The Molecular Biology of FMRP: New Insights into Fragile X Syndrome

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Abstract

The Fragile X Mental Retardation 1 protein FMRP is the product of *FMR1*, a gene whose epigenetic inactivation by a triplet nucleotide repeat expansion causes Fragile X Syndrome (FXS), a neurodevelopmental disorder. FMRP is a widely expressed RNA binding protein whose activity is essential for proper synaptic plasticity and architecture, aspects of neural function that are known to go awry in FXS. For over two decades, FMRP has been known as a translational repressor protein; in its absence, protein synthesis in the brain is excessive, which in turn impairs neuronal circuit formation and higher cognitive function. However, although the neurophysiology of Fragile X Syndrome has been described in remarkable detail, research focusing on the molecular biology of FMRP has only scratched the surface. Recent advances in whole transcriptome and translatome analysis from mouse and human models of the disorder show that FMRP is involved in nearly all aspects of gene expression. Emerging mechanistic details of FMRP regulation offer ways to consider new therapies to treat FXS.

Keywords

FMRP; Fragile X Syndrome; Neurodevelopmental disorder; RNA binding protein

Introduction

Fragile X Syndrome (FXS) is a neurodevelopmental disorder characterized by intellectual disability and behavioral symptoms such as hyperactivity and anxiety. Nearly all FXS cases result from expansion of what are normally ~30 CGG triplet repeats in the 5' untranslated region of the X-linked Fragile X Mental Retardation 1 (*FMR1*) gene to over 200, leading to DNA methylation, transcriptional inactivation, and loss of the gene product Fragile X Mental Retardation Protein (FMRP)[1,2]. FXS afflicts about 1 in 4000 male and 1 in 7000 females and is the most common inherited cause of intellectual impairment[3,4]. There is strong association between FXS and autism; about half of all individuals with FXS and 2 in 3 males with FXS also meet the criteria for this disorder. Consequently, FXS contributes to 2–6% of all autism cases, making it the largest single gene cause of this disability[3,4]. In the

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brain, FMRP is expressed in neural stem cells and probably all neuronal and glial cell types. Its importance is illustrated by the fact that *FMR1* inactivation impairs brain development and cell function at multiple levels including dendrite morphogenesis, circuit integration, and axon targeting (Figure 1).

The discovery of *FMR1* silencing as the cause of FXS has prompted extensive investigations into the functions of FMRP using animal and human stem cell models[5,6]. Studies of FMRP protein have shown that it is a brain-enriched RNA binding protein[7,8,9] and have identified its mRNA targets and molecular activities, particularly as a regulator of translation[9,10]. Through genetic and pharmacologic "rescue" experiments primarily in mice, both upstream and downstream pathways that coalesce on and emanate from FMRP have been identified and suggested as possible therapeutic targets for the treatment of FXS[11,12,13]. However, despite advances towards the mitigation of FXS-relative phenotypes in animal models, recent clinical trials of candidate drugs have been unsuccessful at achieving primary endpoints[5,12,13].

Although enormously useful for understanding FMRP and some aspects of FXS, *Fmr1* mutant mice (one of the most commonly-used animal models of FXS) carry a deletion in the *Fmr1* gene as opposed to CGG expansion-induced gene methylation and silencing, which may be at least partly responsible for the subtly of the behavioral abnormalities found in these mice compared to humans with FXS[13,14]. In addition, it is clear that many aspects of human brain development cannot be modeled in animals[6,15]. Therefore, FXS individual-derived or gene edited human pluripotent stem cells (hPSCs) have been used to mimic FXS pathogenesis in a dish, which may reveal human-specific FMRP functions that can be targeted for therapeutic intervention[6].

Despite being a single gene disorder, individuals with FXS vary widely in intellectual impairment; for example, some males with FXS function nearly normally while others are profoundly disabled[16,17]. Most individuals with FXS have considerable anxiety and about a third of males with FXS exhibit irritability and aggression[18]. Although genetic background is certainly responsible for heterogeneity of symptoms, the molecular function(s) of FMRP may also be a contributing factor. For example, FMRP regulates mRNA splicing in a stoichiometric manner which may lead to differential splicing changes among FXS individuals. In addition, FMRP-targeted mRNAs may be regulated by other proteins (e.g. MTL3 or ADAR) that may be present at various levels among FXS individuals.

