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## The Translation of Translational Control by FMRP: Therapeutic Targets for Fragile X Syndrome

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### Summary

De novo protein synthesis is necessary for long-lasting modifications in synaptic strength and dendritic spine dynamics that underlie cognition<sup>1</sup>. Fragile X syndrome, characterized by intellectual disability and autistic behaviors, holds promise for revealing the molecular basis for these long-term changes in neuronal function. Loss-of-function of FMRP, the fragile X mental retardation protein, results in defects in synaptic plasticity and cognition in many models of the disease. FMRP is a polyribosome-associated RNA binding protein that regulates the synthesis of a set of plasticity-related proteins by stalling ribosomal translocation on target mRNAs. The recent identification of mRNA targets of FMRP and its upstream regulators, and the use of small molecules to stall ribosomes in the absence of FMRP, have the potential to be translated into novel therapeutic avenues for the treatment of FXS.

### Introduction

Many forms of long-term synaptic and spine morphological plasticity require rapid protein synthesis for their expression while the requirement for new transcription is temporally delayed, suggesting that the translation of existing mRNAs is fundamental to the expression of synaptic plasticity. One of the most intriguing examples of a disease characterized by defects in complex behavior and cognition in humans, and defects in synaptic plasticity and circuit function in mouse models, is fragile X syndrome (FXS), which is caused by loss-of-function of the RNA binding protein FMRP (reviewed in<sup>2,3</sup>). Mouse models of FXS are characterized by widespread defects in synaptic plasticity<sup>4</sup>, and both mice and humans have been found to have increased numbers of long, thin dendritic spines relative to the mushroom-shaped spines characteristic of stronger, more mature synapses<sup>5,6</sup>. Recent studies have shown marked alterations in spine dynamics, including an accelerated turnover rate and a failure to respond to input<sup>7,8</sup>. Notably, several protein synthesis-dependent forms of plasticity lose their dependence on new translation in the absence of FMRP<sup>9,10</sup>, suggesting that plasticity-related proteins are already present in adequate levels. Plasticity and spine dynamics are essential for establishing and maintaining normal synaptic communication and for supporting larger groups of neurons that function together in circuits linked by these

synaptic contacts. Defects in several neuronal circuits have been described due to absence of FMRP<sup>11</sup>. These studies underscore the importance of FMRP for normal brain function.

Elucidating FMRP function at the molecular level is key to unraveling its role in more complex processes at the level of synapses, circuits, and behavior. FMRP is expressed in all neurons, but not in mature glia<sup>12</sup>, and within the neuron FMRP is enriched in the cell body, though some is present in granules in dendrites as well as axons during development<sup>13</sup>. The vast majority of FMRP is associated with polyribosomes<sup>14,15</sup> in complexes that contain several stalled ribosomes in addition to its target mRNAs<sup>16</sup>. The major function of FMRP is to repress translation of specific mRNAs and it is widely believed that loss of this form of translational control leads to the deficits seen in human FXS patients and disease models. However, more global effects on translation may be a secondary consequence of dysregulation of the primary mRNA targets of FMRP. Thus, elucidating the molecular details of the function of FMRP in regulating translation as well as identifying primary and secondary changes in protein expression that result from its absence is of critical importance. In addition, activity-dependent mechanisms by which FMRP is regulated are central to understand the both the pathology of FXS as well as how protein synthesis regulates synaptic strength in healthy neurons. This review focuses on presenting and evaluating recent experiments addressing these issues and how recent progress might inform therapeutic development for treatment of individuals with FXS.

## FMRP as a translational regulator

Shortly after the human *FMR1* gene was cloned<sup>17</sup> it was recognized that the encoded protein, FMRP, harbors three canonical RNA binding domains<sup>18</sup>. Although almost all cases of FXS are caused by complete loss of expression of FMRP due to transcriptional silencing, a single patient exists with a point mutation in the second KH domain (KH2) that converts a key hydrophobic amino acid in the heart of the RNA binding pocket to a bulky charged group, referred to as I304N<sup>19,20</sup>. Polysome fractionation of I304N FXS lymphoblastoid cells<sup>21</sup> and brain from an I304N knock-in mouse model<sup>22</sup> revealed that the function of the KH2 RNA-binding domain is essential for normal polyribosome association of FMRP. Furthermore, characterization of endogenous I304N-FMRP in mouse brain demonstrated that the mutation results in loss of RNA binding by FMRP<sup>22</sup>, strongly suggesting that the association of FMRP with polysomes, mediated by RNA binding, is a critical aspect of its function. Coupled with studies demonstrating that impaired memory in an FXS model can be rescued using translational inhibitors<sup>23</sup>, it is likely that understanding translational control by FMRP will inform both the disease and normal neuronal biology.

Mammalian KH domains adopt a very consistent fold necessary for sequencespecific recognition of single-stranded RNA motifs of 4–7 nucleotides<sup>24</sup>. As FMRP harbors two KH domains, one of which (KH2) binds with high affinity to an RNA ligand with both sequence and structural determinants<sup>25</sup>, coupled with reports of the association of FMRP with a subset of mRNAs<sup>26,27</sup> it seemed likely that FMRP bound to specific RNA targets through these motifs. The pursuit of this putative set of FMRP-specific mRNA targets has been reviewed elsewhere<sup>28</sup> but has recently culminated in surprising findings.

## The mRNA targets of FMRP

Recent studies have identified *in vivo* RNA binding sites for RNA binding proteins using UV irradiation to capture endogenous interactions by introducing a covalent crosslink between the protein and bound RNA<sup>29</sup>. After reducing the size of the bound RNA “tags” the RNABP:RNA complex can be stringently purified, usually by immunoprecipitation, and the small RNA ligands cloned and sequenced by high throughput methods. Application of this technique to capture RNA binding by FMRP in mouse brain was performed at P11-P25, a critical period for plasticity. This analysis identified a robust set of 842 FMRP target mRNAs, which were markedly enriched in both pre- and postsynaptic proteins<sup>16</sup>.

