Reversible Inhibition of PSD-95 mRNA Translation by miR-125a, FMRP Phosphorylation, and mGluR Signaling

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DOI 10.1016/j.molcel.2011.05.006

SUMMARY

The molecular mechanism for how RISC and microRNAs selectively and reversibly regulate mRNA translation in response to receptor signaling is unknown but could provide a means for temporal and spatial control of translation. Here we show that miR-125a targeting PSD-95 mRNA allows reversible inhibition of translation and regulation by gp1 mGluR signaling. Inhibition of miR-125a increased PSD-95 levels in dendrites and altered dendritic spine morphology. Bidirectional control of PSD-95 expression depends on miR-125a and FMRP phosphorylation status. miR-125a levels at synapses and its association with AGO2 are reduced in Fmr1 KO. FMRP phosphorylation promotes the formation of an AGO2-miR-125a inhibitory complex on PSD-95 mRNA, whereas mGluR signaling of translation requires FMRP dephosphorylation and release of AGO2 from the mRNA. These findings reveal a mechanism whereby FMRP phosphorylation provides a reversible switch for AGO2 and microRNA to selectively regulate mRNA translation at synapses in response to receptor activation.

INTRODUCTION

MicroRNAs (miRs) are small, conserved, noncoding RNAs that act in association with the RNA-induced silencing complex (RISC) to regulate gene expression posttranscriptionally (Bartel, 2004). miR-mediated translational repression may occur at pre- and/or postinitiation steps (Abu-Elneel et al., 2008; Filipowicz et al., 2008; Lee et al., 1993; Nottrott et al., 2006; Richter, 2008). Numerous miRNAs are expressed at high levels in the nervous system and regulate development (Kosik, 2006), spine morphology, and synapse function (Schratt, 2009b). miRNAs are implicated in neurological and neuropsychiatric disease (Kocerha et al., 2009; Kosik, 2006; Lee et al., 2008). A subset of miRNAs are localized to dendrites (Kye et al., 2007; Schratt et al., 2006; Siegel et al., 2009), where local translation may affect dendritic spine morphology (Schratt, 2009b; Schratt et al., 2006). A critical gap is understanding how the repression of target mRNA translation by RISC components and miRNAs may be dynamically regulated by receptor signaling pathways. The ability of a cell to dynamically regulate RISC interactions with target miRNAs would allow for more precise temporal and spatial control of mRNA function than achieved solely by regulation of miRNA biogenesis. The physiological signals and molecular mechanisms that promote or inhibit the association of RISC complexes onto specific miRNAs are unknown.

A major advance in our understanding of the regulation of miRNA-mediated translation has been the evidence of reversibility in cultured dividing cells deprived of serum. The interaction of mRNA-binding proteins with cis-acting elements can affect miRNA activity (Bhattacharyya et al., 2006; Vasudevan et al., 2007). These studies suggest the hypothesis that mRNA-binding proteins may act as molecular intermediates downstream of receptor signaling pathways to modulate the interactions of RISC-associated miRNAs to target sequences. Since specific mRNA binding proteins can be localized nonuniformly within the cytoplasm to influence mRNA localization, they are uniquely positioned to co-opt RISC as a means to regulate local mRNA translation in polarized cells.

Dynamic regulation of synaptic protein synthesis in response to activation of neurotransmitter receptors, such as group 1 metabotropic glutamate receptors (gp1 mGluRs), plays a key role in long-term synaptic plasticity underlying learning and memory (Bramham and Wells, 2007; Martin and Ephrussi, 2009). MicroRNAs appear to be ideally suited to reversibly inhibit mRNA translation at synapses in response to receptor signaling (Chang et al., 2009; Kosik, 2006). This, in principle, could provide selective, bidirectional, and spatial control for the regulation of mRNA translation. We sought to identify a molecular mechanism whereby a specific mRNA can be selectively silenced by a miRNA through RISC components, and to investigate whether a selective mRNA-binding protein in response to physiological signals may reversibly regulate these interactions.
Here we elucidate the molecular mechanism of reversible and selective regulation of postsynaptic density protein 95 (PSD-95) mRNA translation in response to stimulation of gp1 mGluRs. miR-125a and fragile X mental retardation protein (FMRP) cooperate on the 3′ UTR of PSD-95 mRNA to enable inhibition and confer flexibility allowing for translational activation at synapses in response to receptor activation. Loss of FMRP causes fragile X syndrome (FXS), the most common monogenic form of inherited intellectual disability and autism. FMRP is localized to dendrites and synapses, where it regulates mRNA transport and local protein synthesis necessary for neuronal development and synaptic plasticity (Bassell and Warren, 2008). FMRP interacts with mammalian eIF2C2 (AGO2) and associates with miRNAs (Edbauer et al., 2010; Jin et al., 2004b). We show that FMRP phosphorylation promotes the formation of an AGO2-miR125a inhibitory complex on PSD-95 mRNA. Conversely, mGluR stimulation leads to dephosphorylation of FMRP, release of AGO2 from the mRNA, and activation of translation. The control of translation by mGluR signaling to FMRP and AGO2 is on the timescale of minutes. This demonstrates that the phosphorylation status of an mRNA-binding protein can affect the rapid and reversible control of miRNA-mediated translational regulation. This study provides mechanistic insight into the selective and cooperative interactions that may function as reversible switches to dynamically regulate miRNA function, and has important implications for understanding neuropsychiatric disorders resulting from altered regulation of miRNA pathways.

RESULTS

mGluR-Mediated Translation of PSD-95 Involves MicroRNAs

Activation of gp1 mGluRs rapidly stimulates the translation of PSD-95 mRNA (Muddashetty et al., 2007). PSD-95 is a key component of the postsynaptic molecular architecture whose levels at the synapse are dynamically regulated (Bingol and Schuman, 2004; Colledge et al., 2003). PSD-95 controls AMPAR endocytosis, synaptic strength, and spine stabilization (Bhattacharyya et al., 2009; Xu et al., 2008; De Roo et al., 2008). Gp1 mGluRs are implicated in different forms of mGluR-mediated synaptic plasticity that depend on new protein synthesis (Anwyl, 2009; Auerbach and Bear, 2010; Ronesi and Huber, 2008). In addition, mGluR-driven synthesis of postsynaptic components, such as PSD-95, may play an important role in the growth and/or stabilization of dendritic spines (Schutt et al., 2009; Vanderklish and Edelman, 2002).