To move a field forward sometimes it is useful to a step back. This is what we do in this Review by taking a fresh look at what FMRP does and how its loss drives FXS pathophysiology. This survey, which includes recent advances in next generation sequencing technology, multi-omics gene network analysis, hPSCs, and CRISPR/Cas9 human gene editing, allows us to delve into novel molecular mechanisms regulated by FMRP. We will summarize how new studies on cell type-specific FMRP targets in mouse and human neurons inform us as to how FMRP selects its target transcripts. We will discuss innovative methods that unveil how FMRP regulates translation, alternative splicing, and RNA stability through recognition of mRNA codon bias and m^{6}A modifications. Finally, we will discuss

how this new knowledge base influences our understanding FXS which may lead to innovative therapeutic development.

Identifying FMRP's targets

FMRP domain structure

The most prevalent form of FMRP in humans contains 632 amino acids and is a classic RNA binding protein containing at least three canonical RNA binding motifs (Figure 2) : two hnRNP K homology (KH) domains and an arginine-glycine-glycine (RGG) box[19]. A third putative KH domain, KH_0, has been identified through x-ray crystallography[20], although its RNA binding capabilities have not been tested. The amino terminal region of FMRP contains two Agenet/Tudor domains that interact with RNA, chromatin and other proteins[20,21,22]. FMRP also contains nuclear localization and nuclear export sequences (NLS, NES, respectively) that direct its shuttling between nucleus and cytoplasm[23]. At steady state, however, the protein is predominantly cytoplasmic[23]. FMRP is post-translationally modified by phosphorylation, that is triggered by metabotropic glutamate receptor (mGluR) signaling. This phosphorylation controls FMRP's ubiquitin-mediated destruction at synapses[24], coordination with miRNAs to regulate translation[25], phase separation into liquid droplets by way of its intrinsically disordered domain[26] and association with RNA-containing granules that may influence translational regulation[27].

The modular nature of the FMRP RNA binding domains allows for *in vitro* identification of RNA recognition sequences. For example, assays with FMRP fragment polypeptides show that, unlike other KH motif-containing RNA binding proteins, all three FMRP KH domains bind very weakly to single stranded RNAs and may require higher order secondary structures to confer specificity[28]. The RGG motif preferentially interacts with a G quadruplex (G4) pseudoknot structure in mRNA[28,29,30]. KH domain 2 recognizes a variable sequence within a complex pseudoknot structure that is important for FMRP to associate with polyribosome[31].

FMRP target mRNAs

Nearly three decades have passed since FMRP was shown to bind mRNA[7,8], which has laser-focused the field on answering these questions: what mRNAs are bound by FMRP? Is there cell type-specificity of its mRNA targets? How does FMRP select its mRNA targets? What does FMRP do to its mRNA targets?

In an important initial study, Brown et al[32] co-immunoprecipitated FMRP–RNA complexes (through RNP immunoprecipitation (RIP)) from the mouse brain followed by microarray analysis, which identified 432 FMRP-bound mRNAs. G4 structures were detected in 8 of the 12 top ranked targets. In another assay, FMRP antibody-directed amplification of mRNA sequestered in FMRP-containing mRNPs (APRA) also found G4 structures in many FMRP-bound mRNAs[33]. These early studies suggested that FMRP associated with unique sets of mRNAs and that the presence of G4 structures was one prominent determinant for the interaction; however, because both RIP and APRA lack specificity and are unable to identify protein binding sites on mRNA, a more rigorous and high-resolution method was required.