The postsynaptic targets of FMRP include about 30% of the postsynaptic density (PSD) proteome and include NMDA receptor subunits, the mGluR5 receptor, and many proteins that comprise the NMDA- and mGluR5-receptor interactomes<sup>16</sup>. For example, the NMDAR subunits NR1, 2A, 2B, and 3A, PSD-93 and PSD-95, SAPAP1-4, Shank1-3, Homer1, SynGAP1 and neuroligins 1-3 are FMRP targets. In contrast, FMRP does not appear to regulate expression of the AMPA receptor interactome. None of the 4 AMPAR subunits, none of the 8 stargazin/TARP/Cacng family, neither cornichon homolog (Cnih2/3), Shisa9/CKAMP-44, EphrinB2 nor the newly identified Gsg1L<sup>30</sup> is an FMRP target. This is one example of selective mRNA regulation by FMRP to control expression of specific complexes and pathways within neurons that may have important consequences for synaptic function.

Postsynaptic targets of FMRP were expected based on synaptic plasticity deficits and the EM localization of FMRP to dendrites; however, the observation that FMRP may also regulate as much as 30% of the pre-synaptic proteome was surprising. This includes the large scaffolding proteins bassoon and piccolo, the neurexin family of cell surface adhesion molecules, synapsins 1 and 2, the synaptic vesicle glycoproteins SV2A and SV2B, subunits of the clathrin-associated adaptor complex AP-2, synaptotagmins 1, 7, and 11, unc13, the syntaxin-binding proteins 1 (unc18) and 5 (tomosyn), SNAP-25, and many voltage-gated calcium channels. In addition, several kinesins, which deliver cargo to presynaptic terminals, are FMRP targets (Kif1a/b/c, Kif5a/c, Kif3 and Kif21a/b). A presynaptic role for FMRP (reviewed by<sup>31</sup>) is supported by recent studies in hippocampus<sup>32–34</sup>, cortex<sup>35</sup> and amygdala<sup>36</sup>. A need for rapid synthesis of presynaptic proteins for the formation and maintenance of new synapses has been elegantly described<sup>37</sup>. The recent report of enhanced vesicle recycling and enlarged readily-releasable and reserved vesicle pools found at CA3-CA1 synapses in *Fmr1* KO mice<sup>33</sup> is therefore consistent with the idea that FMRP might normally limit these processes by repressing presynaptic protein synthesis. The presence of FMRP in presynaptic or axonal FMRP-containing granules supports this model<sup>13,38</sup>.

## A role for a general translational increase in fragile X syndrome

Although FMRP directly regulates the translation of a specific subset of target mRNAs, several studies have demonstrated that secondary alterations in general translation may accompany the primary changes due directly to loss of FMRP association with specific mRNAs. In studies using rabbit reticulocyte lysate assays it was shown that FMRP caused a

dose-dependent inhibition of the translation of brain poly(A) RNA<sup>39,40</sup> and translation was increased in cortical synaptoneurosome preparations from *Fmr1* knockout mice as assayed by <sup>35</sup>S-methionine labeling<sup>41,42</sup>. These *in vitro* studies are supported by several types of protein synthesis assays in cell cultures from FXS patients and brain slices from *Fmr1* knockout mice<sup>41,43,44</sup>. Perhaps most importantly, *in vivo* studies with L-[1-<sup>14</sup>C] leucine have shown that *Fmr1* knockout mice exhibit increased rates of brain protein synthesis<sup>45</sup>.

It is impossible to ascertain whether the increased protein synthesis is due exclusively to release of translational repression on specific FMRP targets alone. However, the magnitude of the increased protein synthesis seems inconsistent with an exclusive translation of FMRP target mRNAs. FMRP target mRNAs represent about 5% of all mRNAs and are not particularly abundant; mRNAs for abundant housekeeping genes and ribosomal proteins are not generally associated with FMRP<sup>16</sup>. Moreover, FMRP is not expressed in mature glia and therefore should not directly affect protein synthesis in these cells, which may explain why increased protein synthesis is more apparent in areas of high neuronal cell body density relative to glia<sup>45</sup>. Thus, the increase in translation of specific FMRP target mRNAs due to its absence would have to be substantial to comprise 25% of all new protein synthesis.

Suggestions of specificity in increased protein synthesis do exist; radio-IP assays in labeled hippocampal slices have shown a 25% increase in target CaMKII $\alpha$  synthesis with no change in the non-target GAPDH<sup>46</sup>. However, once again the magnitude of this increase appears inadequate to explain the increased protein synthesis in *Fmr1* knockout mice and suggests that a global increase in translation due to long-term loss of FMRP may be a secondary outcome of loss of direct repression. In support of this possibility, no global increase in <sup>35</sup>S-methionine incorporation was observed due to *acute* loss of polysome association by all three FXRP family members using an RNA decoy approach despite specific increases in FMRP targets<sup>16</sup>. In addition, it is notable that FMRP normally represses expression of the NMDAR and mGluR5 receptors, scaffolding proteins, and several components of the downstream extracellular signal-regulated kinase (ERK) and mammalian target of rapamycin complex 1 (mTORC1) pathways, as well as several potentially limiting initiation and elongation factors. Excess production of these and other proteins in the absence of FMRP could lead to secondary increases in protein synthesis.

