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Multifarious Functions of the Fragile X Mental Retardation Protein

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Abstract

Fragile X syndrome (FXS), a heritable intellectual and autism spectrum disorder, results from loss of Fragile X Mental Retardation Protein (FMRP). This neurodevelopmental disease state exhibits neural circuit hyperconnectivity and hyperexcitability. Canonically, FMRP functions as an mRNA-binding translation suppressor, but recent findings have enormously expanded proposed roles. Although connections between burgeoning FMRP functions remain unknown, recent advances have extended understanding of involvement in RNA-, channel- and protein-binding that modulates calcium signaling, activity-dependent critical period development and excitation-inhibition neural circuitry balance. This article contextualizes three years of FXS model research. Future directions extrapolated from recent advances focus on discovering links between FMRP roles; to determine whether FMRP has a multitude of unrelated functions, or combinatorial mechanisms can explain its multifaceted existence.

Keywords

Fragile X Syndrome (FXS); Autism Spectrum Disorder (ASD); Synapse; Translation Regulation; Activity-Dependent Critical Period

Overview of Expanding FMRP Functions

Fragile X syndrome (FXS), a common genetic root of both intellectual and autism spectrum disorders, is usually caused by a 5'UTR trinucleotide repeat expansion in the FMR1 gene, resulting in loss of the **Fragile X Mental Retardation Protein (FMRP)**. FMRP functions as a master regulator of activity-dependent neurodevelopment, with null mutants manifesting hyperexcitability and reduced activity-dependent modulation of synapse maturation, refinement and plasticity [1]. FMRP is canonically defined as an mRNA-binding translational repressor, with a broad but largely indeterminate range of transcript targets [2], but the scope of FMRP genetic functions continues to explode (Table

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1). From the cytosol, FMRP is classically described to shuttle to/from the nucleus, with a recent *Drosophila* study mapping a novel C-terminus mutation to this nuclear export function [3]. In the nucleus, recent work indicates that FMRP binds chromatin through tandem Tudor (Agenet) domains during the **DNA damage response (DDR)** to regulate genome stability in mice (Table 1) [4]. The involvement with DDR machinery is shown to be important during spermatogenesis, but a requirement in neurodevelopment has not been established. In the *Drosophila* FXS model, FMRP chromatin-binding function mediates replication stress induced H2av phosphorylation, one of the earliest DDR responses to double strand breaks and replication stress [5], but a requirement in neurodevelopment has also not been shown. Downstream of DNA interactions, but prior to canonical translational regulation roles, FMRP is proposed to act at multiple RNA life stages: in mRNA editing, pre-mRNA splicing, and in the microRNA pathway (Table 1). Recent work from zebrafish and mouse FXS models shows FMRP alters RNA-editing via interaction with the **adenosine deaminase ADAR**, supporting earlier *Drosophila* studies (Table 1) [6,7]. In the mouse FXS model, FMRP also works with RNA-binding protein 14 (RBM14) in **pre-mRNA alternative splicing** (Table 1) [8]. FMRP has long been associated with the microRNA pathway, and recent studies suggest key interactions in circuit plasticity and behavioral output in *Drosophila* (Table 1) [9,10]. In both canonical and newly discovered RNA-binding functions of FMRP, the mechanism of FMRP mRNA binding specificity has long been a conundrum, but recent insights into mRNA diversification provide further possible means for FMRP to recognize and regulate target transcripts [11,12].

The FXS field is rife with debates about FMRP roles, including cellular locations, temporal timing and FMRP functions beyond DNA/RNA regulation. A long-term question concerns roles in neurons versus glia. A recent study of human patients shows FMR1 epigenetic alterations silence FMRP specifically in neurons, but not glia or neurons obtained from reprogrammed pluripotent stem cells [13]. In the mouse FXS model, recent work shows FMRP loss changes cell differentiation kinetics for both neurons and glia (Table 1) [14], and astrocyte-specific FMRP knockout in mice increases neuronal dendritic spine density similar to the global FXS condition [15]. Within neurons in all model systems, the soma contains the vast majority of FMRP, yet the lion's share of research and discussion focuses on local FMRP functions at the synapse [2,16–19]. Most study postsynaptic mechanisms, but there appears to be at least as many presynaptic mRNA targets and presynaptic defects in mutants [2,16,20,21]. Yet another long-term debate concerns the timing of FMRP requirements [2]. Although FXS defects persist throughout life in both patients and animal models, this does not necessarily require continuous, maintained FMRP function [22–24]. Indeed, peak FMRP levels in both mouse and *Drosophila* FXS models occur during development, and prominent defects restricted to critical period neural circuit refinement may drive mature dysfunction [1,9,25–27]. Finally, the central role of FMRP mRNA-binding itself has been strongly challenged. In addition to the wide range of newly-discovered FMRP functions discussed above, including nuclear roles such as chromatin-binding, mRNA splicing and mRNA editing (Table 1) [9,28], FMRP shows direct ion channel binding modulating pore conductivity properties, and it is argued that many core FXS neurological defects may be explained by channel-binding alone [20]. These two widely separated biological roles could represent completely divergent FMRP functions in neurons (Fig. 1). Alternatively, FMRP

RNA- and channel-binding functions could be linked in a common mechanism controlling activity-dependent protein synthesis (Fig. 1).