To investigate the molecular mechanism of translational regulation, a firefly luciferase reporter (f-luciferase) with the 3′ untranslated region (UTR) of PSD-95 mRNA was expressed in cultured cortical neurons. The reporter responded to brief (15 min) mGluR activation, indicating the 3′ UTR of PSD-95 mRNA is sufficient to elicit activity-mediated regulation of PSD-95 mRNA translation (Figure 1). (S)-3,5-dihydroxyphenylglycine (DHPG), a specific agonist of group I mGluRs (gpI mGluR), induced a rapid and significant increase in the expression of f-luciferase-PSD-95 UTR (93% ± 32%) in neurons (Figure 1A). To study the involvement of the microRNA pathway in mGluR-mediated translation, we measured the association of luciferase reporter mRNAs with eIF2C2 (mammalian homolog of AGO2) and associates with miRNAs (Edbauer et al., 2010; Jin et al., 2004b). We show that FMRP phosphorylation promotes the formation of an AGO2-miR125a inhibitory complex on PSD-95 mRNA. Conversely, mGluR stimulation leads to dephosphorylation of FMRP, release of AGO2 from the mRNA, and activation of translation. The control of translation by mGluR signaling to FMRP and AGO2 is on the timescale of minutes. This demonstrates that the phosphorylation status of an mRNA-binding protein can affect the rapid and reversible control of miRNA-mediated translational regulation. This study provides mechanistic insight into the selective and cooperative interactions that may function as reversible switches to dynamically regulate miRNA function, and has important implications for understanding neuropsychiatric disorders resulting from altered regulation of miRNA pathways.
of the RISC. Quantitative measurement of f-luciferase mRNA in the AGO2 immunoprecipitate using qPCR showed a significant (44% ± 13%) decrease of f-luciferase-PSD-95 UTR in the pellet from neurons stimulated with DHPG (Figure 1B). A similar DHPG-induced decrease in the association with AGO2 was also observed for endogenous PSD-95 mRNA (61% ± 20%) (Figure 1B), while endogenous β-actin mRNA was unaffected (see Figure S1C available online). Treatment with DHPG had no apparent effect on the stability of β-actin or PSD-95 UTR reporters or endogenous mRNAs, as all mRNA levels tested were unchanged (Figures S1A and S1B). In summary, the 3'UTR of PSD-95 mRNA is sufficient to elicit mGluR-mediated translational activation in neurons. Translational activation coincides with the dissociation of the reporter mRNA (and endogenous PSD-95 mRNA) from AGO2, suggesting a possible role of microRNAs in this process. This led us to search for a microRNA(s) that regulates PSD-95 mRNA translation.

**miR-125a Regulates the Translation of PSD-95 mRNA**

Among the few microRNAs predicted to target the 3'UTR of PSD-95 mRNA (TargetScan 4.1), we initially chose miR-1 and miR-103 because their target site was conserved among species and possessed a higher percent complementarity with PSD-95 mRNA. We also chose to test miR-125a, which was previously predicted to target PSD-95 mRNA 3'UTR (John et al., 2004; and Figure 2A). In neuro2A cells, only the transfection of miR-125a had a significant inhibitory effect on expression of f-luciferase-PSD-95 UTR (−21% ± 6%), while miR-1 and miR-103 had no effect (Figure S2A); hence we pursued only miR-125a for further studies. In cultured cortical neurons, overexpression of miR-125a significantly reduced (−32% ± 4%) the endogenous PSD-95 levels as measured by quantitative immunoblots. Antagonization of miR-125a by transfecting locked nucleic acid (LNA)-antisense RNA oligonucleotides (antimiR-125a) led to 2.7 (±1)-fold increase in endogenous PSD-95 (Figures 2B and 2C). To further validate whether miR-125a targets PSD-95 mRNA, we generated f-luciferase-PSD-95 UTRmt125a by mutating two nucleotides in the seed region of the UTR which was expected to disable miR-125a binding to this mRNA (Figure 2D). In neuro2A cells miR-125a transfection had no effect on the expression of f-luciferase-PSD-95 UTRmt125a, while it significantly reduced the translation of f-luciferase-PSD-95 UTR (Figure 2E), indicating that the two-nucleotide mutation rendered the reporter unresponsive to miR-125a. Blocking endogenous miR-125a by transfecting antimiR-125a significantly increased expression of f-luciferase-PSD-95 UTRmt125a (94% ± 47%), while it had no effect on f-luciferase-PSD-95 UTRmt125a (Figure 2F). These data on the endogenous PSD-95 and luciferase reporter with PSD-95 3'UTR suggest that antimiR-125a relieved the translational inhibition imposed on the PSD-95 3'UTR containing an intact binding site for miR-125a. The effect of antimiR-125a was dose dependent (Figure S2C) and specific to PSD-95 3'UTR, as it had no effect on f-luciferase-β-actin UTR (Figure S2B). Mutation of the miR-125a-binding site (f-luciferase-PSD-95 UTRmt125a) also resulted in a marked reduction of this mRNA association with AGO2 (59% ± 20) compared to the wild-type UTR, as measured by AGO2 immunoprecipitation and q-PCR analysis of mRNA (Figure 2G). However, total levels of the mutant mRNA reporter did not differ from wild-type (data not shown). This suggests that a single miR125a target site may be largely responsible for AGO2-mediated translational regulation in the context of the PSD-95 3'UTR.

Next, we investigated whether miR-125a had a role in mGluR-mediated activation of PSD-95 mRNA translation in primary neurons. DHPG treatment induced a significant increase in the relative luciferase activity of a reporter with the PSD-95 3'UTR in cultured cortical neurons, but when the reporters were cotransfected with antimiR-125a this translational activation was blocked (Figure 2H). Activation of mGluRs with DHPG had no effect on the translation of mutant f-luciferase-PSD-95mt125a (Figure 2H) in cultured cortical neurons. DHPG-induced dissociation of PSD-95 UTR from AGO2 was also specifically abolished by antimiR-125a (Figure 2I). These results indicate miR-125a specifically targets the 3'UTR of PSD-95 mRNA and is essential for mGluR-mediated translation in neurons.

