. We tested these constructs extensively *in vivo*. Unfortunately, these tagged constructs did not performed per our expectations. First, when expression of transgenic PrA-tagged FMRP was driven in the adult *Drosophila* brain, we did not observe an expected shift in size when Western blots were probed with an anti-FMRP antibody (**Figure 1A**). Second, when blots were probed with an IgG antibody (which should bind to the PrA tag), we could not see the PrA tag (**Figure 1C**). Finally, when PrA-tagged FMRP was immunoprecipitated from brain extract using IgG-conjugated magnetic beads (Dynabeads; see below), the FMRP protein was pulled down in both control and PrA-tagged FMRP expressing extracts (**Figure 1B**). While there was clearly more FMRP in the PrA-tagged FMRP expressing extracts, the additional protein runs appears to run at exactly the same size as endogenous FMRP. We believe that the PrA tag is likely being cleaved from FMRP during processing of the transgenic protein *in vivo*. **Based on these data, we decided to shift to an alternative strategy for the purification of FMRP.** It is important to note that each of these strategies must be done on a much smaller scale and require larger volumes of expensive antibody (anti-FMRP, -HA).

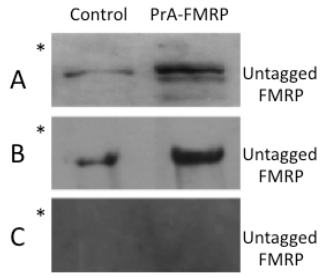

Figure 1. PrA-tagged FMRP. (A) Bands are the size of untagged FMRP. * indicates the expected size of PrA-FMRP (B) Endogenous FMRP is IP'd flies both the control and PrA-tagged FMRP flies. (C) The PrA tag is not recognized by specific antibodies.

Next, we developed a HA-FLAG-tagged FMRP protein. We initially **validated** the construct in *Drosophila* S2 cells to confirm that the tagged version of the protein was expressed (**Figure 2A**). Next, we confirmed that the HA-FLAG-tagged FMRP could be immunoprecipitated from S2 cell extract (**Figure 2B**). Unfortunately, we were not able to confirm that the HA-FLAG-tagged FMRP protein can consistently co-immunoprecipiate proteins that are expected to interact directly with FMRP (**Figure 2C**) [2,3]. That said, we did develop a stably transfected cell line that could be useful for large-scale purification. We had also constructed a transgenic *Drosophila* line that constitutively expresses the HA-FLAG-tagged FMRP protein. This line is still available but has not been tested any further - primarily because of advantages of the approaches described below (**Figures 6 and 7**).

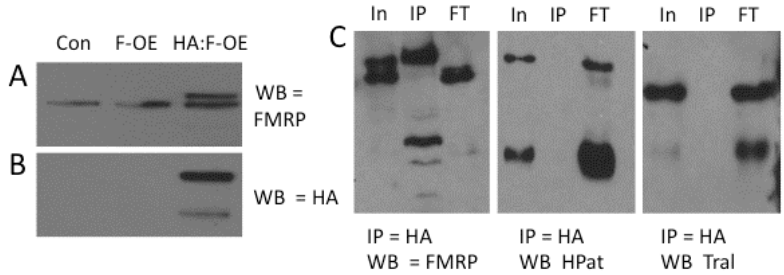

Figure 2. HA-FLAG-tagged FMRP. (A-B) Con = control; F-OE = untagged FMRP overexpression; HA:F-OE = HA:FLAG-tagged FMRP overexpression. Western blots against (A) FMRP and (B) the HA tag. (C) In = input; IP = immunoprecipitation; FT = flow-through. IP'd with antibodies against HA. Western blots against FMRP and the P body components, HPat and Tral. These should co-IP with FMRP [2, 3].

We next tried FMRP antibody covalently crosslinked to magnetic Dynabeads (Invitrogen). Dynabeads are very small and should provide a significant improvement in immunoprecipitation efficiency [1]. In our hands, crosslinked FMRP antibody did not always efficiently pull down FMRP from fly brain extract (as shown in **Figure 3A**) and inconsistently pulled down proteins we have previously shown to co-immunoprecipitate with FMRP such as Me31B and Tral (**Figure 3B-C**) [2]. This could be due to FMRP consistently crosslinking to Dynabeads in such a way that the epitopes that target FMRP are masked (Invitrogen). Based on the complications described above, we **deviated** from this approach in the following ways. First, we used a proven (albeit much more costly) approach to immunoprecipitate FMRP protein using FMRP antibodies that are non-covalently bound to commercially available Protein A/G-conjugated magnetic Dynabeads (Invitrogen). This approach much more consistently pulls down FMRP as well as proteins that should co-immunoprecipate

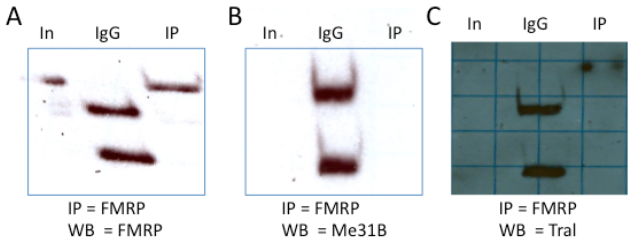

Figure 3. FMRP antibody covalently bound to Dynabeads. (A-C) In = input; IgG = IP with a normal mouse IgG; IP = IP with a FMRP antibody. Western blots against (A) FMRP, (B) Me31B, and (C) Tral. Again, these proteins have been shown to interact with FMRP [2].