This article provides an update on major advances in the FXS field over the past three years, and has been divided into five parts grouped by FMRP biological functions. While FXS phenotypes present similarly in different genetic models, we carefully define the animal system used in each body of work, and note when any contradictory findings have been found between models or with human patients. Part I addresses recent progress on FMRP RNA-binding roles, predominantly in the suppression of protein translation, although translational activation has also been documented in some cases. We discuss all the newly identified FMRP mRNA targets, involved in a diverse range of biological functions including neuronal signal transduction, internal cellular architecture and intercellular signaling mechanisms. New FMRP roles are summarized in Table 1, new mRNA targets of FMRP canonical function in translationally regulation are shown in Table 2, and protein partners recently identified to interact with FMRP in translation control are shown in Table 3. We also briefly discuss new advances in non-mRNA binding translational regulation mediated by FMRP. Part II addresses recent progress on FMRP channel-binding roles, primarily in the control of neuronal excitability. We also discuss calcium-signaling mechanisms, downstream of direct channel-binding as well as indirect FMRP regulation. Calcium signaling functions highlight the role of FMRP as a translation activator in some contexts. Part III addresses new insights into FMRP synaptic development roles, including signaling, structural and functional requirements. We also discuss the activity-dependent regulation of critical period synaptic remodeling during the refinement of neural circuitry. Part IV briefly addresses excitatory/inhibitory (E/I) imbalance in the FXS disease state. We discuss recent advances in understanding FMRP roles regulating the balance between strengthened excitatory and repressed inhibitory connections in model neural circuits. Finally, in part V, we highlight important future directions for FXS research. We discuss logical extensions of the above ongoing work, and also emphasize key objectives that fall outside the scope of these areas.

Part I: New Progress in RNA-Binding/Translation Suppression Mechanisms

The canonical FMRP role is direct mRNA-binding translation suppression (Fig. 1), although FMRP has long been associated with mRNA throughout its lifecycle, including splicing, editing, trafficking and stability [6,11,12,29,30]. FMRP selectively associates with a subset of mRNAs: the candidate target list is long, but altered protein levels have been established for only a tiny handful of proteins (Table 2) [29,31]. Indeed, proteomic screens suggest that the number of protein changes in both mouse and *Drosophila* FXS models is surprisingly small, and many of these changes may be indirect, since they don't align well with mRNA-binding data [32]. Given synaptic defects in FXS patients and models, there is a particular bias towards mRNA targets encoding synaptic proteins [33]. However, it is clear that FMRP also binds many non-synaptic transcripts, and that translational regulation of most synaptic transcripts does not occur exclusively (or even predominantly) locally at synapses [5,12,30]. Although many mechanisms have been proposed to explain the RNA-binding specificity of FMRP, including both secondary structure (G-quartet) and consensus nucleotide sequences [34], there is little compelling evidence for any universal mechanism [35]. Therefore, it

remains unclear by which mechanism(s) FMRP specifically binds target mRNAs, or which mRNAs contribute predominantly to FXS neuropathology. The basis of inherent RNA-binding specificity, or perhaps lack thereof, is an outstanding question in need of continued investigation. Here, we focus only on new FMRP mRNA targets and translational control mechanisms identified over the past three years (Tables 2,3).

FMRP represses translation via interaction with translation machinery, including RNA-binding proteins and microRNA components. A newly-defined FMRP partner is the ribosomal L5 protein (Table 3) [36]. In *Drosophila*, FMRP-L5 binding prevents tRNA and elongation factor ribosome association to suppress translation. A recent study in human FXS patient cells suggests translation repression also arises from FMR1 5'UTR CGG binding to **activity-regulated cytoskeleton associated protein (ARC)** transcripts in mobile ribosome granules, inhibiting mRNA localization/translation (Table 2) [29,37,38]. In mice, telomere repeat-binding factor 2 (TRF2-S) binding to target mRNAs facilitates axonal delivery, which is antagonized by FMRP binding the TRF2-S GAR domain [39]. FMRP knockdown promotes mRNA localization to enhance growth and presynaptic transmitter release (Table 3). FMRP has long been known to partner with multiple other RNA-binding proteins (e.g. Staufen, Pumilio, Lark and many others; Table 3), which may provide mRNA-binding specificity and/or coupled regulation of joint targets [40–43]. This co-regulation has striking effects on neural circuit plasticity. For example, it was recently shown in *Drosophila* that FMRP partners with mRNA-binding Ataxin-2 in the microRNA-dependent plasticity mechanism of **long-term olfactory habituation (LTH)** [10]. FMRP, Ataxin-2 or microRNA component knockdown elevates CAMKII translation reporter activity during LTH. FMRP and Ataxin-2 both display transdominant genetic interactions with two **microRNA-mediated translational suppression** proteins, RNA-induced silencing complex (RISC) component Ago1 and deadbox helicase me31B, required for **long-term central adaptation (LTCA)** from odorant exposure [9,10].

FMRP has long been known to regulate core signal transduction pathways through multiple mechanisms. A recent mouse study establishes a newly-defined FMRP mRNA target for **diacylglycerol (DAG) kinase**, which converts DAG to phosphatidic acid (PA) during lipid signaling (Table 2). Loss of DAG kinase translation repression by FMRP of causes postsynaptic signaling and spine defects [33,44]. FMRP also binds signaling proteins, including Janus kinase and microtubule interacting protein 1 (JAKMIP1) and **cytoplasmic FMR1 interaction protein 1 (CYFIP1**; Table 3). A recent gene expression study shows JAKMIP1 modulates microtubule transport and $GABA_BR$ levels [32]. WAVE complex CYFIP1 links FMRP to actin regulation, and mechanistic target of rapamycin (mTor) signaling in both mice and *Drosophila* [45]. A FMRP/CYFIP1/Staufen complex recruited by **TAR DNA binding protein 43kDa (TDP-43)** represses the translation of Ras-related C3 botulinum toxin substrate 1 (Rac1), microtubule-associated protein 1B (MAP1B) and glutamate receptor 1 (GluR1) to limit spinogenesis in mice and human cell lines (Table 2) [34,40]. Downstream of CYFIP1, the mTor target ribosomal protein s6 is hyperphosphorylated by p90-ribosomal S6 kinase (RSK), downstream of both **extracellular signal-regulated kinase (ERK)** and phosphatidylinositide 3 kinase (PI3K) signaling elevated in FXS model mice [46,47]. A recent study found Acamprosate decreases elevated

ERK signaling to attenuate functional and behavioral defects [48]. Finally, in mice FMRP also binds **adenylyl cyclase type 1 (ADCY1)** mRNA to suppress translation, and elevated ADCY1 expression in the FXS condition also activates the above pathway to promote postsynaptic spine overgrowth (Table 2) [47].