To test the effect of miR-125a on the expression of endogenous PSD-95 protein in dendrites, cultured hippocampal neurons were transfected with antisense oligonucleotides against miR-125a (antimiR-125a) at DIV 10, and cells were fixed and immunostained for PSD-95 protein after 48 hr (Figure 2J). There was a significant increase (45% ± 13%) in the expression of endogenous PSD-95 immunofluorescent signal in the distal dendrites of neurons exposed to antimiR-125a, while transfection with scrambled sequence oligonucleotides or nonspecific antimiR-124 did not cause any significant changes (Figure 2K).

We note that the miR-125a target site on the human 3'UTR of PSD-95 mRNA does not comply with the strict "seed rule," as there is a single nucleotide bulge on the microRNA and only five out of six matches in the two to seven seed region (Figure 2A, Figure S3A). Such bulges are reported in functional miR-mRNA interactions previously (Brodersen and Voinnet, 2009; Ha et al., 1996). Also of note, in the mouse 3'UTR there is a "g instead of a" compared to the seed region of human resulting in a tolerated G.U wobble (Figure S3A). The luciferase reporter bearing the mouse PSD-95 UTR responded similarly to the luciferase reporter with the human PSD-95 3'UTR, both showing increased translation in primary neurons in response to either antimiR-125a or DHPG stimulation (Figure S3B). We also tested the effect of restoring the perfect seed match for miR-125a in the mouse PSD-95 3'UTR (Figure S3C). The construct with a perfect seed match responded to antimiR-125a and DHPG stimulation similar to wild-type mouse and human PSD-95 3'UTRs, indicating the mismatch in the seed region resulting in a G.U wobble behaves similarly (Figure S3C).

**miR-125a Is Localized to Dendrites**

Only a small subset of neuronal microRNAs are localized to dendrites and synapses (Kye et al., 2007). miR-125a is highly expressed in neurons and abundant in synaptoneurosomal fractions from mouse cerebral cortex (Figure 3A) compared to several other microRNAs which are previously reported in dendrites and at synapses (Figure 3A and Kye et al., 2007; Schratt et al., 2006). There was a small but significant reduction in miR-125a levels (−37% ± 23%) from synaptoneurosomes upon stimulation with DHPG (15 min) indicating possible mGluR-induced turnover (Figure 3B). Fluorescence in situ

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(A) Predicted miR-125a-binding site in the human 3' UTR of PSD-95 RNA.

(B) Immunoblots depicting the effect of miR-125a overexpression (lanes 4 and 5) and antagonization (lanes 2 and 3) on the levels of endogenous PSD-95 in primary cortical neurons. miR-124a is the negative control. Tubulin (lower panel) was the loading control.

(C) Quantification of endogenous PSD-95 levels in primary cortical neurons, normalized to tubulin in each lane (n = 3, p = 0.003, one-way ANOVA, Dunnett test ±SD).

(D) The 3'UTR PSD-95 mRNA reporter (FL, full length) was mutated in the seed region by site-directed mutagenesis to generate PSD-95-UTRmt125a which would be insensitive to miR-125a (f-luciferase-PSD-95 UTRmt125a).

(E) Relative luciferase activity from neuro2A cells transfected with either f-luciferase-PSD-95 UTR or f-luciferase-PSD-95 UTRmt125a (n = 3, p = 0.01, one-way ANOVA, Dunnett's test ±SD). miR-125a or miR-124 was cotransfected with luciferase reporters; values are normalized to the control (no miR overexpression).

(F) Relative luciferase activity from neuro2A cells transfected with either f-luciferase-PSD-95 UTR or f-luciferase-PSD-95 UTRmt125a (n = 3, p = 0.02, one-way ANOVA, Dunnett's test ±SD). Antisense miR-125a or miR-124 oligonucleotides were cotransfected with luciferase reporters; values are normalized to the control (no anti-miR).

(G) f-luciferase mRNA ratios of pellet/supernatant from the AGO2 immunoprecipitate of neuro2A cells that were transfected with either f-luciferase-PSD-95 UTR or f-luciferase-PSD-95 UTRmt125a, analyzed by qPCR. Values are normalized to f-luciferase-PSD-95-UTR (n = 3, p = 0.01, paired t test, ±SD).
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hybridization (FISH) showed that miR-125a is present in distal dendrites of cultured hippocampal neurons (Figure 3C). Quantitative in situ hybridization also revealed a reduced miR125a signal in dendrites after 15 min stimulation of the neurons with DHPG (Figure 3D).

Dendritic localization of miR-125a was further validated by FISH on sections of mouse brain. miR-125a was visualized clearly in the dendritic layers of hippocampus in CA1 region (Figure 3E), in contrast to miR-298, which was restricted to the cell body layer with almost undetectable signal in the dendrites (Figure 3E). Our results are consistent with a previous report that miR-298 is highly expressed in neurons, but almost absent from dendrites (Kye et al., 2007). The specificity of the miR125a signal in dendrites was demonstrated by FISH experiments using LNA probes that carried mismatch mutations at only two positions (miR-125a-MM2) within the 125a-specific probe (Figure S3D). This led to significant decrease (−50% ± 5%) in fluorescent intensity compared to miR-125a probe (Figure S3E). Mismatch probes showed strong reduction of dendritic signal in cultured cells (Figure S3D) and on brain slices, respectively (Figure S3F). These results suggest the possibility that miR-125a may function locally in dendrites and at synapses.