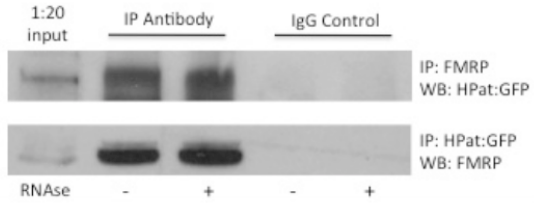

Figure 4. FMRP antibody pulled down with PrA/G Dynabeads. IPs from adult fly brain extract with antibodies against FMRP (top panel) or GFP (to pull down a HPat:GFP fusion protein; bottom panel). Western blots probed with either FMRP or GFP. These particular samples were also treated with RNAse as indicated.

such as HPat (**Figure 4**). Second, to help prevent non-specific interactions can be problematic with hydrophobic magnetic Dynabeads, we also using FMRP antibodies bound (non-covalently) to Protein A Sepharose [2,4]. This is a modification of a technique that has been used to pull down FMRP-associated mRNAs [5].

We next attempted to use the FMRP antibody-Dynabead approach described above to co-immunopurify *Drosophila* miRNAs that we predicted (generally based on homology with mammalian miRNAs) would associate in RNP complexes with FMRP [4]. Surprisingly, we found that there was no miRNA enrichment in FMRP co-immuno-precipitates compared to controls (**Figure 5**). In fact, miRNAs tended to associate non-specifically with magnetic Dynabeads. We believe this is likely due to the hydrophobic nature of the particle. This hydrophobicity unfortunately seems to make it very difficult to block non-specific binding of small RNAs.

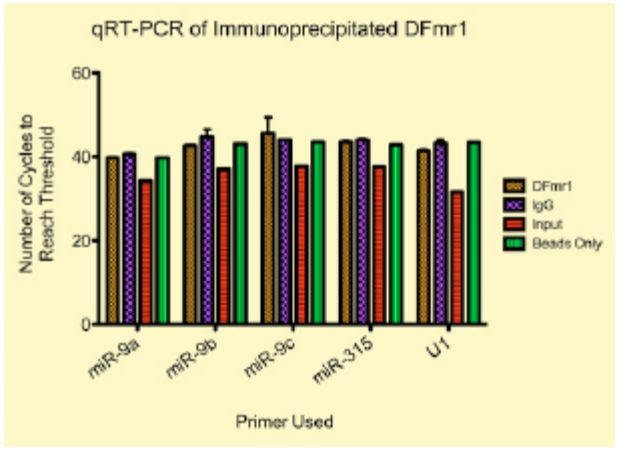

Figure 5. FMRP linked Dynabeads do not specifically pull down predicted FMRP associated miRNAs. IPs from adult fly brain extract with antibodies against FMRP, normal mouse IgG, and Dyabeads only. Also shown is a 10% volume of input to confirm the presence of miRNAs. RNA was purified from IPs and subjected to quantitative real-time PCR (RT-qPCR).

We then took a step back and determined if we could pull down mRNAs that are known to associate with FMRP using FMRP antibodies bound to Protein A Sepharose [6]. After some minor optimization, we were successfully able to pull down four known FMRP binding partners of FMRP from *Drosophila* S2 cells (two are shown in **Figure 6**; specificity of FMRP pulldown is shown in **Figure 4**). To enrich for FMRP associated miRNAs, we transfected cells with GFP-tagged FMRP and used Nano-Trap technology (GFP Traps; Chromotek) to pull down miRNAs [7]. GFP Traps are magnetic beads coated with Separose (to avoid non-specific binding problems associated with hydrophobic particles) covalently linked to a GFP-binding protein. We have successfully used this approach to enrich for known FMRP-associated miRNAs (**Figure 7**). Importantly, these pull down approaches have finally been optimized and will allow us to pull down FMRP-associated RNAs for sequencing.