In addition to core signal transduction and cytoskeletal regulation within the cell, FMRP also binds mRNA targets encoding proteins involved in intercellular interactions. Recent work shows FMRP binds **Down syndrome cell adhesion molecule (DSCAM)** mRNA to suppress translation in *Drosophila* (Table 2) [49]. In the *Drosophila* FXS model, enlarged synaptic termini are associated with DSCAM misregulation via an Abelson kinase 1 (Ab1) mechanism, and removal of just one Ab1 gene copy restores synapse structure in the disease state [49]. In the same *Drosophila* model, FMRP binds eukaryotic elongation factor 1 (EF1α) mRNA to suppress translation (Table 2) [50]. Increased EF1α levels in the FXS condition causes elevated nuclear localization of hyperphosphorylated ubiquitin ligase murine double minute-2 (Mdm2), which is resistant to myocyte enhancer factor 2 (MEF2) control (Table 1). When activated by MEF2, Mdm2 normally disassembles synaptic scaffolds to limit excitatory synapse number, so this defect drives FXS hyperexcitability [50]. In both *Drosophila* and mouse models, FMRP binds mRNA encoding **bone morphogenic protein receptor type II (BMPR2)**, which activates LIM-domain kinase 1 (LIMK1) to inhibit actin-binding Cofilin and cause actin reorganization that promotes synaptogenesis in the FXS condition (Table 2) [35]. Genetic or pharmacological correction of BMPR2-LIMK signaling rectifies synaptic and movement defects in the *Drosophila* FXS model [51]. The FMRP C-terminal domain (CTD) binds BMPR2 mRNA, highlighting the question of which RNA-binding domains mediate different interactions, and whether they act alone or in combination.

Part II: New Progress in Channel-Binding and Calcium Signaling Mechanisms

In addition to translation roles, FMRP directly binds ion channels (Fig. 1), and modulates Ca^{2+} signaling to control neural activity [1]. FMRP binds multiple classes of K^+ channels, including Na^+-activated Slack [16] and Ca^{2+}-activated Slowpoke (Slo) BK channels [17]. FMRP-Slack binding regulates gating to shape action potential kinetics, especially during high-frequency activity. FMRP binding to presynaptic BK channels modulates Ca^{2+} influx and neurotransmitter release (Fig. 1). In addition to direct channel binding, FMRP controls channel levels by translational regulation, including repression of Kv3.1b K^+ channels in mice and activation of L-type Ca^{2+} channels in human cells (Table 2) [52,53]. Contradictory studies report Kv4.2 K^+ channels are translationally repressed [54] or activated [55] by FMRP in mice (Table 2). FMRP indirectly controls presynaptic N-type Ca^{2+} channel levels ($Ca_v2.2$) via proteasomal degradation in human cells [18]. Downstream of channels, FMRP also regulates calcium binding protein (CaBP) function, through both RNA- and protein-binding mechanisms [10,29,56,57]. Moreover, the *Drosophila* FXS model shows reduced Calmodulin and Calbindin CaBP mRNA levels, resulting in the impaired sequestration of cytosolic Ca^{2+} (Table 2) [58]. This transcript change could be the result of mRNA-binding mechanisms or impaired transcriptional regulation. Thus, FMRP controls channel/CaBP

expression and function via multiple overlapping direct and indirect avenues, providing a rich and confusing tapestry of activity misregulation in the FXS disease state.

Recent work continues to identify mechanisms by which FMRP controls channels via both RNA- and protein-binding mechanisms (Fig. 1). A human patient-derived FMR1 missense mutation (R138Q) has helped to discriminate these two FMRP functions by blocking channel-binding while preserving mRNA-binding translational control [20]. In the *Drosophila* FXS model, this mutant variant fails to rescue synaptic structural defects, suggesting growth phenotypes are separable from channel functions directly modulating presynaptic activity [20]. A caveat to this conclusion is that the R138Q mutation blocks FMRP interaction with Ca^{2+}-gated BK channels, but may not impair other channel binding. Moreover, *Drosophila* FMRP has not yet been shown to bind ion channels, and it remains critical to determine whether this is an evolutionarily conserved mechanism, as suggested by the binding of FMRP to Slack K^+ channels in *Aplysia* [59]. Furthermore, it is unknown whether channel-binding activity regulation and RNA-binding translational suppression can actually be separated, as they may form two parts of a coupled activity-dependent regulatory mechanism (Fig. 1). Recent studies into activity regulation via channel expression may result in new therapeutic treatments. In the *Drosophila* FXS model, a recent study shows that overexpression of N-type Ca^{2+} channels can be corrected by lowering channel activity with chronic magnetic nanoparticle stimulation [60]. This modulation also rescues action potential duration, intensity and frequency, and may enhance $GABA_A$ receptor expression in the disease state [60].