Inhibiting miR-125a Affects Spine Density and Branching
The localization of miR-125a to dendrites, its abundance in synaptoneuroosomes, and its involvement in an mGlur pathway suggest miR-125a might function to regulate local protein synthesis affecting dendritic spine-synapse morphology (Schratt, 2009b; Schratt et al., 2006). Blocking miR-125a by transfecting anti-miR-125a had a marked effect on the spine morphology of cultured hippocampal neurons (Figure 4A). Analysis of dendritic spines in cultured hippocampal neurons (transfected at DIV 10, fixed, and immunostained at DIV 14) showed a 33% (±3%) increase in spine density (Figure S4A) and a 3-fold increase in spine branches (300.9% ± 14.0%) in neurons transfected with anti-miR-125a compared to neurons transfected with scrambled RNA (Figures 4D and 4E). Neurons transfected with anti-miR-125a also showed increased number of synapsin punctae compared to scrambled RNA transfected neurons, suggesting increased synapse number in these neurons (Figure S4C).

Since miR-125a targets PSD-95 mRNA and transfection of anti-miR-125a leads to increased endogenous PSD-95 (Figures 2A–2C), we tested the effect of PSD-95 knockdown on the spine phenotype observed by antagonization of miR-125a. Specific siRNAs against PSD-95 mRNA (Fan et al., 2009) significantly reduced PSD-95 protein levels in hippocampal neurons (Figures 4B and 4C), while the control (scrambled) siRNA had no effect. The reduced PSD-95 completely rescued the increased spine branching phenotype caused by anti-miR-125a (Figures 4B, 4D, and 4E). It also significantly lowered the spine density increase caused by anti-miR-125a (Figure S4B). Control siRNA had no effect on either spine density or branching. These data suggest that the excessive spine density and spine branching observed by antagonization of miR-125a is mediated by the increased synthesis of PSD-95.

FMRP Controls miR-125a-Mediated Regulation of PSD-95 mRNA Translation
FMRP, the deficiency of which causes FXS, is reported to regulate the mGluR-mediated translation of postsynaptic proteins (Schutt et al., 2009), including PSD-95 (Muddashetty et al., 2007). Based on previous reports that FMRP directly binds to the 3′UTR of PSD-95 mRNA (Zalfa et al., 2007), we confirmed that FMRP is associated with the f-luciferase with the 3′UTR of PSD-95 mRNA (Figure S5F). We then tested the effect of FMRP on the translation of this reporter by depleting FMRP in neuro2A cells by specific siRNA (Figure S5J). FMRP knockdown significantly increased the translation of f-luciferase-PSD-95 3′UTR (Figure S5K), while it had no effect on f-luciferase-β-actin UTR. These data show that FMRP is necessary for translational inhibition imposed on the 3′UTR of PSD-95 mRNA. In primary cortical neurons from Fmr1 KO mice, translation of the transfected f-luciferase-PSD-95 3′UTR was significantly higher than (+205% ± 50%) wild-type neurons, but coexpression of FMRP-WT in KO neurons sharply reduced the translation of the reporter (Figure S5E). This indicates an increased translation of PSD-95 mRNA in Fmr1 KO neurons on transient transfection of the f-luciferase-PSD-95 3′UTR reporter, an effect that can be rescued by coexpression of FMRP-WT.

The interaction between FMRP and PSD-95 mRNA 3′UTR in cells, as shown by an IP and qPCR assay, was validated by additional experiments. When FMRP and PSD-95 3′UTR were expressed in separate cultures, lysed, and mixed before immunoprecipitation (Figure S5G), their interaction was significantly lower (−78% ± 12%) compared to when they are expressed together in the same cells. We also tested whether FMRP interacts directly with the region of the 3′UTR in vivo, by using a stringent UV-crosslink-IP (CLIP) method (Figures S8H and S8I) (Ule et al., 2005). A significant reduction (−64% ± 5%) in the binding of f-luciferase mRNA where the FMRP binding region is deleted (f-luci-PSD-95 3′UTRΔGR) compared to full-length UTR indicates that this region (which includes the miR-125a binding site) is essential for FMRP binding to PSD-95 mRNA in vivo prior to lysis.
Surprisingly, we observed that the two-nucleotide mutation inserted in the PSD-95 3' UTR to disable the miR-125a interaction had no significant effect on FMRP binding (Figure 5A); however, this mutation completely removed the effect of FMRP on translational inhibition of the 3' UTR reporter. While FMRP knockdown in neuro2A cells resulted in a significant increase in the
expression of f-luciferase-PSD-95 UTR (59% ± 16%), it had no effect on f-luciferase-PSD-95 UTRm125a (Figure 5B). These experiments indicate that the 3'UTR of PSD-95 mRNA is sufficient to elicit FMRP mediated regulation of translation, but it requires a functional miR-125a interaction. These data suggest that FMRP binding to the 3'UTR on its own is insufficient to regulate translation. The mGluR and miR-125a mediated regulation of PSD-95 translation was also disrupted in the absence of FMRP in a mouse model of FXS (Figures 5D and 5E). In cultured cortical neurons from Fmr1 KO mice, unlike WT neurons (Figure 5D), treatment with DHPG or antimiR-125a did not increase the translation of f-luciferase-PSD-95 UTR (Figure 5E). Interestingly, DHPG had no effect on the interaction of FMRP with endogenous PSD-95 mRNA (Figure 5C and Figure S5L), in contrast to the DHPG-induced dissociation of endogenous PSD-95 mRNA from AGO2 shown earlier (Figure 1B). Taken together, these data show that FMRP association with the mRNA is essential for mGluR-mediated regulation translation which is executed by relieving miR-125a imposed inhibition on the 3'UTR.