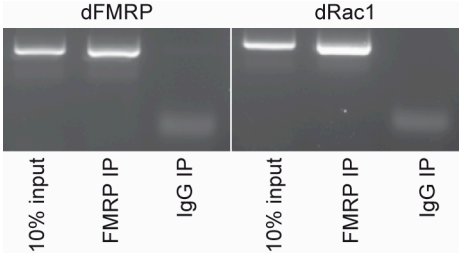

Figure 6. Known FMRP-associated mRNAs co-immunoprecipitate with FMRP in S2 cell extract. METHODS: FMRP has been shown to physically interact with several *Drosophila* mRNAs. Briefly, FMRP-containing RNPs were pulled down from S2 cell extract using a RIP protocol we optimized for fly FMRP antibodies. RNA was isolated directly from immunoprecipitates and was subjected to RT-PCR using probes targeting conserved (between isoforms) exonic sequence. The mRNAs tested encoded for fly FMRP (*dfmr1*), *dRac* (both shown here), Chickadee (*chic*), and Pickpocket (*ppk*). We observed similar results with. *ppk* is not expressed in S2 cells.

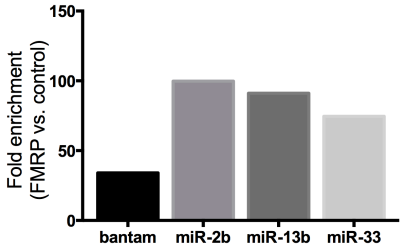

Figure 7. Known FMRP-associated miRNAs co-immunoprecipitate with FMRP in S2 cell extract. METHODS: FMRP has been shown to physically interact with several *Drosophila* miRNAs including bantam and miR-2b (both highly expressed in S2 cells; **Table 1**). Briefly, FMRP-containing RNPs were pulled down from S2 cell extract using a RIP protocol we optimized for the GFP trap system RNA was isolated directly from immunoprecipitates and was then analyzed by quantitative real-time PCR (qRT-PCR) using probes targeting mature miRNAs. miRNA levels in immunoprecipitates were normalized to levels in the input and then to results of a paired untransfected control. While these data represent the results of a single experiment (thus no error bars), the enrichment trend is repeatable (data not shown).

Aim 1a_1b. Purification of FMRP-associated sRNAs (months 16-18).

Per the **revised** statement of work, we have purified FMRP-associated RNAs for sequencing using approaches described above**.** The efficiency of immunoprecipitation using these approaches is shown in **Figures 6 and 7**.

Aim 1a_2. Deep sequencing of sRNA libraries and data analysis (months 18-19).

Despite finding an alternative vendor that will provide high quality RNA-seq data, we ran out of funds to perform this analysis. However, funds to complete this sequencing (using preliminary data described in this report) have been requested from both intramural (University of Denver) and extramural (NIH) sources.

As **proof of concept** (and to establish a base-line for all miRNAs found in the *Drosophila* brain), we have done RNA-seq experiments to identify miRNAs found in explanted adult fly brains (**Table 1**). In summary, we identified a total of 169 miRNAs in the adult brain. 80 of these miRNAs met the cutoff outlined in our original grant proposal of 100 reads-per-million (RPM). This relative abundance data will be essential when assessing whether a particular FMRP-associated miRNA might have a biological function in the brain. For example, a miRNA that has a very low copy number may not be biologically relevant. We have also done comparative analysis to identify miRNAs that are enriched in the adult fly brain compared to the larval CNS (**Figure 8**). Again, we have shown that miRNAs predicted to associate with FMRP can be co-IP'd using the approaches described in **Figure 7** above.

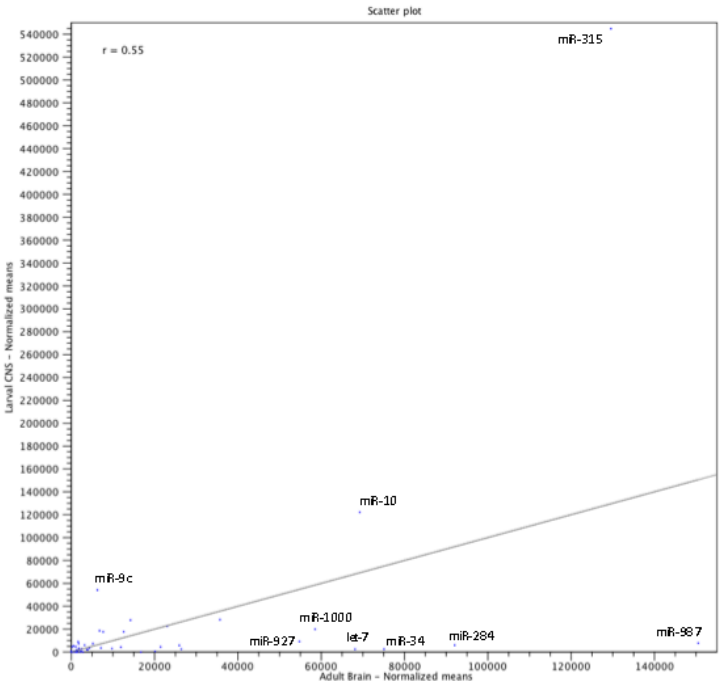

Figure 8. Relative expression of miRNAs in the adult brain compared to the larval CNS. Adult and larval brains share the expression of 147 miRNAs. Of those, three are enriched in the larval CNS (miRs-9c, -10, and -315). Six are enriched in the adult brain (miRs-927, -1000, -34, -284, -987, and let-7).