## Outcome of loss of translational repression

Once a high confidence list of FMRP mRNA targets and an equally robust list of non-targets controlled for length and neuronal abundance were identified it was possible to link mRNA binding with a functional outcome. Using a brain polyribosome-programmed *in vitro* translation system it was shown quantitatively that ribosome stalling occurs on FMRP target transcripts<sup>16</sup> (Figure 1). Two genetic models for FMRP loss-of-function and an acute loss-of-function model were used to demonstrate that absence of FMRP resulted in relief of ribosome stalling and increased synthesis of the target proteins. Thus, the simplest model predicting the outcome of loss of translational repression by FMRP *in vivo* is an increase in the synthesis of its specific mRNA targets resulting in higher than normal levels of the encoded proteins, although compensatory mechanisms such as turnover may restore normal levels of some. Because increased levels of proteins are not always pathogenic, it is

necessary to identify which of the many target proteins contribute to FXS pathogenesis. A logical first step is to use either Western blot or more global proteomic approaches to identify protein level changes in FXS models. Many such experiments have been performed with often-conflicting results, including reports of decreased levels of FMRP targets. In this section we review this data in light of the idea that an increase in global protein synthesis may accompany loss of FMRP.

Western blots have been used to detect increases in FMRP target proteins in its absence. Perhaps the best example of this is the FMRP target MAP1B. In *dfmr1*-null flies that have only one FMRP paralog a robust bidirectional regulation in MAP1B levels is evident due to loss or overexpression of *dfmr1*<sup>47</sup>. In mice, in which three potentially redundant paralogs exist, a 75% increase in MAP1B is observed in *Fmr1* knockout hippocampal slices<sup>10</sup> and a 30% increase is seen in total brain extract<sup>48</sup>. Increased levels of other FMRP target proteins also have been observed in mice including CaMKII $\alpha$ <sup>10,48</sup>, the protein tyrosine phosphatase STEP<sup>49</sup>, the potassium channels Kv3.1<sup>50</sup> and Kv4.2<sup>51</sup> and the upstream regulator of phosphoinositide 3 kinase (PI3K), PI3K enhancer (PIKE)<sup>52</sup>. Examination of 17 known PSD proteins in purified PSD preparations revealed many FMRP targets are increased at either 2 weeks or 2 months of age in either neocortex or hippocampus including SAPAPs1-3 (dlgap1-3), Shanks 1 and 3, and NMDA receptor subunits<sup>53</sup>. In human samples, the target CYFIP2 was increased in FXS patients relative to normal controls<sup>54</sup>.

However, in many cases steady state levels of FMRP target proteins appear to be unchanged in the absence of FMRP. Although this might be explained by the age or tissue examined (see examples in<sup>12,53</sup>), activation state, or increased protein turnover that offsets increased synthesis, recent examples of apparently *decreased* levels of FMRP targets (an 8–15% decrease in FMRP targets NR1, NR2A, NR2B, and SAPAP3<sup>55</sup>, a decrease in SYNGAP1 and NR2B<sup>12</sup>, as well as altered levels of non-targets further muddy the correlation between association of an mRNA with FMRP and increased protein synthesis in FMRP's absence. These findings underscore the importance of complementary proteomic approaches in understanding the functional outcome of FMRP loss-of-function.

## Functions encoded by FMRP-bound transcripts suggest new therapeutic targets

High throughput methods including HITS-CLIP have demonstrated that RNABPs can regulate functionally related sets of target RNAs in the mammalian nervous system<sup>29</sup>. Ingenuity functional pathway analysis suggests that this is true for the FMRP-regulated target transcripts as well<sup>16</sup>. Such analysis may be of special benefit considering that components of these pathways may be overexpressed in FXS, focusing efforts on their inhibition. Pathway analysis suggests that glutamate receptor signaling and synaptic plasticity might be impacted by loss of FMRP. These findings are consistent with and extend reports that in the absence of FMRP protein synthesis is exaggerated<sup>56</sup> that either genetic or pharmacologic inhibition of mGluR5 rescues phenotype in *Fmr1* knockout mice<sup>43,57</sup>, and that mGluR antagonists show evidence of clinical efficacy<sup>58</sup>. In addition to mGluR antagonists, the identification of the NMDAR pathway as an FMRP target is significant as

NMDAR antagonists are available that might be considered for clinical evaluation as either single agents or in conjunction with mGluR5 antagonists.

mGluRs and NMDARs mediate changes in synaptic plasticity by signaling through the PI3K/Akt/mTORC1 and MEK/ERK pathways to increase cap-dependent translation<sup>59</sup>. Recent data suggests that FMRP may also regulate the activity of these translational control pathways directly. For example, the 6<sup>th</sup> ranked FMRP target is PIKE also known as centaurin-gamma1), which is overexpressed in the *Fmr1* knockout mouse, resulting in elevated PI3K signaling to the mTORC1 pathway<sup>52</sup>. Moreover, FMRP target mRNAs that encode phosphatase and tensin homolog deleted from chromosome 10 (PTEN), neurofibromatosis 1 (NF1), and tuberlin (TSC2), all of which negatively regulate mTORC1 signaling, suggest a critical role for FMRP in regulating the balance of cap-dependent translation. In addition, ERK1, all three eIF4G isoforms, eEF2 and eEF1, Ago1/2 and Dicer are FMRP targets. Therefore, it is possible that loss of FMRP may have secondary effects on neuronal translation. As will be discussed below, these findings add support to the idea that pharmacologic agents that act on the mTORC1 and ERK pathways may be worth considering therapeutically<sup>60,61</sup>.