To further explore the overlap in the FMRP- and miR-125a-mediated regulation of PSD-95 mRNA translation, we studied the polysomal distribution of endogenous PSD-95 mRNA and miR-125a from WT and Fmr1 KO synaptoneurosomes. Actively translating polysomes in the synaptoneurosomes can be clearly distinguished by separating them on linear sucrose gradient and analysis of the differential effects of cyclohexamide, puromycin and EDTA treatment (Figures S5 A–S5D and Muddashetty et al., 2007; Stefani et al., 2004). In WT synaptoneurosomes, activation of gpI mGluR by DHPG resulted in significant shift (fractions 7–10) of endogenous PSD-95 mRNA into polysomes (Figure 5F). DHPG treatment also led to a decrease in the miR-125a in fraction 1, which represents the mRNP s (Figure 5G). The reason for this shift is not entirely clear, but we speculate that the translational activation induced by mGluR stimulation leads to the dissociation of PSD-95:miR-125a inhibitory complex...
and a concomitant shift of PSD-95 mRNA into polysomes and also a resultant decrease in mir-125a in the mRNP fraction. In Fmr1 KO synaptoneurosomes, PSD-95 mRNA is elevated significantly in polysomal fractions (fraction 5–10) compared to WT at basal state (Figure 5F and Muddashetty et al., 2007). This may be due to the inability to form a mir-125a-mediated inhibitory complex on PSD-95 mRNA in the absence of FMRP (discussed further below). mGluR-mediated translational activation of PSD-95 mRNA is absent in Fmr1 KO synaptoneurosomes and neurons (Muddashetty et al., 2007; Todd et al., 2003). Interestingly, the distribution of both PSD-95 mRNA and mir-125a on linear sucrose gradients from unstimulated KO synaptoneurosomes resembled that of DHPG treated synaptoneurosomes from WT (Figures 5F and 5G). In summary, both FMRP and mir-125a are essential for mGluR-mediated regulation of PSD-95 mRNA translation, and FMRP seems to control the execution of mir-125a inhibition on PSD-95 mRNA translation in a reversible manner.

**FMRP Is Essential for the Interaction of AGO2 with PSD-95 mRNA at Synapses**

The microRNA-mediated translational inhibition occurs by formation of a RISC on mRNAs (Bartel, 2004). The components of this inhibitory complex are reported to be present at synapses (Lugli et al., 2005). Here we show dendritic localization of AGO2 in cultured hippocampal neurons and their colocalization with piccolo, a synaptic marker (Figure S4D). The necessity of FMRP and mir-125a for mGluR-mediated translation of PSD-95 mRNA and its dysregulation in Fmr1 KO synaptoneurosomes opens the possibility of failure to form RISC on PSD-95 mRNA at synapses in the absence of FMRP. Supporting this hypothesis, in Fmr1 KO synaptoneurosomes, a significantly reduced (~66% ± 12.2%) amount of mir-125a was found in precipitated AGO2 (Figure 5H) compared to WT. This effect was specific to mir-125a, since there was no difference in the mir-124 precipitated by AGO2 between WT and Fmr1 KO, both in total extract and synaptoneurosomes (Figure S6A). Interestingly, mir-125a itself was significantly decreased in Fmr1 KO synaptoneurosomes compared to WT (Figure 5I). The ratio of mir-125a in Fmr1 KO synaptoneurosomes (S) to the total (T) extract (Figure 5I) was less than 50% of WT (~53.1% ± 10.4). This effect was specific to mir-125a, while mir-124, another synaptic microRNA (Figure 5I), and several other microRNAs tested (data not shown) were unaffected. Endogenous PSD-95 mRNA precipitated with AGO2 was also significantly reduced (~58% ± 13%) in Fmr1 KO synaptoneurosomes (Figure 5J) compared to WT, while precipitation of β-actin mRNA was unaffected (Figure S6B). These data suggest that FMRP is necessary to recruit AGO2 complexes containing mir-125a onto PSD-95 mRNA at synapses.

**Phosphorylation of FMRP Modulates mir-125a Regulation of PSD-95 mRNA Translation**

Thus far our results indicate that FMRP acts as a modulator between the mGluR signaling pathway and mir-125a-mediated regulation of PSD-95 mRNA translation. To understand how FMRP provides flexibility to miR-mediated translational regulation, we investigated the phosphorylation status of FMRP as a likely mode of control. FMRP is phosphorylated primarily on the conserved S499 (human S500) (Ceman et al., 2003), which is thought to be critical for regulating the translation of its target mRNAs (Ceman et al., 2003; Narayanan et al., 2007, 2008). In our study, we used the overexpression of phosphomutants S499D (mimics the phosphorylated form) S499A (mimics the dephosphorylated form) (Narayanan et al., 2007) or FMRP-WT in Fmr1 KO neurons and murine L-M(TK−) cells in which have low endogenous FMRP levels (Ceman et al., 1999). Comparable amounts of FMRP-WT, FMRP-S499D, and FMRP-S499A were expressed in these cells (Figure 6A and Figure S7E), and the phosphomutants had no effect on the mRNA levels of transfected f-luciferase-PSD-95 UTR constructs (Figure S7F). In Fmr1 KO neurons, overexpression of FMRP-WT and FMRP-S499D...
Figure 6. FMRP Phosphorylation Inhibits PSD-95 mRNA Translation through miR-125a

(A) Immunoblot showing the effect of overexpressing FMRP-WT, FMRP-S499D (mimics constitutively phosphorylated FMRP), or FMRP-S499A (constitutively dephosphorylated FMRP) in cultured cortical neurons from Fmr1 KO mice. Upper panel shows levels of endogenous PSD-95 protein, middle panel is tubulin (loading control), and the lower panel is Flag-FMRP.
significantly reduced the endogenous PSD-95 protein levels, while overexpression of FMRP-S499A had no effect (Figures 6A and 6B). Coexpression of FMRP-S499D with f-luciferase-PSD-95 UTR in neurons significantly reduced (~74% ± 14%) the relative luciferase activity compared to coexpression with FMRP-WT (Figure 57B). Coexpression of FMRP-S499D had no effect on f-luciferase-PSD-95 UTR mt125a. Antagonizing mir-125a by anti-mir-125a blocked the inhibitory effect of FMRP-S499D on f-luciferase-PSD-95 UTR in cortical neurons (Figure 6C). Overexpression of FMRP-S499D leads to significantly reduced expression of f-luciferase-PSD-95 UTR in primary cortical neurons at basal state, and the DHPG-induced increase in the luciferase activity was also abolished (Figure 6D).

These data indicate the critical role of S499 of FMRP in mediating the inhibition and mGluR-mediated activation of PSD-95 mRNA translation involving mir-125a.