Milestone. Quality sRNA libraries have been generated and novel FMRP-associated miRNAs identified.

This milestone was not reached for the reasons described above. Primarily, this was due to: 1) significant difficulty optimizing FMRP co-IP protocols in our system; and 2) not having sufficient grant funds remaining to complete the sequencing portion of the project. However, we are currently pursuing funding opportunities to complete RNA sequencing.

Aim 2. Identification and validation of target mRNAs (months 6-24).

*Aim 2a_1. Characterization of FMRP-miRISC interactions (months 6-18).

We made a **serendipitous discovery** that will allow us to rapidly identify FMRP-associated mRNAs that are translationally repressed via the miRNA pathway. These studies will be discussed in more detail below. The bioinformatic analysis initially proposed in Aim 2 was **biased** to target mRNAs with **annotated functions** in the control of synapse structure or functions. In contrast, using results of these novel findings, our new focused approach will allow for the **unbiased identification of FMRP and miRNA-regulated mRNAs** and could potentially lead to **new gene discovery**.

We developed a novel *in vitro* reporter assay that allowed us to screen for genetic modifiers of FMRP-mediated translation repression. These experiments were based on a previously described tethering assay [8]. Briefly, *Drosophila* FMRP was fused to the λN protein that binds with high affinity to five BoxB stem loop sites (5xBoxB) cloned into the 3' UTR of a firefly luciferase reporter mRNA (FLuc-5xBoxB; **Figure 9A**). *Drosophila* S2 cells transiently transfected with the FLuc-5xBoxB reporter, a plasmid expressing λN-FMRP (or the λN peptide control), and a plasmid expressing *Renilla* luciferase (RLuc) as a transfection control. We observed a > 3-fold decrease in FLuc activity when λN-FMRP was expressed relative to the λN peptide control (**Figure 9C-D**). Surprisingly, this repression was almost completely eliminated when GW182, a core component of the miRNA pathway, is depleted from transfected cells by RNAi (**Figure 9D)**. Bearing this in mind, **GW182 RNAi** will be used to determine if a target of FMRP-mediated repression is co-repressed by miRNAs.

Name	Reads	RPM
mir-184	212927	127871.87
mir-276b	202977	121896.47
mir-276a	201745	121156.6
mir-957	184563	110838.06
mir-14	133097	79930.505
bantam	86482	51936.181
mir-317	82009	49249.952
mir-999	65214	39163.828
mir-8	54554	32762.037
mir-277	40735	24463.129
mir-34	36987	22212.293
mir-987	31715	19046.229
mir-210	29284	17586.309
mir-315	27416	16464.494
mir-11	21702	13032.99
mir-133	21650	13001.761
mir-284	20288	12183.821
mir-10	17448	10478.279
let-7	15851	9519.2111
mir-981	15594	9364.8715
mir-927	12991	7801.6574
mir-1000	12326	7402.2961
mir-995	10293	6181.3917
mir-305	9982	5994.6227
mir-970	9870	5927.3619
mir-2b-2	7653	4595.9575
mir-278	7467	4484.2565
mir-2b-1	7331	4402.5826
mir-932	6060	3639.2921
mir-252	5512	3310.1944
mir-125	5078	3049.5586
mir-31a	4920	2954.6728
mir-990	4490	2696.4392
mir-1010	4010	2408.1784
mir-274	3531	2120.5182
mir-993	3256	1955.3688
mir-2a-2	3063	1839.464
mir-2a-1	2884	1731.9667
mir-263a	2880	1729.5646
mir-124	2670	1603.4505

Name	Reads	RPM
mir-33	2503	1503.1598
mir-279	2264	1359.6299
mir-13b-1	2097	1259.3392
mir-281-2	2092	1256.3365
mir-13b-2	2028	1217.9017
mir-190	1857	1115.2088
mir-306	1714	1029.3311
mir-9a	1664	999.30397
mir-7	1536	922.43443
mir-2c	1531	919.43172
mir-9c	1329	798.12198
mir-956	1181	709.24158
mir-1012	969	581.92641
mir-283	925	555.50251
mir-996	858	515.26611
mir-285	740	444.40201
mir-307a	736	441.99983
mir-1004	687	412.57321
mir-12	636	381.94551
mir-998	587	352.51889
mir-13a	583	350.11672
mir-929	568	341.10857
mir-137	507	304.47543
mir-1017	479	287.66022
mir-219	466	279.85316
mir-275	401	240.81784
mir-1003	350	210.19014
mir-100	340	204.18471
mir-2535b	319	191.5733
mir-92b	305	183.16569
mir-193	281	168.75265
mir-31b	272	163.34776
mir-9b	264	158.54342
mir-1001	263	157.94288
mir-375	253	151.93744
mir-281-1	238	142.92929
mir-1	235	141.12766
mir-971	223	133.92115
mir-1006	216	129.71734
mir-1009	179	107.49724

Table 1. RNA-seq analysis of miRNAs found in the adult *Drosophila* brain. The brain was explanted from young (~3 day old) adult wild-type flies *(*genotype is w^{1118} *Iso31*). Small RNA-seq was done at the University of Colorado Microarray Core facility (Aurora, Colorado). miRNAs are ranked in order of abundance in the adult fly brain. Reads (or tags) indicate the number of sequence reads that mapped to any variant of the indicated miRNA. Reads were then normalized to reads-per-million (RPM). As outlined in the original grant proposal, miRNAs with an abundance of less than 100 RPM are excluded from this table. The table shows the 80 most abundant miRNAs (out of 169 mapped reads in this tissue).