FMRP target mRNAs also encode proteins involved in the regulation of cAMP levels, including adenylate cyclases and phosphodiesterases<sup>16</sup>. These findings are consistent with observations that *dfmr1*-null flies, *Fmr1* knockout mice<sup>62</sup> and human patients<sup>63</sup> have altered basal or stimulated cAMP levels. In addition, a large number of proteins regulating small GTPases are targeted by FMRP, including at least 11 GTPase activating proteins (GAPs) and 12 guanine nucleotide exchange factors (GEFs). The striking enrichment of these proteins as FMRP targets is significant as some have been found to regulate the actin cytoskeleton in an activity-dependent manner in neurons<sup>64</sup> and perturbing their function alters dendritic spine morphology and is linked with neurologic disease<sup>65</sup>. A prominent example is the target SYNGAP1, which is stimulated by NMDARs to regulate ERK<sup>66</sup> and is both a cause of non-syndromic mental retardation and strongly linked to autism<sup>67</sup>.

## Receptors that regulate FMRP and their potential as therapeutics for FXS

Experiments examining synaptic plasticity in *Fmr1* knockout mice were the first to identify potential molecular targets for therapeutics to treat individuals with FXS. The initial studies largely focused on protein synthesis-dependent forms of long-lasting synaptic plasticity because of the role of FMRP as a translational repressor. The first breakthrough was the finding that hippocampal metabotropic glutamate receptor-dependent long-term depression (mGluR-LTD), which requires dendritic protein synthesis<sup>68</sup>, is enhanced in *Fmr1* knockout mice<sup>69</sup>. This gave rise to the "mGluR theory"<sup>56</sup>, which posited that abnormal synaptic function and aberrant behavior in FXS was due to exaggerated mGluR-dependent protein synthesis. Consistent with this theory, developmental defects and aberrant behavior in *dfmr1* flies were reversed by the mGluR5 antagonist MPEP<sup>70</sup>. Moreover, genetic reduction of mGluR5 was shown to correct abnormal dendritic spine morphology, exaggerated protein synthesis, enhanced mGluR-LTD, and behavioral phenotypes displayed by *Fmr1* knockout mice<sup>43</sup>. More recently, it was shown that acute treatment of young adult *Fmr1* knockout mice with the selective mGluR5 inhibitor CTEP can reverse exaggerated protein synthesis,

enhanced mGluR-LTD, and susceptibility to audiogenic seizures and chronic treatment reverses abnormal dendritic spine morphology and cognitive deficits<sup>57</sup>. These studies are particularly important when considering therapeutic treatments for FXS individuals because CTEP corrected FXS-associated phenotypes after their onset. There are numerous other examples demonstrating that blocking group I mGluRs can correct a broad array of neuronal, synaptic, and behavioral phenotypes associated with FXS<sup>2</sup>.

*Fmr1* knockout mice also have impaired  $\gamma$ -aminobutyric acid (GABA) receptor function that is likely caused by decreased expression of GABA<sub>A</sub> receptor subunits and/or enzymes that synthesize GABA<sup>71-73</sup>, suggesting that inhibitory synaptic transmission is decreased in FXS. Indeed, in *dfmr1* null flies, GABA treatment rescued multiple phenotypes, including abnormal behavior<sup>74</sup>, and it was demonstrated recently that GABA<sub>A</sub> receptor agonists reduce audiogenic seizures in *Fmr1* knockout mice<sup>75</sup>. Moreover, exaggerated protein synthesis, susceptibility to audiogenic seizures, and repetitive behavior are normalized in *Fmr1* knockout mice treated with arbaclofen, a GABA<sub>B</sub> receptor agonist<sup>76</sup>. These studies are particularly intriguing because they have been translated to FXS individuals, where arbaclofen improved social avoidance, one of the main clinical symptoms of the syndrome<sup>77</sup>. It remains to be determined whether GABA<sub>B</sub> receptor agonists can reverse FXS-associated phenotypes via the dampening of excessive mGluR5-mediated protein synthesis.

The therapeutic targets for FXS mentioned above are neurotransmitter receptors, which are the most common targets for the treatment neurological and psychiatric disorders. The advantage of targeting neurotransmitter receptors is that there are likely to be fewer peripheral side effects in individuals treated with the compounds. However, G-protein coupled receptors such as group I mGluRs regulate a plethora of downstream neuronal signaling pathways in the brain, and thus, receptor-targeted compounds could inhibit pathways that are not impacted in FXS individuals. Recent studies have begun to delineate the signaling pathways that couple group I mGluRs to the translation machinery, providing a new set of potential therapeutic targets for treatment of FXS.

## Translational control pathways and their potential as therapeutics for FXS

Two protein kinase signaling pathways that regulate translation initiation, mTORC1 and ERK, have been implicated in the pathophysiology of FXS. mTORC1 phosphorylates eIF4E-binding protein (4E-BP) and p70 S6 kinase 1 (S6K1), both of which bind Raptor<sup>78</sup>. Unphosphorylated 4E-BPs bind tightly to eIF4E, whereas 4E-BPs phosphorylated by mTORC1 do not, thereby permitting eIF4E to bind eIF4G to form the eIF4F initiation complex allowing initiation to proceed. mTORC1 also impacts initiation by phosphorylating S6K1, which then phosphorylates downstream targets such as ribosomal protein S6 and eIF4B. Importantly, phosphorylation of eIF4B by S6K1 increases the helicase activity of eIF4A, as does the interaction of eIF4E with eIF4G. ERK phosphorylates the kinase Mnk1 that subsequently phosphorylates eIF4E, which is correlated with enhanced translation (Figure 2a<sup>79,80</sup>).