Overexpression of FMRP-S499D in L-M(TK−) cells had a significant effect on the distribution of transfected f-luciferase-PSD-95 UTR mRNA on a linear sucrose gradient (Figure 6E). There was a significant shift of the reporter mRNA into the mRNP fraction and a resultant decrease in the polysomal fractions (Figures 6E and 6F) when compared to cells overexpressing FMRP-WT (47% ± 1.6%) or FMRP-S499A. The overexpression of FMRP-S499D also resulted in a significant increase of mir-125a in fraction 1–2 (mRNP), compared to FMRP-S499A, when lysates from L-M(TK−) cells were separated on a linear sucrose gradient (Figure S7G). The sucrose gradient and luciferase activity experiments taken together suggest that FMRP-S499D forms an inhibitory complex on PSD-95 mRNA involving mir-125a which cannot be relieved by mGluR activation because of the constitutively phosphorylated form of FMRP (Figures 6A–6D). Further evidence for an inhibitory complex is suggested by increased association of FMRP-S499D (G) Ratio of miR-125a in AGO-2 pellet to supernatant from cortisol neurons significantly reduced (−48% ± 28%) of AGO2 was present in FMRP immunoprecipitates (Figures 7C and 7D). In contrast, DHPG had no effect on the interaction of AGO2 with overexpressed FMRP-S499D (phospho-FMRP mimic). We showed earlier that DHPG dissociates AGO2 (Figure 1B) but not FMRP (Figure 5C) from PSD-95 mRNA. Taken together, these results suggest that dephosphorylation of FMRP is the essential step for subsequent dissociation of RISC from FMRP-bound PSD-95 mRNA and activates mRNA translation. The interaction between AGO2 and FMRP appears to be RNA dependent, as RNase treatment significantly reduced the myc-FMRP precipitated with Flag-AGO2 expressed in neuro2A cells (Figure 7E). Further work is needed to elucidate the molecular details of this interaction. In summary, the phosphorylated form of FMRP preferentially forms the inhibitory complex on PSD-95 mRNA involving mir-125a, whereas dephosphorylation of FMRP is the critical switch required for relieving mir-mediated inhibition of translation.

**DISCUSSION**

**MicroRNA and FMRP Partner to Control Translation in the mGluR Signaling Pathway**

This study reveals a molecular mechanism for the cooperative involvement of mir-125a and FMRP in the reversible regulation of PSD-95 mRNA translation at synapses downstream of gp1 mGluR signaling. Translational inhibition of PSD-95 mRNA by mir-125a is relieved by mGluR activation, which rapidly induces the dissociation of the RISC containing AGO2 from PSD-95 mRNA. Inhibition of PSD-95 mRNA by mir-125a was shown to have bidirectional control of endogenous PSD-95 expression in dendrites and at synapses. FMRP is necessary for both mir-125a-mediated inhibition and mGluR-induced translation of PSD-95 mRNA in neurons. Conversely, mir-125a binding to the 3′ UTR of PSD-95 is necessary for FMRP-mediated

(B) Quantification of effect of overexpression of FMRP-WT and its phosphomutants (FMRP-S499D and FMRP-S499A) on level of endogenous PSD-95 protein in cultured cortical neurons from Fmr1 KO; values were normalized to tubulin (n = 4, p = 0.007, one-way ANOVA, ±SD).

(C) Relative luciferase activity from primary cortical neurons in which f-luciferase-PSD-95 UTR was cotransfected with FMRP-WT or FMRP-S499D (n = 3, p = 0.01, one-way ANOVA, Bonferroni’s test ±SD), anti-mir-125a or anti-mir-124 was cotransfected where indicated.

(D) Relative luciferase activity from WT cortical neurons in which f-luciferase-PSD-95 UTR was cotransfected with FMRP-WT or FMRP-S499D before DHPG or mock treatment (n = 3, p = 0.017, one-way ANOVA, Bonferroni’s test ±SD).

(E) Distribution of f-luciferase-PSD-95 UTR on linear sucrose gradients (15%–45%) from L-M(TK−) cells transfected with FMRP-WT or FMRP-S499D or FMRP-S499A. Values show the percentage of the total f-luciferase-PSD-95 UTR mRNA in each fraction (representative graph).

(F) Percentage of f-luciferase-PSD-95 UTR and β-actin mRNA in the polysomal fraction of the linear sucrose gradient from the lysate of L-M(TK−) cells transfected with FMRP-WT or FMRP-S499D or FMRP-S499A (n = 3, p = 0.005, one-way ANOVA, Bonferroni’s test ±SD).

(G) Ratio of mir-125a in AGO-2 pellet to supernatant from Fmr1 KO cortical neurons which were transfected with FMRP-WT, FMRP-S499D, or FMRP-S499A (n = 3, p = 0.001, one-way ANOVA, Dunnett test ±SD).

(H) Ratio of PSD-95 mRNA in Flag-FMRP pellet to supernatant from cortical neurons which were transfected with FMRP-WT, FMRP-S499D, or FMRP-S499A (n = 3, p = 0.02, one-way ANOVA, Dunnett test ±SD) (also see Figure S7).
Figure 7. Dephosphorylation of FMRP Is Essential for Dissociation of PSD-95 mRNA from AGO2

(A) Ratio of PSD-95 mRNA in AGO2 immunoprecipitate from cortical neurons (n = 3, p = 0.01, one-way ANOVA, Bonferroni’s test, ±SD) at basal state or after DHPG treatment. Cells were preincubated with okadaic acid where indicated.

(B) Ratio of luciferase mRNA in AGO2 immunoprecipitate from cortical neurons which were cotransfected with f-luciferase-PSD-95 UTR and FMRP-WT or FMRP-S499D (n = 3, p = 0.005, paired t test, ±SD) and analyzed at basal state or after DHPG stimulation.

(C) Immunoblots depicting the AGO2 and Flag-FMRP immunoprecipitated with anti-Flag antibody. Upper panel represents the input and the lower panel the precipitate. Lanes 1 and 2 are cells transfected with FMRP-WT (1-basal, 2-DHPG treated), lanes 3 and 4 are cells transfected with FMRP-S499D (3-basal, 4-DHPG treated).