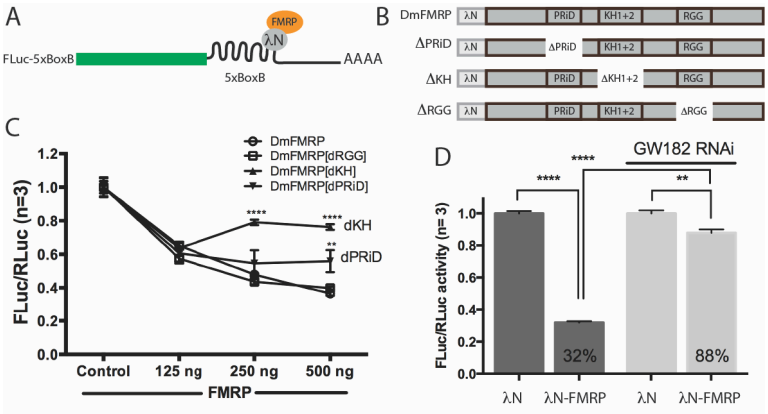

Figure 9. Repression of a luciferase reporter by FMRP requires a core component of GW/P bodies and the miRISC. METHODS: The transfection of S2 cells including RNAi was done exactly as we have described except that experiments were scaled for 12-well dishes (Nesler et al., 2013). FLuc values were normalized to RLuc and then to their respective λN peptide control. (A) is a schematic showing the tethering assay. (B) shows which regions were deleted from the fly FMRP protein: ΔPRiD [amino acids 116-212]; ΔKH [226-335; deletes both KH domains]; and ΔRGG [470-507]. These deletions are described in (Gareau et al., 2013). (C) Increasing amounts of wild type and mutant FMRP were added to the *in vitro* translational reporter assay. (D) **500 ng of λN-FMRP plasmid** was added to this assay. GW182 depletion was confirmed by Western blotting (data not shown). STATS: (C) Two-way ANOVA with a Dunnett's post-hoc test. Significance was determined between groups. (D) One-way ANOVA with a Tukey's post-hoc test. Significance was determined within groups. In both cases, ** $p < 0.01$; **** $p < 0.0001$. Values within columns indicate the % of control activity.

Next, we asked if known functional domains of FMRP were required for repression. Two of these domains (KH and RGG) have been implicated in binding to both miRNAs and mRNAs and a third (the PRiD) has been shown mediate protein-protein interactions [9]. Interestingly, we found that only the KH domain was required for repression (**Figure 9C).** This is relevant to the future directions of this project because these deletion constructs can be used in RNA-immunoprecipitation and coIP experiments to determine which regions of FMRP are required for miRNA and mRNA interactions.

As an additional control for the λN-FMRP tethering assay, we asked if FMRP could repress FLuc-5xBoxB reporter activity in the absence of the λN protein tag. Surprisingly, we found that untethered FMRP was equally capable of repressing FLuc-5xBoxB reporter expression (**Figure 10A)**. We next asked what was the minimal number of BoxB binding sites required for FMRP-mediated reporter repression. We found that one BoxB site was sufficient to confer repression; 40% of control expression vs. 30% with the FLuc-5xBoxB reporter; $p = 0.75$). FMRP was capable of repressing activity of the FLuc-1xBoxB reporter in a concentration dependent manner (**Figure 10A**). In contrast, when the BoxB sites were removed, the ability to repress was eliminated. Together, these data suggest that FMRP can bind directly to one copy of a **stable stem loop**. We predict this represents a **novel FMRP binding site** in target mRNAs.

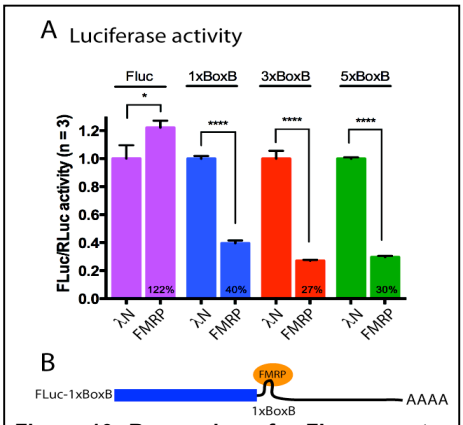

Figure 10. Repression of a FLuc reporter by untethered FMRP. (A) Untethered FMRP is capable of repressing 1x, 3x, and 5x BoxB-containing reporters. FLuc contains no BoxB stem loop sequences. (B) Structure of the 1xBoxB reporter. * $p < 0.05$; **** $p < 0.0001$.