In a seminal study, Darnell et al[9] covalently crosslinked FMRP to RNA by ultraviolet irradiation, which was followed by stringent FMRP immunoprecipitation and high throughput sequencing (HITS-CLIP, also called CLIP-seq) to identify associated RNAs. They identified 842 high confidence FMRP target RNAs in juvenile mouse forebrain, 76% of which were not identified by RIP[32]. Surprisingly, the RNAs identified by HITS-CLIP did not contain noticeable G4 structures. However, nearly a third encoded postsynaptic proteins such as metabotropic glutamate and N-methyl-D-aspartate (NMDA) receptors, whose signaling is impaired in FXS. The FMRP HITS-CLIP target list has been an enormously useful resource for investigators analyzing the biology of FMRP (Table 1). Two additional observations by Darnell et al[9] have catalyzed studies in the molecular biology of RNA-protein interactions. First, there was no clearly identifiable FMRP interacting sequence motif within mRNAs (i.e., cis elements), contradicting the previous studies using RIP[9,32]. Second, FMRP predominantly bound to the coding regions (CDS) of mRNAs rather than 5' or 3'-UTRs, unlike most other RNA binding proteins.

Because FMRP is thought to be expressed in all cells in the brain[6,11], a key question is whether it binds mRNAs with cell-type specificity. FMRP HITS-CLIP of juvenile mouse cortex, hippocampus, and cerebellum showed that the target RNAs in each region generally overlapped and that the FMRP binding sequences were mostly in CDS[34]. In a refinement of HITS-CLIP, Van Driesche et al[35] generated a conditional FMRP-GFP mouse line and through crossing with different Cre driver lines, created mice expressing a FMRP-GFP fusion protein in specific cell types. Then using antibodies against GFP for HITS-CLIP, they identified both common and unique FMRP-bound RNAs in cerebellar Purkinje and granule cell neurons as well as in hippocampal area CA1 neurons[35, 36]. Among the new FMRP target RNAs identified in hippocampal CA1 neurons are several that control circadian rhythms especially those that influence behaviors associated with *Fmr1*-deficient mice such as contextual fear memory[36]. These results support an earlier study showing dysregulation of circadian rhythms in FMRP-deficient mice[37]. Confirming the findings of the earlier FMRP mouse forebrain CLIP study[9], Van Driesche et al[35] and Sawicka et al[36] found that many FMRP CLIP targets are encoded by autism spectrum disorder (ASD) high risk genes. The CLIP RNAs identified in these two studies are also particularly long, although the biological significance of this observation is unclear. Most importantly, the FMRP CLIP studies generally found that a number of FMRP's target RNAs are translationally dysregulated in *Fmr1*-deficient animals[9, 35,36].

Studies showing that *Fmr1* deficiency in mouse models does not faithfully recapitulate all aspects of brain biology of FXS in humans[38] and that mouse embryonic stem cells do not completely mimic their human counterparts[39,40,41] suggest that interspecies differences in brain development and FMRP function might be significant. One question is whether the FMRP targets identified in mouse are the same as those in human cells. To assess this, ectopic expression of tagged FMRP in human embryonic kidney (HEK293) cells was followed by a modified CLIP procedure (PAR-CLIP, where cells are metabolically labeled with the photoactivatable nucleoside analog 4-thiouridine (4-TU)), which revealed that half of the expressed mRNAs (>6000) were bound by FMRP[42], raising concerns about specificity and biological relevance. More recent work using an antibody against endogenous FMRP and a modified CLIP-seq (eCLIP) method identified FMRP targets in

post-mortem adult human frontal cortex[43], although the drawbacks here are mixed cell types and questionable applicability to neurodevelopment. In a recent study to interrogate developing neural cells, FLAG-tagged FMRP knock-in hPSC lines differentiated into dorsal forebrain excitatory neuroprogenitors and neurons (dNPC, dNeurons) or ventral forebrain inhibitory linages (vNPCs, vNeurons) were generated[44]. FLAG antibody immunoprecipitation and an optimized CLIP protocol for small numbers of primary cells identified FMRP target RNAs in four human neural cell types: vNPCs, vNeurons, dNPCs, dNeurons. As in the mouse brain, human FMRP preferentially bound long transcripts and mostly to CDS. Furthermore, many FMRP targets (39%–77%) were shared among the four cell types, suggesting a common FMRP function during neurogenesis. However, some FMRP targets were specific to NPCs, including those involved in cell cycle regulation, which may be relevant to the macrocephaly observed in some individuals with FXS and autism. FMRP targets enriched in the ventral but not dorsal neural cells encoded proteins involved in cell junction, ATP binding, and cytoskeleton pathways, suggesting that *FMR1* might play a role in the unique tangential migration of the ventrally developed interneurons. The majority of FMRP targets were not shared with those identified in adult human brains, which might be attributed to differences in both cell-type specificity and stage of development. Interestingly, a previous application of network-based integrative analysis[45] for reconciling FMRP targets with transcriptomic changes revealed that FMRP regulates the production of transcription factors, which in turn could coordinate the synthesis of the downstream RNAs observed in RNA-seq datasets (Table 1)