Recent studies show calcium signaling is misregulated at multiple levels to alter FXS model synapse and circuit excitability. As referenced above, *Drosophila* FMRP binds CAMKII mRNA, and FMRP knockdown in the *Drosophila* FXS model activates a CAMKII Ca^{2+}-dependent translational reporter [10]. *Drosophila* FMRP also binds to the transcripts of neuronal calcium sensor 1 (NCS-1) and the associated guanine exchange factor (GEF) Ric8a, which operate in another Ca^{2+}-sensing mechanism. NCS-1 and Ric8a antagonistically regulate neuronal activity and synapse number downstream of voltage-gated Ca^{2+} channels (Table 2) [56]. In the *Drosophila* FXS model, the drug phenothiazine inhibits the NCS-1/Ric8a interaction to prevent the assembly of this Ca^{2+} sensing complex and restore normal synapse architecture [57]. FMRP also modulates calcium homeostasis in non-canonical mechanisms. As referenced above, in human cells the FMR1 5'UTR CGG expansion interacts with ARC mRNA in mobile ribosomal granules, which also disrupts Ca^{2+} homeostasis by misregulating CaBP mRNAs that localize to these granules in a similar translation misregulation mechanism [29]. Using a drug that destabilizes the FMR1 CGG repeats (TMPyP4), normal Ca^{2+} homeostasis can be restored, indicating some relationship between the FXS trinucleotide expansion and neural Ca^{2+} dynamics [29]. Added to this complexity, FMRP-dependent Ca^{2+} signaling is altered differentially in excitatory versus inhibitory neurons as they mature during the early-use critical period in the *Drosophila* FXS model [1].

Part III: New Progress in Activity-Dependent Synapse Development Mechanisms

The critical question of FMRP roles during neurodevelopment versus maturity is centrally important for determining optimal FXS treatment strategies [1,2,21,25,26]. Recent FXS clinical trials have been greatly disappointing, but this may be in large part due to targeting adult patients [21]. Moreover, many mouse FXS model interpretations may be complicated by "adult" studies initiated in animals at ~4-weeks, which overlaps the neurodevelopmental period [61]. In the *Drosophila* FXS model, studies over the last decade have revealed clear, selective FMRP roles restricted to early-use critical periods [1,25,26]. This is not to say that there are not maintained roles at maturity, but only that FMRP clearly has specialized functions during neurodevelopment. Recent work shows that depleting FMRP in mouse embryonic stem cells alters the normal kinetics of both neuronal and glial differentiation [14]. Later on in the *Drosophila* model, multiple aspects of sensory- and activity-dependent synaptic remodeling and calcium signaling refinement are limited to FMRP functions during early-use critical periods, without any apparent persisting roles at adult maturity [1]. Indeed, FMRP expression has been suggested to functionally delineate temporal windows of activity-dependent neural circuitry optimization, with sharply dropping FMRP levels marking the end of the critical period in *Drosophila* [1]. FMRP has been proposed to act as a core component of the critical period activity-sensing mechanism during this developmental window, but much more investigation is needed to rigorously test this hypothesis.

Recent work in the *Drosophila* FXS model shows FMRP is absolutely required in activity-dependent remodeling of synaptic connectivity during an early-use critical period [25,26]. Although FMRP is similarly required in both excitatory and inhibitory neurons, the direction of activity-dependent changes appears opposite in these neuronal classes, including both synaptic architecture and calcium signaling refinement [1]. Optogenetic manipulations in *Drosophila* result in opposing changes in dendritic arborization in these two neuron classes, but either directional change absolutely requires FMRP during the developmental critical period [25]. In the *Drosophila* FXS model, glial phagocytosis is delayed in this same circuitry, suggesting a possible synaptic pruning mechanism [62]. However, FMRP may not work in glia but rather within neurons, which may signal glial phagocytosis through extracellular protein expression. Interestingly, neuronal FMRP regulates synaptic **matrix metalloproteinase (MMP)** classes in *Drosophila*, which sculpt intercellular signaling via proteolytic cleavage of extracellular proteins [63,64,65]. In the *Drosophila* FXS model, MMP levels are elevated, resulting in altered intercellular signaling, including *trans*-synaptic Wnt signaling [66,67]. This mechanism depends on regulation of the cell surface Wnt co-receptor Heparan Sulfate Proteoglycan (HSPG) Dally-like Protein (Dlp). Future studies will be needed to test whether activity-dependent MMP/Dlp intersections underlie FXS synaptogenic defects, especially in the context of excitatory versus inhibitory neuron classes.

Part IV: New Progress in Excitatory/Inhibitory (E/I) Balance Mechanisms

The **metabotropic glutamate receptor (mGluR)** theory of FXS hyperexcitability [68,69] is developmentally restricted, with early defects later corrected [22,24]. In mice, FMRP loss

causes mGluR type 1 and/or 5 (mGluR1/5) pathway activation. In the *Drosophila* FXS model, elevated **phosphatidylinositide 3-kinase enhancer (PIKE)** levels exaggerate mGluR1/5 signaling to both impair synaptic plasticity and cause hyperexcitability [70]. One result of this mGluR hyperexcitability is the well-documented decrease in cAMP levels in both mouse and *Drosophila* FXS models [71]. Interestingly, **phosphodiesterase 4 (PDE4)** inhibition in adult *Drosophila* reportedly restores mGluR-dependent learning/memory defects, independent of correcting brain learning/memory circuitry defects, showing therapies administered after the developmental critical period can still have efficacy [71]. FMRP and mGluR5 also may modulate excitability by acting together with **amyloid precursor protein (APP)** to alter signaling pathways, including mTor and ERK discussed above [14,68]. *Drosophila* FMRP binds APP mRNA, and APP translation is elevated in the *Drosophila* FXS model. Increased APP levels are proposed to mediate hyperexcitation via a variety of mechanisms, including mGluR5 redistribution at synapses via Aβ oligomers metabolites of APP. In the mouse FXS model, genetically reducing APP levels prevents hyperexcitability and corrects behavioral, postsynaptic dendritic spine and glutamate signaling defects [14,68]. Dual dysregulation of APP and α-secretase ADAM10 in the mouse FXS model during the critical period activates the MAP kinase pathway to cause synaptogenic and behavioral deficits [72].