Why is GW182, an evolutionarily conserved effector of miRNA-mediated target silencing, required to regulate FMRP-mediated repression of the FLuc-5xBoxB reporter? It is possible that GW182 has a novel function in the control of general mRNA translation. That said, we instead hypothesized that it was more likely that FMRP was somehow repressing reporter expression via the miRNA pathway. The 3' UTR used in the FLuc-5xBoxB reporter is from the Simian virus 40 (SV40) small t antigen. We ran the SV40 3'UTR through the PITA algorithm against all known *Drosophila* miRNAs (miRBase v20). PITA identifies miRNA/mRNA target interactions based on both seed paring and thermodynamics [10]. Among these predicted miRNAs we identified miR-958-5p as a candidate (**Table 2; Figure 11A)**. Note that most of the miRNAs in **Table 2** have been successfully coIP'd with FMRP (**Figure 7**). We were interested in miR-958 because: 1) **its predicted binding site was very close (~50 nt)** to the 5xBoxB sequence; 2) the predicted interaction energy was relatively low ($\Delta\Delta G$ = -12.27; the fourth strongest predicted interaction); and 3) we had already identified miR-958 as an activity-regulated miRNA required for activity-dependent axon terminal growth at the *Drosophila* NMJ [11]. First, we asked if miR-958 was capable of repressing the FLuc-5xBoxB reporter (**Figure 11B**). When we co-transfected S2 cells with a plasmid expressing the miR-958 primary transcript (pri-miRNA), we observed a > 3-fold decrease in FLuc activity relative to an empty vector control ($p < 0.01$). As we had predicted, this repression was completely eliminated when FMRP was depleted from transfected cells by RNAi (FMRP is highly expressed in S2 cells). Finally, we asked if FMRP binding to the BoxB stem loop is required to facilitate this repression. We found that miR-958 was incapable of repressing the activity of a reporter lacking the BoxB site. Together, these data strongly suggest that

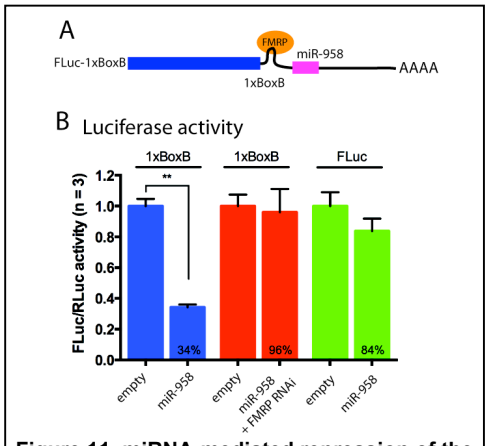

Figure 11. miRNA-mediated repression of the FLuc reporter requires binding of FMRP. FMRP is endogenously expressed in S2 cells. (A) Structure of the 1xBoxB reporter indicating the proximity of the miR-958 binding site. (B) co-transfected miR-958 is capable of repressing the 1xBoxB reporter. This repression is eliminated when FMRP expression is knocked down by RNAi or when the FMRP binding site is eliminated (in the FLuc reporter). ** $p < 0.01$.

binding of endogenous FMRP to the **stable stem loop** is required for miR-958-mediated repression. The **proximity** of the miRNA and FMRP binding sites could potentially be used to quickly screen candidate mRNAs for further analysis.

miRNA	Position	Seed	ΔΔG	RPM
miR-958	80	7:0:0	-12.27	250
bantam	73	6:0:1	-4.98	6.0×10^5
miR-13b-3p	271	6:0:0	-4.11	1.9×10^5
miR-33-5p	237	6:0:0	-3.3	7.7×10^3
miR-2b-3p	271	6:0:0	-2.91	9.3×10^4
miR-2a-3p	271	6:0:0	-2.61	1.1×10^5

Table 2. miRNAs predicted to bind to *FLuc-BoxB* 3'UTR. Columns: 1) miRNA name and strand; 2) Position relative to the first nucleotide in the 3'UTR sequence; For reference, the 1xBoxB sequence corresponds to **position 7-28**; 3) Seed match; 4) predicted binding energy; and 5) reads per million. RPM was taken from **GEO Accession GSM371638**.