(D) Quantification of AGO2 and FMRP immunoprecipitated with anti-Flag antibody. Cortical neurons were either transfected with Flag-tagged FMRP-WT or FMRP-S499D followed by IP with Flag antibody (n = 3, p = 0.04, paired t test, ±SD) at basal state or after DHPG stimulation.

(E) Immunoblot showing the effect of RNase treatment on the FMRP-AGO2 interaction in neuro2A cells. Upper panel shows the expression of myc-FMRP. Middle panel shows AGO2 and FMRP in Flag-AGO2 pellet. Bottom panel is the RNA gel (ethidium bromide staining) showing the effect of RNase treatment. (+, +) indicates RNase treatment. First two lanes are from the cells not expressing Flag-AGO-2.

(F) Model depicting the role of FMRP phosphorylation and miR-125a in the reversible regulation of PSD-95 mRNA translation in response to mGluR activation at dendritic spines.
repression. FMRP binding to the 3'UTR of PSD-95 mRNA is insufficient to regulate translation on its own, but was enabled by the recruitment of RISC (AGO2) and miR-125a. Taken together, these data suggest that FMRP co-opts RISC complexes containing miR-125a onto PSD-95 mRNA at synapses. Devoid of this mechanism in the absence of FMRP, translation of PSD-95 mRNA at synapses is dysregulated at basal state and occludes activation of translation by mGluRs. miR-125a is significantly reduced in Fmr1 KO synapto-neuromeres, a possible outcome due to its inability to form and/or stabilize an inhibitory complex on PSD-95 mRNA in the absence of FMRP. The association of AGO2 with both PSD-95 mRNA and miR-125a is significantly reduced in Fmr1 KO, which further supports the model that FMRP is essential for the formation of a RISC-mediated inhibitory complex on PSD-95 mRNA and its regulation at synapses. With the lack of such a complex, miR-125a is likely degraded, hence the reduced levels in synapto-neuromeres from the Fmr1 KO. Future work may uncover whether this is a general mechanism for FMRP to guide specific miRNAs onto target mRNAs at synapses. A recent study has identified that many miRNAs in the brain including miR-125a are associated with FMRP and demonstrated that FMRP is necessary for miR-125b and miR-132 to affect dendritic spine morphology (Edbauer et al., 2010). Further work may reveal a common molecular mechanism for FMRP to regulate translation of target mRNAs using microRNAs.

**Phospho-FMRP Forms the Inhibitory Complex on PSD-95 mRNA with miR-125a and AGO2**

Reversible inhibition of PSD-95 mRNA was shown to involve the phosphorylated form of FMRP forming an inhibitory miRNA complex with miR-125a and AGO2. Phosphorylation of FMRP has been previously shown to play a critical role in mGluR-mediated translation, although the mechanism was not known (Narayanan et al., 2007, 2008). Based on our data, we propose a model (Figure 7F) whereby phosphorylated FMRP recruits RISC-miR-125a onto PSD-95 mRNA and inhibits translation. Dephosphorylation of FMRP at a single serine appears to be the critical step to reverse this miR-mediated translation inhibition. Activation of gp1 mGluR induces the dissociation of RISC from PSD-95 mRNA at synapse and initiates rapid translation in a process mediated by FMRP dephosphorylation. The phosphorylation status of FMRP may determine the accessibility of miR-125a to PSD-95 mRNA, thus conferring additional specificity to their interaction. We hypothesize that phosphorylation of FMRP exposes the miR-125a binding site on PSD-95 mRNA, while dephosphorylation destabilizes this interaction and thus leads to miR-125a dissociation from PSD-95 mRNA. Since FMRP remains bound to the mRNA following stimulation, we speculate that FMRP rephosphorylation would lead to re-recruitment of RISC and inhibition of translation. There has been prior evidence for activity-regulated removal of RISC components. Degradation of MOV10 (a RISC protein) occurs in NMDA-receptor-mediated activity-dependent manner at synapses, which may be a general translational control point (Banerjee et al., 2009). While this study shows the importance of MOV10 as a general checkpoint for translation, it does not decode the mRNA specificity of receptor-induced translation. Our findings suggest that involvement of RNA-binding proteins such as FMRP provides specificity and flexibility to this process. The actual influence of FMRP on the molecular interactions of microRNA with its target mRNAs and its possible direct interaction with AGO2 or other components of RISC, such as GW182 and MOV10, needs to be further explored.

**Localized MicroRNAs Affecting Spine Morphology Have Implications for FXS**

The discovery of several localized miRNAs in neurites of cultured neurons (Kye et al., 2007) and their enrichment in synaptic fractions (Lugli et al., 2005; Siegel et al., 2009), followed by the demonstrated role of miR-134 in the local translation of Limk1 in vitro (Schatt et al., 2006), led to studies showing the widespread presence of a localized system of microRNAs affecting synaptic structure and function (Schatt, 2009a; Siegel et al., 2009). Here we visualize the dendritic localization of miR-125a in vitro and in vivo and demonstrate its abundance in synaptic fractions. We observed a role for miR-125a in regulating the morphology and density of dendritic spines. Inhibition of endogenous miR-125a function resulted in a marked increase in spine density and branching. We also demonstrated that the spine phenotype observed by miR-125a inhibition is due to excess PSD-95 mRNA translation and could be rescued by PSD-95 knockdown. These data suggest that miR-125a-mediated inhibition of PSD-95 mRNA translation may restrain spine growth and/or branching. This is consistent with observations that overexpression of PSD-95 resulted in the formation of branched and multi-innervated spines (Nikonenko et al., 2008), which bears some similarities to the spine phenotype observed by miR-125a inhibition. Further work is needed to assess the contribution of localized miR-125a and PSD-95 mRNA on spine morphology. Additional work is also needed to assess how spine phenotypes in FXS may be due to loss of microRNA-mediated control of local mRNA translation. An exuberant excess of spines having an immature, long and thin morphology is a well-described phenotype of human FXS patients and the Fmr1 KO mouse model (Bagni and Greenough, 2005). We speculate that the spine phenotype in FXS may be partly due to excess basal expression of PSD-95 and other mRNAs, which have lost their regulation by localized microRNAs. FMRP- and miR-125a-mediated translation of PSD-95 mRNA at synapses, in response to mGluR activation, may normally regulate formation of the PSD and stabilization of newly formed dendritic spines. It is known that mGluRs stimulate protein-synthesis-dependent growth of spines (Vanderklish and Edelman, 2002) and that PSD-95 is important for stabilization of newly formed dendrites (De Roo et al., 2008). Recent work shows that spines in Fmr1 KO mice have increased turnover and delayed stabilization (Pan et al., 2010), which could be due to impaired synthesis of PSD-95 and other PSD proteins (Muddashetty et al., 2007; Schutt et al., 2009). Since FMRP associates with many miRNAs, and this association is necessary for FMRP’s role on spine morphology (Edbauer et al., 2010), it will be interesting to assess a possible general role for FMRP-miRNA interactions in local protein synthesis underlying spine stabilization. The present study provides further motivation to investigate whether miRNA-mediated regulation of mRNA translation through FMRP...
and/or other mRNA binding proteins may represent a general mechanism to achieve dynamic and bidirectional control of local protein synthesis affecting synapse structure and function. This work provides mechanistic insight into the role that dysregulated miRNAs play in FXS, which has broader implications for the role of miRNAs in other neurological diseases (Kocerha et al., 2009) and autism spectrum disorders (Abu-Elneel et al., 2008). Future research may consider therapeutic strategies to restore microRNA function.