In addition to the mGluR hyperexcitability theory, GABAergic hypoinhibition may underlie FXS symptoms [27]. Earlier work in both mouse and *Drosophila* FXS models showed impaired GABAergic circuit assembly and function. Recent mouse work shows FMRP binds multiple $GABA_AR$ mRNAs (although this is debated [73]), resulting in delay of the developmental GABA inhibitory switch in FMR1 knockouts [68]. In FXS mice, underdevelopment and reduction of many components of the GABAergic system occurs at an early age [73]. For example, a $GABA_bR$ subunit (R1a) mediating presynaptic inhibition is reduced in FMR1 knockout mice [73]. Similarly, the *Drosophila* FXS model shows deficits in $GABA_bR$ suppression of presynaptic glutamate release [19]. This early delay in GABA signaling is followed by later overelaboration of GABAergic neurons in a *Drosophila* brain learning/memory center [27]. In the mouse FXS model, recent work shows that both pre- and postsynaptic alterations cause abnormal $GABA_AR$-mediated phasic inhibition [73], although GABAergic defects vary widely across brain regions [73]. In the *Drosophila* FXS model, GABAergic neuron structure and Ca^{2+} signaling are both impaired, although correction of hypoinhibition is not sufficient to restore learning [27]. A recent *Drosophila* study shows altered GABAergic signaling reduces lateral inhibition across olfactory projection neurons [74]. However, in this case, GABAergic neurons are actually more active in the FXS condition, although postsynaptic neurons are less sensitive to inhibitory signaling. A recent mouse study shows glycinergic inhibitory signaling is unaffected but excitatory input elevated in the lateral superior olive [75], suggesting E/I imbalance also underlies FXS auditory processing defects.

Part V: Future Directions

Canonically, FMRP is an mRNA-binding translational suppressor. However, at a foundational level, we still fail to grasp the binding mechanism to specific mRNA targets. This question is complicated by our ever-increasing grasp of mRNA complexity [11,12], as

well as by examples of FMRP acting as a translational activator [55]. FMRP also has multiple mRNA-binding domains [35], but it is not clear whether they work together, separably or sequentially. Moreover, FMRP interacts with multiple other RNA-binding translational regulators [40–43], microRNA [10,42] and ribosomal components [36], highlighting unanswered questions about FMRP mechanisms of translation regulation. Partnerships with other mRNA-binding proteins could provide the long-sought specificity for coupled control of transcript targets in neuron class-specific mechanisms [40–43]. FMRP roles in glia may also contribute to FXS [13–15], and this remains an important question. Intercellular glial-neuron signaling, as well as *trans*-synaptic signaling, may have central roles in FXS neuropathology, which are just coming to light [63–67]. FMRP roles in the extracellular space, particularly in MMP regulation, may regulate intercellular signaling. Studies in this area may help address the historical divide in the FXS field between pre- and postsynaptic mechanisms. Although the bulk of research remains focused on postsynaptic roles, there are at least as many FMRP targets encoding presynaptic proteins [2,16,20,21]. Future work needs to build a consensus view of FMRP roles on both sides of the synapse, as well as within the synaptic cleft itself.

As we begin to bridge the gap between pre- and postsynaptic mechanisms, there remains a wide divide between FMRP roles in development vs. maturity. At the earliest time point, some work suggests FMRP roles in neuron/glia differentiation [14]. However, the majority of developmental studies focus on early-use critical periods of peak FMRP expression, theorizing that critical period defects may set the stage for dysfunction at maturity [1,9,25–27]. It is vital to define how critical periods are delineated, possibly by FMRP itself, and test FMRP roles in activity-dependent synaptic and circuit refinement [1,22,24–26]. On the other hand, FMRP function is maintained at maturity. In both mouse and *Drosophila* FXS models, genetic and pharmacological interventions in mature animals have proven efficacious in some cases [68,71]. For example, PDE4 inhibitors in *Drosophila* adults rescue FXS learning/memory deficits, despite persistent malformation of underlying brain circuitry [71]. In the mouse FXS model, many argue that persistent changes in mGluR signaling must be taken into account in therapies [22]. E/I imbalance theories, including mGluR hyperexcitability and GABA hypoinhibition, are entangled in this ongoing question of development versus mature functions. Although debate continues over the relative importance of hyperexcitability and hypoinhibition in the FXS condition [27], the two theories have recently been framed more compatibly [67,68]. Future work needs to build a consensus view of FMRP roles in different neuron classes and in different temporal windows of requirement.

The biggest recent change in the FXS field has been the challenge to the predominant function of FMRP as an mRNA-binding translational suppressor. While most recent studies continue to identify new FMRP mRNA targets (Table 2), other work reveals new FMRP protein partners in an ever-broadening arena of biology (Table 3). Although some interactions support canonical roles in translation control, others have expanded FMRP biology to a dizzying scale spanning from chromatin-binding to channel-binding (Table 1) [3,4,6,7,8]. Considerable work has shown the importance of channel-binding interactions in FXS, especially for disease state hyperexcitability. There is a critical need to address whether channel-binding is a wholly separate FMRP function, or rather part of an integrated mechanism driving activity-dependent translation control (Fig. 1). How the other, more

diverse FMRP functions may contribute to FXS neuropathology remains largely unknown. Future work on the downstream effects of these broad changes, and identifying key alterations in the FXS disease state, will be crucial to deciphering unfolding non-canonical roles. We should also question whether FMRP is truly a multifarious protein with diverse, unrelated functions adopted through the long course of evolution, or rather a central link in an integrated mechanism linking activity input to appropriate protein translation. Occam's razor supports a combinatorial mechanism of FMRP requirement at the heart of the sensory input- and activity-dependent optimization of neural circuitry.