It was reported that *Fmr1* knockout mice exhibit increased eIF4E-eIF4G interactions and phosphorylation of S6K1 in the hippocampus<sup>52</sup>, which is indicative of excessive mTORC1 signaling. What is the upstream signaling that couples mGluR5 to mTORC1? It appears to involve disrupted Homer scaffolds and increased PI3K signaling. In *Fmr1* knockout mice mGluR5 was less associated with long Homer isoforms and more associated with the shorter Homer1a<sup>81</sup>. In addition, genetic deletion of Homer1a in *Fmr1* knockout mice reduced increased eIF4F complex formation, exaggerated protein synthesis, and susceptibility to audiogenic seizures<sup>81</sup>. Interestingly, Homer interacts with PIKE, which, as mentioned above, is an FMRP target mRNA<sup>16</sup> and whose protein expression is increased in *Fmr1* knockout mice<sup>41,52</sup>. Moreover, the expression of the p110 $\beta$  subunit of PI3K, which also is an FMRP target mRNA<sup>16</sup> is elevated in *Fmr1* knockout mice. Inhibitors of PI3K corrected exaggerated protein synthesis and abnormal dendritic spine morphology in *Fmr1* knockout mice<sup>41</sup> as well as exaggerated protein synthesis in lymphoblastoid cells from FXS patients<sup>42</sup>. All together, these findings indicate that disrupted Homer scaffolds and excessive PI3K signaling contribute to exaggerated protein synthesis, as well as molecular, cellular, and behavioral phenotypes in FXS.

Because PI3K is typically an upstream regulator of mTORC1<sup>60</sup>, one might predict that disruption of mTORC1 would correct multiple aspects of FXS. However, acute treatment with rapamycin to disrupt mTORC1 does not correct exaggerated protein synthesis and audiogenic seizures displayed by *Fmr1* knockout mice<sup>46</sup>. In contrast, it recently was demonstrated that genetic deletion of S6K1 can correct exaggerated protein synthesis, enhanced mGluR-LTD, and several behavioral phenotypes displayed by *Fmr1* knockout mice<sup>44</sup>. How can one reconcile these seemingly contradictory findings? A simple explanation is that chronic treatments of *Fmr1* knockout mice with rapamycin are required to reduce exaggerated protein synthesis. Because both PIKE and the p110 $\beta$  subunit of PI3K are elevated in *Fmr1* knockout mice, it is likely that PIKE/PI3K triggers the excessive activation of PDK1/2, and subsequently mTORC1 (Figure 2). However, in addition to mTORC1, S6K1 receives direct stimulatory input from PDK1<sup>82</sup>, as well as ERK<sup>79</sup>. Thus, S6K1 activity could be stimulated by either PDK1 or ERK, the former being consistent with the observation of elevated PIKE/p110 $\beta$  levels in FXS mice<sup>52</sup> and latter being consistent with the observation that hypersensitivity to ERK contributes to exaggerated protein synthesis and audiogenic seizures in *Fmr1* knockout mice<sup>46</sup>. Moreover, lovastatin, which interferes with Ras activation, recently was shown to prevent ERK activation, exaggerated protein synthesis, and susceptibility to audiogenic seizures in *Fmr1* knockout mice<sup>83</sup>. Thus, S6K1 is a potential integrator of signaling from both increased levels of PI3K and hypersensitivity to ERK in FXS (Figure 2b).

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder caused by mutations in either hamartin (TSC1) or TSC2<sup>84</sup>. Because inactivation of TSC1/2 upregulates mTORC1 signaling, one would predict that TSC model mice, similar to *Fmr1* knockout mice, would exhibit enhanced translation. However, it was shown that TSC2 mutant mice have decreased translation in the hippocampus that can be blocked by rapamycin<sup>85</sup>, which is likely due to feedback inhibition of pathways upstream of mTORC1<sup>60</sup> and/or upregulation of the unfolded protein response<sup>86</sup>. Moreover, the TSC model mice, in contrast to *Fmr1* knockout



mice, displayed impaired mGluR-LTD. Finally, when TSC model mice were bred with *Fmr1* knockout mice, protein synthesis, mGluR-LTD, and cognitive phenotypes that were present in both lines of mutant mice were normalized<sup>85</sup>. As mentioned earlier, TSC2 mRNA is a target of FMRP<sup>16</sup>, so it is possible that TSC2 protein expression is altered in *Fmr1* knockout mice. An additional unknown is the molecular signaling responsible for the normalization of the phenotypes displayed by the TSC/FXS double mutant mice.

The mRNAs that encode PIKE, PI3K, and TSC2 are all associated with FMRP and thus, their protein products are potential therapeutic targets for FXS (Figure 2b). Other signaling molecules that may be targets include striatal-enriched protein tyrosine phosphatase (STEP)<sup>49</sup> and GSK3 $\beta$ <sup>87,88</sup> whose transcripts are also FMRP targets<sup>16</sup>. Thus, downstream signaling pathways that are impacted by the reduction of STEP and GSK3 $\beta$  and whether they interact with the mTORC1 and ERK pathways in FXS remains to be elucidated.

The idea that the lack of FMRP results in exaggerated mGluR-dependent protein synthesis is the leading theory for the molecular basis of FXS, and both genetic reduction<sup>43</sup> and pharmacological inhibition of mGluR5<sup>57</sup> reduced exaggerated protein synthesis and a broad range of phenotypes displayed by *Fmr1* knockout mice. However, it also was reported that mGluR-LTD is protein synthesis-*independent* in *Fmr1* knockout mice<sup>9,10</sup>. Moreover, activation of group 1 mGluRs is uncoupled from the activation of PI3K<sup>41,89</sup>, mTORC1<sup>52,81,89</sup>, and ERK<sup>10</sup>, the translation of FMRP target mRNAs, including MAP1B<sup>10</sup>, p110 $\beta$ <sup>41</sup>, and STEP<sup>49</sup>, the translation of Arc<sup>81</sup>, and general protein synthesis<sup>41,46</sup>. These findings suggest that the loss of FMRP limits the dynamic range for mGluR-dependent protein synthesis and that dampening mGluR-dependent translational control, via either an mGluR antagonist or by targeting downstream effectors such as PI3K and S6K1 is required to reset translational homeostasis in *Fmr1* knockout mice (Figure 2b).