EXPERIMENTAL PROCEDURES

Cell Culture

Primary neuronal cultures were prepared from cerebral cortices and hippocampi of E18 mouse (C57BL/6 and Fmr1 knockout, backcrossed in C57BL/6J) or rat (Sprague dawley) as described previously (Kaecy and Banker, 2009). High-density cultures were used for biochemical experiments, while low-density cocultures with glia were used for imaging experiments. Neuro2A and L-M(TK–) cells were cultured in DMEM (HyClone) with 10% FBS (Sigma).

DNA Constructs, Transfection, and MicroRNA Inhibitors

The 3'UTR of mouse and human PSD-95 mRNA was amplified from cDNA. Human PSD-95 3'UTR was used for all the experiments unless specified. F-UCi-PSD-95 UTm-125a and other mutant constructs were obtained by site-directed mutagenesis of the hPSD-95 3'UTR using overlapping primers. Pre-microRNA (Invitrogen) and GFP-miR expression vectors (GeneCopeia) were used for the overexpression of microRNAs. LNA antisense-RNA oligonucleotides (Ambion) were used for inhibiting/antagonizing microRNAs. Double-stranded stealth siRNA (Invitrogen) was used for knocking down Fmr1 mRNA, and a pool of three target-specific siRNAs (Santa Cruz Biotechnology) were used for PSD-95 mRNA knockdown. DNA and RNA were transfected to neuro2A and L-M(TK–) cells by Lipofectamine2000 (Invitrogen) and primary cultured neurons by magnetofection using Neuromag (OZ Bioscience) according to manufacturer’s instructions.

Fluorescence In Situ Hybridization and Immunocytochemistry

Mature (DIV 14–21) hippocampal neurons were fixed and processed for in situ Fluorescence In Situ Hybridization and Immunocytochemistry according to manufacturer’s instructions.

Spine and Synapsin Analysis

Cultured hippocampal neurons at DIV 10 were transfected with LNA-anti-miR oligonucleotides or scrambled RNA (Ambion) by magnetofection using neumag reagent (OZbioscience) according to manufacturer’s protocol. For PSD-95 knockdown, siRNAs against PSD-95 mRNA were cotransfected with antimiRs at DIV 10. At DIV 14, neurons were stained to excess rhodamine-phalloidin to label spines and anti-synapsin antibody to label synapses.

Synaptoneurosome Preparation

Synaptoneurosomes were prepared either by differential filtration (Muddashetty et al., 2007) (Milliopore) or separation on density gradient as described earlier (Gardoni et al., 1998). Stimulation was carried out as described previously (Muddashetty et al., 2007).

Immunoprecipitation and Western Blot

Immunoprecipitation was performed as described previously (Muddashetty et al., 2007) using protein G Sepharose (Roche). Monoclonal antibody 7G1 for FMRP (Developmental Studies Hybridoma Bank) and AGO2 (Abnova) were used. Flag-FMRP Immunoprecipitation was performed using anti-Flag agarose (Sigma). Samples were processed for quantitative real time PCR or western blotting following the immunoprecipitation. PSD-95 (Chemicon), FMRP (Sigma), Flag (Sigma), AGO-2 (Abnova), and α-tubulin (Sigma) antibodies were used for western blots.

Polysome Assay

Synaptoneurosomes were prepared from P21 WT and Fmr1 knockout mice as described previously (Muddashetty et al., 2007).

MicroRNA Isolation and Quantification

MicroRNAs were isolated using miRvana kit (Ambion) according to the manufacturer’s protocol. Quantification was carried out by real-time PCR by either TaqMan (Applied Biosystem) probes (Kye et al., 2007) or a simpler SYBR-based detection method (Roche) as described earlier (Ro et al., 2006).

Luciferase Assay

Neuro2A and L-M(TK–) cells were transfected with Lipofectamine 2000 (Invitrogen), cultured neurons were transfected by magnetofection (OZ Bioscience), and luciferase activity was measured by dual luciferase assay (Promega).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at doi:10.1016/j.molcel.2011.05.006.

ACKNOWLEDGMENTS

The authors thank Stephanie Ceman for FMRP constructs; Custom Cloning Core facility (Emory University) for preparing constructs; and Andrew Swanson, Tamika Malone, and Yukio Sasaki for technical assistance. This work was supported by FRAXA and NFXF postdoctoral fellowships (R.S.M.); National Institutes of Health grants MH085617 (G.J.B.), DA027080 (G.J.B.), and HD020521 (S.T.W.); Baylor–Emory Fragile X Research Center (P30HD024064); and Emory Neuroscience–National Institute of Neurological Disorders and Stroke Core Facilities Grant P30NS055077.

Received: October 21, 2010
Revised: February 22, 2011
Accepted: March 25, 2011
Published: June 9, 2011

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