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Glossary Box

Activity-Regulated Cytoskeleton-Associated Protein (ARC)
An immediate-early gene (IEG) displaying activity-dependent mRNA localization to the synapse, where local translation is involved in synaptic plasticity, learning and memory.

Adenosine Deaminase Acting on RNA (ADAR)
A class of RNA-editing enzymes that bind double stranded RNA to convert adenosine to inosine by direct deamination.

Adenylyl Cyclase (ADCY1)
Converts adenosine triphosphate (ATP) into the second messenger cyclic adenosine monophosphate (cAMP).

Amyloid Precursor Protein (APP)
An integral membrane protein concentrated at neuronal synapses.

Bone Morphogenetic Protein Receptor Type 2 (BMPR2)
A serine-threonine receptor kinase of the transforming growth factor-β superfamily.

Calcium/Calmodulin-Dependent Protein Kinase II (CAMKII)
A serine-threonine protein kinase regulated by calcium-calmodulin complexes, involved in many signaling cascades at synapses.

Cyclic Adenosine Monophosphate (cAMP)
Derivative of adenosine triphosphate (ATP) and a common second messenger in intracellular signaling at synapses.

Cytoplasmic FMR1-interacting Protein 1 (CYFIP1)
Serves as a Rac-1 (an activating Ras GTPase) binding site in the WAVE Complex to facilitate actin nucleation through ARP2/3 complex activation.

Diacylglycerol Kinase (DAG Kinase)
Converts Diacylglycerol (DAG) into Phosphatidic Acid (PA) during lipid signaling.

Down Syndrome Cell Adhesion Molecule (DSCAM)
A transmembrane protein regulating synaptic growth. In Down Syndrome patients, Dscam is over-expressed owing to chromosome 21 trisomy.

DNA Damage Response (DDR)
Phosphatidylinositol 3 kinases are activated by double-stranded DNA breaks, and open replication forks to enable histone phosphorylation driving chromatin folding changes.

Extracellular Signal-Regulated Kinases (ERK)
A core component of the Ras-Raf-MEK-ERK signaling pathway that regulates many functions; also known as Mitogen-Activated Protein Kinase (MAPK).

Fragile X Mental Retardation Protein (FMRP)
Classically defined as an mRNA-binding translation regulator, FMRP also binds multiple cytosolic proteins and ion channels to regulate neural excitation, calcium signaling, excitatory/inhibitory balance, and has a wide range of other proposed mechanistic functions discussed in this article.

Fragile X Syndrome (FXS)
A leading heritable cause of intellectual disability and autism spectrum disorders. Typically caused by a trinucleotide repeat in the 5’ untranslated region (UTR) of the *fragile X mental retardation 1* (FMR1) gene, leading to hypermethylation and transcriptional silencing.

Long-Term Central Adaptation (LTCA) / Long-Term Habituation (LTH)
Two terms both describing sensory input-dependent plasticity in *Drosophila* olfactory neural circuits.

Matrix Metalloproteinase (MMP)
Class of extracellular proteases responsible for proteolytic cleavage of a wide range of extracellular protein targets.

Metabotropic Glutamate Receptor (mGluR)

A class of G-protein coupled receptor (GPCR) that binds the excitatory neurotransmitter glutamate.

MicroRNA-Mediated Translational Suppression
MicroRNAs bind Argonaute proteins to form RNA-Induced Silencing Complexes (RISC) that associate with complementary mRNAs to silence expression.

Phosphodiesterase 4 (PDE4)
An enzyme that hydrolyzes and degrades the second messenger cyclic adenosine monophosphate (cAMP), terminating cAMP signaling.

Phosphatidylinositide 3 Kinase Enhancer (PIKE)
Enhances activity of the phosphatidylinositide 3 kinase (PI3K) enzyme family, which phosphorylates the 3-hydroxyl group on the phosphatidylinositol inositol ring.

Pre-mRNA Alternative Splicing
The process by which transcripts are spliced into different mRNAs by the differential inclusion and exclusion of exon transcript sequences.

TAR DNA Binding Protein, 43 Kilodaltons (TDP-43)
An mRNA-binding protein functioning during development; involved in cell cycle progression, apoptosis, RNA processing and alternative splicing.

Trends Box

- Fragile X syndrome (FXS), a leading heritable autism, is caused by a 5’UTR trinucleotide repeat expansion in the gene encoding Fragile X Mental Retardation Protein (FMRP)
- The disease presents with stereotypical hyperexcitability and synaptic overelaboration, which are well replicated across a broad range of neural circuits in genetic models
- Canonically, FMRP is a mRNA-binding translational suppressor, but genetic roles range from chromatin-binding to mRNA splicing/editing to other forms of translation control
- Additional FMRP roles include direct ion channel-binding to regulate neural excitability, which may be an independent function or linked to activity-dependent translation control
- FXS cell-type specific defects in neurons and glia, include altered calcium signaling, critical period synapse refinement and excitation/inhibition balance in neural circuitry