## Novel therapy for fragile X syndrome directed at its molecular function rather than upstream or downstream targets

Although targeting signaling pathways has the advantage of being downstream of neurotransmitter receptors, many of these kinases, including PI3K and ERK, are still far enough upstream from the translational machinery that their inhibition could impact signaling cascades unaffected in FXS. Thus, if excessive translation of FMRP target mRNAs and/or exaggerated general protein synthesis is the causative factor in FXS, then the direct targeting of the translational machinery might be the most effective way to reset translational homeostasis. Evidence for the efficacy of this approach was first provided by studies in *dfrm1*-null flies, which showed that the general protein synthesis inhibitors cycloheximide and puromycin rescue impaired long-term memory<sup>23</sup>. Thus, direct targeting of the translational machinery may be a means for reversing abnormal behaviors associated with FXS.

There is speculation that exaggerated translation is causative not only for FXS, but also for other ASDs<sup>60,61</sup>, and there is evidence for a genetic link between ASD and the cap-binding translation initiation factor eIF4E<sup>90</sup>. Moreover, microdeletions in the Prader-Willi/Angelman critical region contain the gene for CYFIP1, which encodes a non-canonical

eIF4E-binding protein that also binds to FMRP<sup>91</sup>. Importantly, it recently was shown that transgenic mice overexpressing eIF4E exhibit increased eIF4F complex formation, exaggerated protein synthesis, enhanced mGluR-LTD in the striatum and hippocampus, and aberrant autistic-like behaviors, all of which were reversed by 4EGI-1, an inhibitor of eIF4E-eIF4G interactions<sup>92</sup>. These findings have provided direct evidence for the idea that exaggerated protein synthesis can cause synaptic and behavioral aberrations associated with ASD. Moreover, because *Fmr1* knockout mice exhibit increased eIF4E-eIF4G interactions<sup>52,81</sup>, these findings suggest that exaggerated protein synthesis, enhanced mGluR-LTD, and FXS-associated behaviors in *Fmr1* knockout mice might be reversible by 4EGI-1.

As discussed above, FMRP functions to stall ribosomal translocation on its target mRNAs, suggesting that compounds that inhibit translation elongation might alleviate phenotypes associated with the loss of FMRP (Figure 1). Many small molecules, primarily used as antibiotics because they stall the translocation of bacterial ribosomes, already have been tested in relevant assays. Interestingly, minocycline, a second generation tetracycline analog, reversed abnormal synaptic structure, spine morphology, and aberrant behaviors exhibited by *Fmr1* knockout mice<sup>93</sup> and *dfmr1* null flies<sup>94</sup>. Minocycline was tested in these models because it inhibits matrix metalloproteinase-9 (MMP-9), which is overexpressed in *Fmr1* KO mice<sup>93</sup> and flies<sup>94</sup>. However, one has to wonder whether all or part of the effect of minocycline is due to its ability to slow down ribosomes, taking the edge off excess translation as previously suggested<sup>16</sup>. Minocycline is an antibiotic used in humans that is quite lipid-soluble and crosses the blood brain barrier well<sup>95</sup>. Minocycline and other tetracyclines inhibit protein synthesis via A-site occupation and stall mammalian ribosomes with 7–20% of the efficacy with which they inhibit bacterial translation<sup>96</sup>. Preliminary open-label add-on clinical trials with minocycline have shown positive results for cognitive and behavioral measures in 20 FXS patients<sup>97</sup>, which warrants further research on minocycline and other inhibitors of ribosomal elongation.

## Summary and Conclusions

Knowledge concerning the function of FMRP and how its loss alters receptor-mediated signaling and translational control leading to aberrant dendritic spine dynamics, synaptic plasticity, circuit function, and complex behavior in FXS has expanded exponentially in the last decade. Nonetheless, questions central to the development of therapeutics for treating individuals with FXS remain unanswered. Traditional methods of drug discovery for brain disorders focus on neurotransmitter receptors with good reason, and there is considerable optimism that pharmaceuticals targeting metabotropic glutamate receptors and GABA receptors will provide benefits for FXS patients. In addition, the identification of the mRNA targets of FMRP that encode synaptic proteins and components of signaling pathways involved translational control, many of which overlap with autism and other neurodevelopmental disorders, have provided a rich list of potential therapeutic targets for FXS. However, this plethora of targets implies that one should be very cautious in inferring the normal function of FMRP from studying the chronic loss-of-function state in *Fmr1* mutant animals and cell lines from individuals with FXS. We argue that the main function of FMRP is to regulate protein synthesis and as such, FXS should be considered a disease of

dysregulated translation. Finally, because the lack of FMRP appears to alter the expression of a multitude of proteins involved in translational control and synaptic function, we propose that a rational approach for effective treatment of FXS would be to reset translational homeostasis by targeting initiation and/or elongation, thereby translating translation to the clinic.