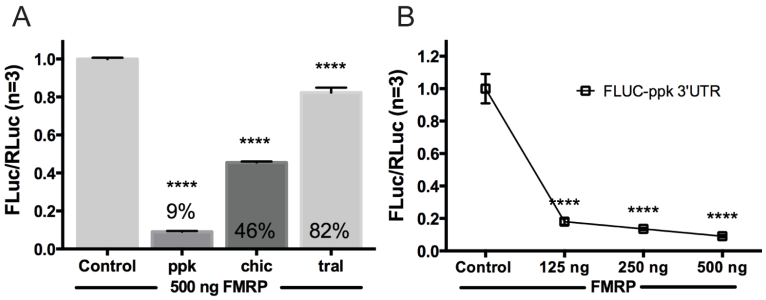

Figure 12. Untethered FMRP can repress the translation of reporters for known mRNA targets. METHODS: The entire regulatory 3'UTR of *ppk*, *chic,* and *tral* (from the stop codon to just beyond the polyA signal) was cloned downstream of the FLuc reporter. Simply put, these sequences replace the entire *SV40 3'UTR* with BoxB sequences. (A) These reporters were co-transfected with RLuc, and the λN peptide (control) or **500 ng of FMRP plasmid** as in **Figure 3-4**. (B) The *ppk* reporter was co-transfected with increasing concentrations of FMRP to show concentration-dependence. STATS: One-way ANOVA with a Tukey's post-hoc test. **** $p < 0.0001$. Values within

As **proof of concept**, we identified known mRNA targets of FMRP that: 1) encode for proteins involved in the control of synapse structure or function; and 2) have been shown to interact directly with FMRP. Pickpocket (PPK1) is a member of the DEG/EnaC superfamily of proteins and functions as a conserved Na^{2+} channel subunit [12]. *Drosophila* FMRP can **directly bind** to and downregulate levels of the *PPK1* mRNA *in vivo* [13]. This work also suggested that reduction of *PPK1* mRNA levels involved Ago2 (and potentially Ago1), the effector in the siRNA-induced silencing complex (siRISC). Thus, we asked if untethered FMRP was capable of repressing the activity of a FLuc-ppk 3'UTR reporter (**Figure 12A-B)**. As observed with the FLuc-1xBoxB reporter, we found that increasing concentrations of co-transfected FMRP plasmid reduced FLuc-ppk 3'UTR reporter activity in a concentration-dependent manner. Interestingly, the *PPK1* 3'UTR is predicted to form **two highly stable stem loops** that are very similar to the BoxB site. We are in the process of confirming that FMRP targets the *PPK1* mRNA for miRNA-mediated translational repression and/or decay.

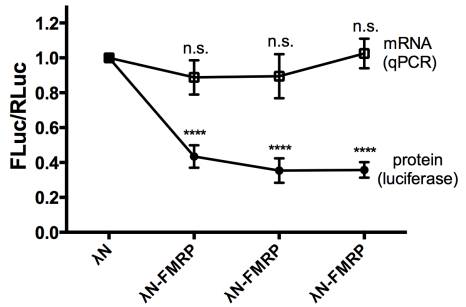

Figure 13. FMRP-mediated repression does not result in mRNA decay. METHODS: The *FLuc-5xBoxB* reporter was co-transfected with increasing concentrations of λN-FMRP. The abundance of FLuc and RLuc mRNA was assayed by qPCR. Repression of the reporter was assayed by FLuc and RLuc luminescence. All samples were normalized to λN controls. STATS: One-way ANOVA with a Tukey's post-hoc test. **** $p < 0.0001$; n.s. = not significant.

Finally, we asked what the fate of the 5xBoxB reporter is following FMRP binding. There are two possibilities (both of which are consistent with miRNA-mediated regulation). First, the mRNA could be targeted for 5' to 3' degradation. Second, the mRNA could be targeted for translational repression. Our evidence suggests that FMRP binds to the 5xBoxB reporter and targets it for translational repression and not degradation (**Figure 13**).

To provide further support for the idea that GW182 is required for FMRP mediated translational repression, we have begun to determine if FRMP and GW182 colocalize to FMRP-containing RNPs both *in vivo* and *in vivo*. As expected, we have observed a significant amount of overlap in *Drosophila* neurons, wing imaginal discs, and S2 cells (**Figures 14 and 15**).

Aim 2a_2. *In silico* identification of target mRNAs for all miRNAs identified in Aim 1a (months 19-20).

Experiments associated with this subaim have not been initiated because they absolutely required completion of Aim 1a_2 above. We intend to complete these experiments in the near future.

Aim 2b. Validation of miRNA/mRNA interactions for candidates identified in Aim 2a (months 20-24).

Again, the experiments associated with this subaim have not been initiated because they absolutely required completion of Aim 1a_2 and Aim 2a_2 above. We intend to complete these experiments in the near future.

Milestone. *Bona fide* mRNA targets for regulation by FMRP-associated miRNAs have been identified.

Do to unforeseeable complications with Aim 1a_1a (above) this milestone has not yet been reached.