Outstanding Questions

- How does FMRP manifest RNA-binding specificity to repress the translation of a select subset of transcripts?
- Do FMRP RNA-binding domains work together, sequentially or separably to mediate translation control?
- Why does FMRP interact with the microRNA pathway, and what role does this play in translation control?
- How does FMRP work with other RNA-binding proteins in translation control; together, sequentially or separably?
- What percentage of FMRP translation control occurs locally at the synapse as compared to the soma?
- Does FMRP involved in RNA- vs. channel-binding represent a single protein pool for both binding functions?
- Is channel-binding a separate function of FMRP, or is it mechanistically tied to RNA-binding functions?
- How does lack of FMRP in glia contribute to the FXS disease state, and what processes are impacted?
- Does FMRP in neurons and/or glia regulate intercellular signaling required for synaptic pruning/refinement?
- How does *trans*-synaptic signaling depend on FMRP, and defects in this process contribute to the FXS state?
- Are FMRP-dependent pre/postsynaptic changes independent or coupled to affect synaptic connectivity?
- What MMP roles in the extracellular space are regulated by FMRP, and is this a direct translation mechanism?
- How are activity-dependent critical periods delineated, and does FMRP play a role in this temporal restriction?
- Do FXS developmental defects cause defects at maturity, or are these separable stages of requirement?
- How do FMRP roles differ by neuron classes and within different temporal windows of requirements?
- What is the role of FMRP in establishing or modulating excitation/inhibition balance in neural circuits?
- Are the hyperexcitation and hypoinhibition characterizing FXS mechanistically linked or separable defects?
- How does the apparently diverse range of FMRP functions contribute to FXS neurological phenotypes?

- Does FMRP have many unrelated functions, or is there an integrated mechanism of FMRP requirement?

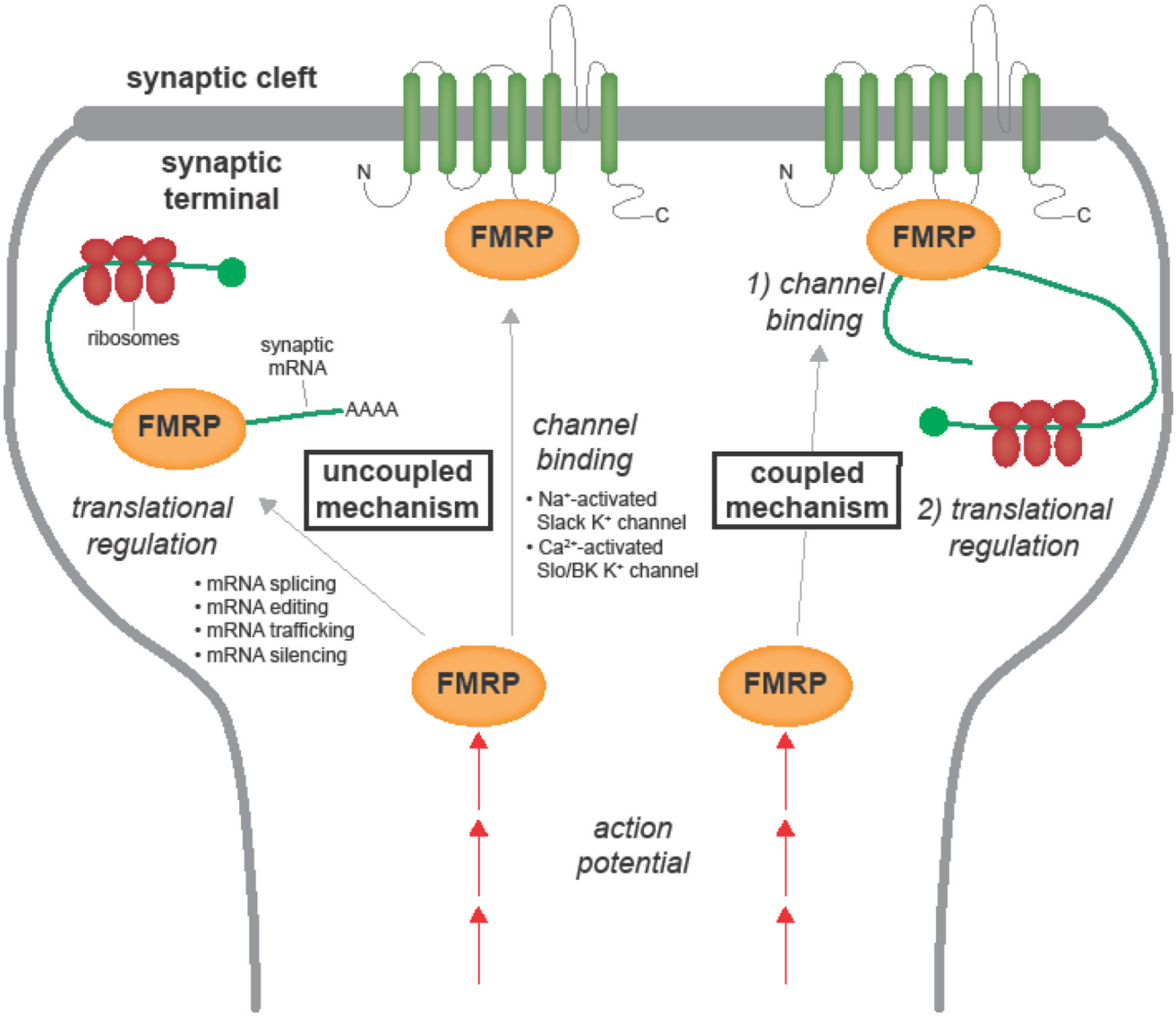

Figure 1. FMRP roles in RNA- and channel-binding at the neuronal synapse

Activity-dependent functions of FMRP in RNA-binding translation regulation and direct channel-binding activity regulation. In the uncoupled mechanism (left), RNA- and channel-binding roles are unrelated, representing two evolutionarily divergent functions. In the coupled mechanism (right), channel-binding is an integral activity-sensing step in the translational regulation of FMRP-bound transcripts.

Table 1
Recently defined FMRP genetic functions

The table lists recently validated roles of FMRP (column 1), the FXS model system of the work (column 2), and the primary reference(s) discussed in the text (column 3).