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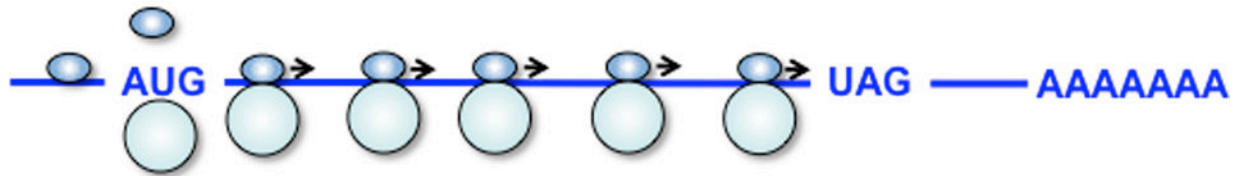
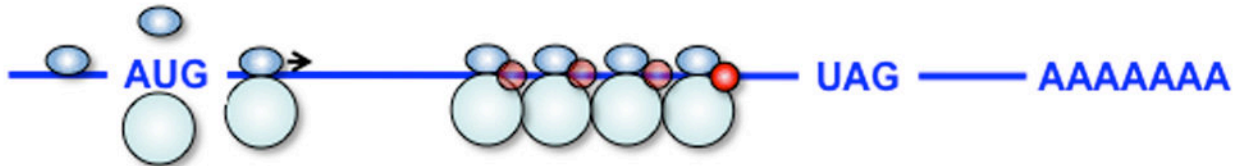
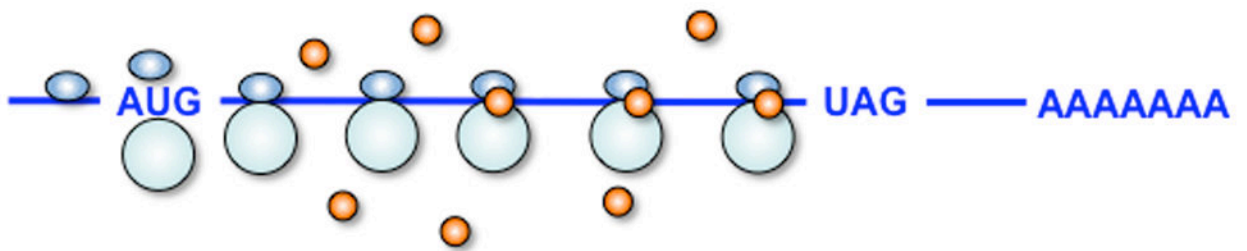
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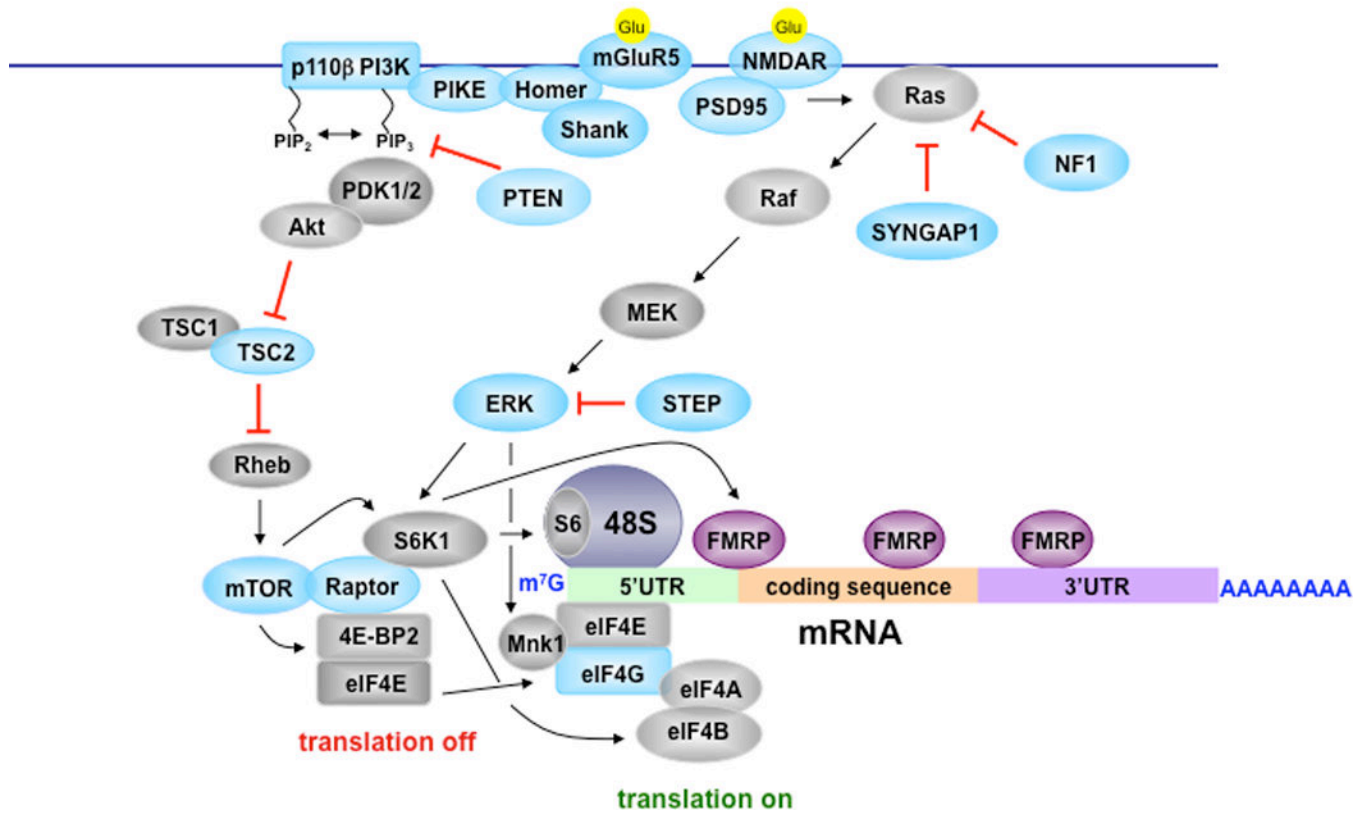
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**active translation****repressed translation by FMRP** ●**translational repression restored by antibiotics** ●  
(minocycline)**Figure 1. FMRP and small molecules act to stall ribosomal elongation**