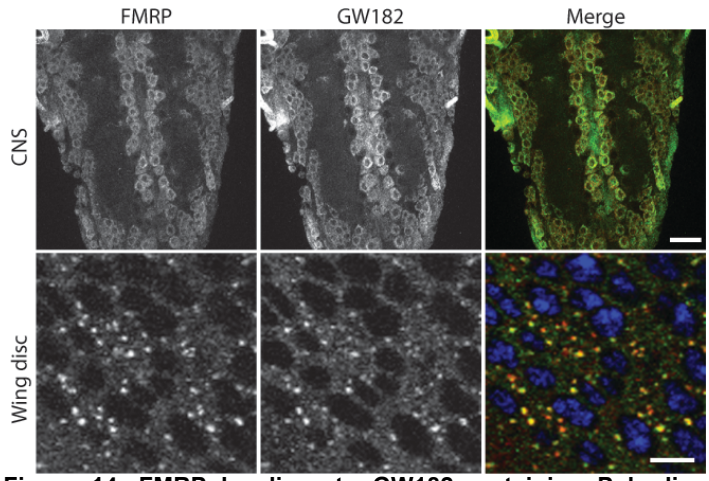

Figure 14. FMRP localizes to GW182-containing P bodies. METHODS: (Top panels) Larval ventral ganglion stained with mouse anti-FMRP 6A15 (Abcam) and guinea pig anti-GW182 (Schneider et al., 2006) as we have previously described (Pradhan et al., 2012). Panels represent a single optical section acquired by **scanning confocal microscopy**. Note there is a significant amount of overlap between cells expressing FMRP (red) and GW182 (green). Scale = 20 μm. (Bottom panels). Larval *Drosophila* wing imaginal discs stained with the antibodies described in above. Yellow punctae in the merged image show significant co-localization between FMRP and GW182. Scale = 5 μm.

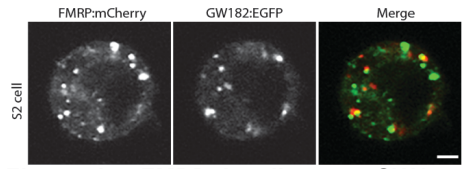

Figure 15. FMRP localizes to GW182-containing P bodies. METHODS: Representative S2 cells transiently transfected with an mCherry-tagged FMRP and EGFP-tagged GW182. The image was acquired by **scanning confocal microscopy**. Note there clearly distinct popu-lations of particles that contain either just FMRP or just GW182 (red and green respectively). There is also a reasonable amount of overlap (yellow). Scale = 1 μm.

KEY ACCOMPLISHMENTS:

1. We have overcome significant difficulties purifying FMRP-containing RNPs in flies **(**see **Figures 1-5**)

2. We have developed an efficient small-scale FMRP immunopurification technique for mRNA and miRNA purification (see **Figures 6 and 7**).

3. We have established miRNA sequencing and data analysis pipeline (see **Figure 8** and **Table 1**).

4. We have developed a rapid assay to confirm that FMRP target mRNAs are co-regulated by both FMRP and the miRNA pathway (see **Figures 9 and 11**).

5. A structural motif required for FMRP binding and recruitment of the miRISC has been identified (see **Figure 10**).

6. A proximity requirement for FMRP and miRNA binding sites (~100 nt) has been identified (see **Figure 11**).

7. The structural motif (stable stem loops) has been validated in a fly mRNA (3'UTR) that has been shown to directly interact with FMRP *in vivo*. This 3'UTR does not contain any other known structural motifs (see **Figure 12**).

8. We have identified the mechanism by which FMRP and the miRNA pathway regulate target mRNAs (see **Figure 13**). This mechanism appears to be through translational repression and not mRNA decay.

9. We have shown that FMRP and GW182 colocalize to RNPs both *in vivo* and *in vitro* (see **Figures 14 and 15**).

CONCLUSIONS:

Despite not completing the RNA sequencing portion of this project (due primarily to unforeseeable complications with FMRP immunoprecipitations, a very tight budget, and very short time frame that did not really account for problems), we have made **significant advances** in the understanding of FMRP-mediated translational repression as it related to the **molecular pathogenesis of Fragile X Syndrome**. Most importantly, we have developed a novel reporter assay that will allow us to fairly quickly dissect the molecular mechanisms involved in FMRP- and miRNA-mediated mRNA regulation. To the best of our knowledge, these novel reagents do not currently exist outside of our lab.

The primary objective of this grant application has always been to generate sufficient preliminary data to secure funding from the NIH in order to complete this project. We have recently submitted an R15 AREA grant submission to NIH that focuses on exploring the mechanisms underlying FMRP- and miRNA-mediated control of translation. As part of this NIH grant submission, we propose the complete the RNA sequencing portion of this project**.** In the event that this is not funded, we have also submitted an intramural grant application (to the University of Denver) to complete sequencing.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Manuscripts are in preparation but have not been submitted.

Grant applications have been submitted but not yet funded.

Therefore, there is nothing to report at this time.

INVENTIONS, PATENTS, AND LICENSES:

Nothing to report.

REPORTABLE OUTCOMES:

Nothing to report.

OTHER ACHIEVEMENTS:

Nothing to report.

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