Proposed FMRP Roles	FXS Model	References
mRNA-binding translational regulator; canonically suppressing translation	Mouse and *Drosophila*	[2,33,34,46]
Chromatin-binding; regulates genome stability, required for early DDR	*Drosophila* and Mouse	[4,5]
ADAR-binding RNA editing regulator; alters RNA editing of neural genes	*Drosophila*, Zebrafish, Mouse	[6,7]
RBM14-binding in pre-mRNA splicing; promotes mRNA target binding	Mouse	[8]
microRNA pathway regulation; neural circuit plasticity and behavioral output	*Drosophila*	[9,10]
Regulation of cell differentiation kinetics; effects both neurons and glia	Mouse	[14]
Ion channel-binding to regulate gating; circuit excitability and plasticity	Mouse	[16,17]

Table 2

Recently defined FMRP direct RNA-binding targets

The table lists recently validated mRNA targets bound by FMRP (column 1), encoded protein function (column 2), impacted signaling pathway (column 3), disease state effect (column 4), and the FXS model system of the work (column 5). Primary reference(s) discussed in text are listed in the rightmost column (column 6).

RNA transcript	Related Protein Function	Related Signal Pathway	Disease State Impact	FXS	References
ARC	Synaptic ARC facilitates removal of AMPA receptors	Depends on MAPK signal cascade for activation	Spine overgrowth, Ca^{2+} signaling misregulation	Human cell lines	[29,38,39]
CAMKII reporter construct	Translational reporter binds Ca^{2+}-Calmodulin	Activity-dependent Ca^{2+} signaling at the synapse	Elevated activity underlying LTH	*Drosophila*	[10]
DAG Kinase	Converts DAG to PA for lipid signaling and homeostasis	Lipid signaling during vesicle trafficking	Postsynaptic spine deformation	Mouse	[33,44]
Rac1, MAPB1, GluR1	Rho GTPase, microtubule stability, AMPA-R subunit	Wide range of central signaling pathways	Defective hippocampal spinogenesis	Mouse and human	[34]
ADCY1	Adenylyl cyclase produces cAMP second messenger	cAMP upregulates ERK & PI3K, which activate RSK	Hyperphosphorylates s6, spine overgrowth	Mouse	[47]
DSCAM	Cell adhesion and repulsion during synaptic growth	Relies on FMRP binding with Abelson kinase (Ab-1)	Unregulated presynaptic growth	*Drosophila*	[49]
EF1α	Overexpression of hyperphosphorylated Mdm2	MEF2 transcription factor resistant Mdm2	Eliminates excitatory synapse scaffold	*Drosophila*	[50]
BMPR2	Receptor S/T kinase in growth and differentiation	Activates LIMK1, inhibiting cofilin, an actin destabilizer	Actin reorganization for dendritic overgrowth	Mouse and *Drosophila*	[35]
Kv3.1b mRNA	Voltage-gated K+ channels in brainstem	K^+ influx to regulate auditory circuit processing	Disrupts processing of auditory information	Mouse	[52]
Kv4.2 mRNA	Voltage-gated K^+ channels in hippocampus	Major K^+ channel regulating hippocampal excitability	Channel expression repression/activation	Mouse	[54,55]
L-type Ca^{2+} channel	Voltage-dependent, slow L-type Ca^{2+} channel	Ca^{2+} influx to modulate gene transcription	Defective synaptic architecture	Human	[53]
Calmodulin, Calbindin	Ca^{2+}-binding proteins act as second messengers	Ca^{2+} signaling activates many signaling pathways	Impaired sequestration, Impaired E/I Balance	*Drosophila*	[58]
NCS-1, Ric8a	Ca^{2+} signaling to regulate synaptic mechanisms	Coregulate synapse activity and synapse number	Increased activity and synapse number	*Drosophila*	[56]

Table 3
Recently defined FMRP direct protein-binding partners

The table lists recently validated direct protein partners bound by FMRP (column 1), mechanisms involved in FMRP-binding functions (column 2), and the FXS model system of the work (column 3). Primary reference(s) discussed in text are listed in the rightmost column (column 4).

Protein Partner	FMRP-binding Function	FXS Model	References
Adenosine deaminase acting on RNA (ADAR)	RNA editing; binds double stranded RNA to convert adenosine to inosine by deamination	*Drosophila*, Zebrafish, Mouse	[6]
RNA-binding protein 14 (RBM14)	Alternative splicing; binds pre-mRNA for splicing, results in differential mRNA expression	Mouse	[8]
L5 protein of 80s ribosome	FMRP blocks tRNAs and elongation factors from binding 80s ribosome, suppressing translation	*Drosophila*	[36]
Telomere repeat-binding factor 2 (TRF2-S)	FMRP antagonizes TRF2-S target mRNA binding to limit axonal mRNA localization and function	Mouse	[37]
Staufen, Pumilio, Lark and other translational regulators	Translation regulation via RNA-binding in direct or indirect partnership with FMRP function	Human, Mouse, & *Drosophila*	[40–43]
Ataxin-2 mRNA-binding translational regulator	RNA-binding: microRNA pathway-induced RNA silencing for regulating & dampening LTH	*Drosophila*	[10,42]
Cytoplasmic FMR1-interacting protein 1 (CYFIP1)	Actin cytoskeleton and mTor signaling; binds UG/GU RNA with TDR-43 and Staufen	Human, Mouse, & *Drosophila*	[32,45–47]
Janus kinase and microtubule interacting protein 1 (JAKMIP1)	Microtubule regulation and GABAB receptor expression regulation in inhibitory neurons	Human	[32]
TAR DNA-binding protein of 43kDa (TDP-43)	Complex with FMRP, Staufen and CYFIP1 binds mRNA to suppress hippocampal spinogenesis	Mouse and human	[34,40]
Slack and Slowpoke (Slo) BK K^+ channels	FMRP regulates gating to shape AP kinetics, modulate Ca^{2+} influx and neurotransmitter release	Mouse and *Xenopus*	[16,17,20]