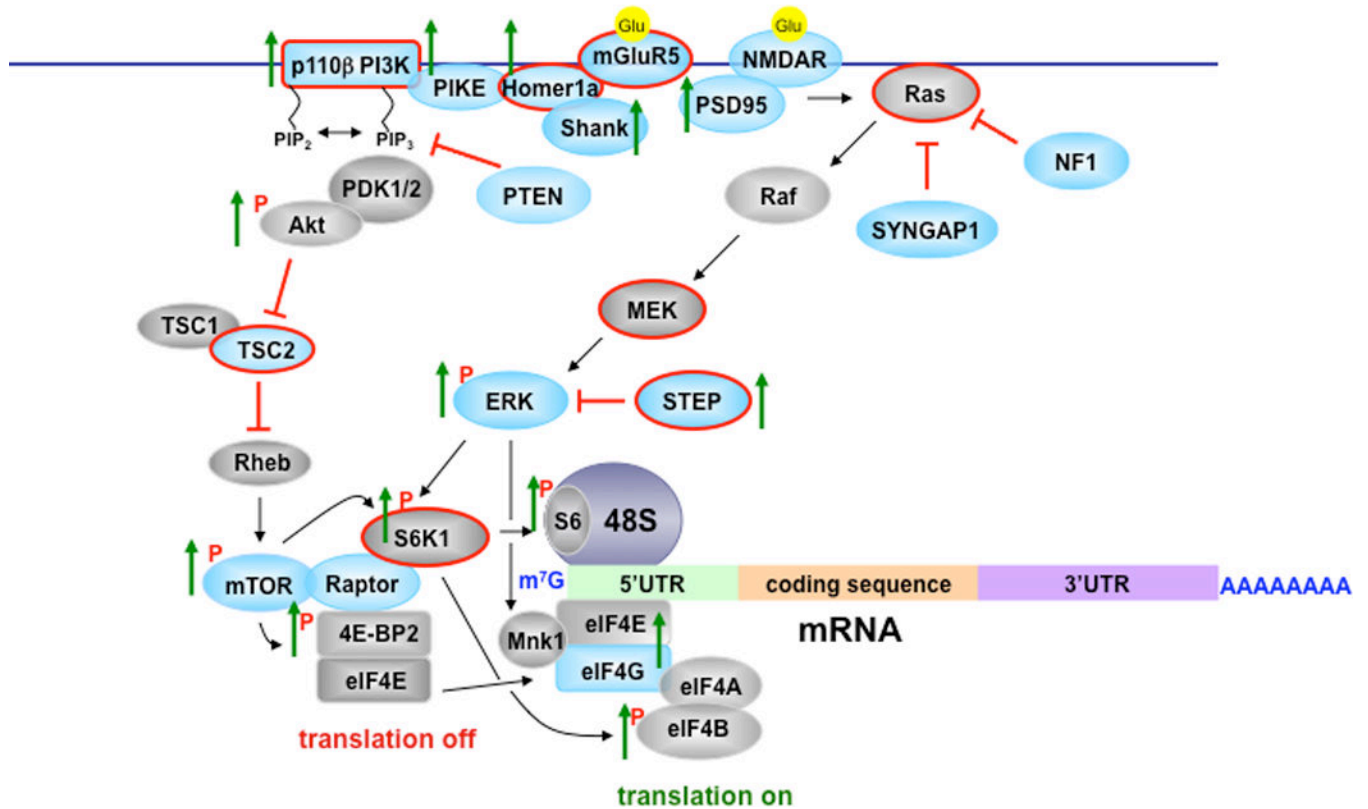
Upper panel, active translation: mRNAs are translated into protein by translocating ribosomes (40S and 60S subunits are shown in light blue) that assemble at the start codon (AUG) and dissociate at the stop codon (for example, UAG). Middle panel, FMRP-repressed translation: FMRP inhibits ribosomal translocation on specific mRNAs in a complex consisting of target mRNA and several stacked or condensed ribosomes. As the stoichiometry of FMRP to ribosomes to target mRNAs is not known, a minimum of one FMRP molecule (red sphere) is depicted in the stalled complex, recognizing the possibility that additional FMRP molecules (illustrated by transparent red spheres) may exist in the stalled complex. Lower panel, minocycline-repressed translation: Minocycline, a tetracycline analog, (orange spheres) and many other small molecules inhibit translation by interfering with ribosomal translocation in different ways. Because of the similarity in proposed action between FMRP and such small molecules it is possible that they might partially replace FMRP functions lost in fragile X syndrome and be therapeutically beneficial.



**a**



b



**Figure 2. Signaling Pathways Controlling Translation in Normal and FXS Model Mice**

**A)** Translational control in neurons of normal mice. Metabotropic glutamate receptor 5 (mGluR5) and NMDA receptors are coupled to PI3K/Akt/mTORC1 and MEK/ERK signaling to regulate cap-dependent translation (for detailed reviews see<sup>59–61,78–80</sup>). Blue colored proteins are those whose mRNAs are FMRP targets. **B)** Translational control in neurons of FXS model mice that lack FMRP. Green arrows indicate either total or phosphorylated levels (indicated by red P) of proteins that have been reported to increase in either FXS model mice or cells from FXS individuals<sup>41,42,44,48,49,52,54,81</sup>. Proteins with red lines around them indicate those that have been successfully targeted either pharmacologically or genetically to reverse molecular, morphological, synaptic, and/or behavioral phenotypes in cellular and/or animal models of FXS<sup>41–44,46,49,52,57,70,74,81,83</sup>.