How does FMRP select its target RNAs? Recognition of the G4 structure reported in mouse brain[34] is not a consistent preference of human FMRP[44]. A more detailed analysis of the original mouse and human FMRP CLIP studies[9,42] has identified WGGA and UGGA among the top preferred binding motifs of FMRP[46,47], which was also observed in new CLIP-seq studies of mouse brain[34] and human neuron studies[44]. In general, FMRP binding motifs are more degenerate than those of many other RNA binding proteins and are thus poor predictors of FMRP target transcripts. Table 1 summarizes the key features of FMRP target mRNAs identified using high throughput methods. The top biological processes that are regulated by these mRNAs include cellular signaling, synaptic development and function, axonal and dendritic development, cytoskeleton and microtubule, RNA transport, as well as transcriptional and epigenetic regulations (Figure 2).

Several consensus observations can be drawn from the CLIP studies discussed above. First, FMRP preferentially binds to mRNAs that are implicated in autism, indicating a molecular convergence between these developmental neural disorders[9,43]. Second, FMRP preferentially binds to long RNAs[35,36,44]. Although this in and of itself does not indicate a biological function, it is worth noting that the topoisomerase TOP1 is particularly important for the transcription of long genes associated with autism[48], and there is a direct interaction between topoisomerase TOP3beta and FMRP[49,50,51]. Third, FMRP predominantly binds to coding regions of mRNAs, which suggests that it mediates a novel translational control mechanism[9].

FMRP and the regulation of translation

FMRP loss alters translation

The identification of FMRP CLIP targets does not necessarily indicate that the binding of FMRP to a particular target has a molecular function. The observation that hippocampal slices derived from *Fmr1* knockout mice incorporate 15–20% more ^{35}S-methionine into protein compared to wild type mice has experimentally supported original suggestions that FMRP is primarily a translational inhibitor[52,53,54]. However, changes in transcription or in RNA stability can also lead to increased protein levels.

The first of two high-resolution methods that have been used to assess FMRP-regulated translation is TRAP-seq (translating ribosome affinity purification; also call RiboTag-seq) where an epitope-tagged ribosomal protein (rp) introduced into animals (genetically or by viral injection) is incorporated into translating ribosomes followed by immunoprecipitation of ribosome-mRNA complexes and RNA-seq (Figure 3). Because the epitope tagged rp can be expressed in particular cell types, this method is useful for assessing the translatome in, for example, excitatory neurons derived from a whole brain extract. In one TRAP-seq study, GFP-tagged rpL10a expressed in mouse hippocampal CA1 pyramidal neurons exhibited increased co-precipitation with *Chrm4* mRNA in *Fmr1*-KO versus control WT mice. Chrm4 encodes muscarinic acetylcholine receptor 4 (M4), a protein that is translated excessively upon *Fmr1* depletion[55]. Another TRAP-seq study (with Cre-dependent HA-tagged rpL22) was combined with Cre-mediated CLIP in mouse CA1 pyramidal neurons to assess both FMRP binding and translation in the same cells[36]. These two experiments reported in references[36] and[55] revealed that in *Fmr1*-deficient neurons, the levels of many FMRP target mRNAs are reduced (as measured by the amount of RNA co-precipitated with HA-tagged ribosomes) and that the stronger the FMRP binding to a particular mRNA in wild type neurons, the greater the reduction of the RNA level in *Fmr1*-lacking neurons (for related studies cf[35,56]).

Although TRAP-seq is a valuable method for identifying ribosome-bound mRNAs in specific cell types, it is perhaps less useful for examining detailed mechanisms of translational control. For example, the amount of any particular RNA immunoprecipitated by, say 4 or 8 epitope-tagged ribosomes might be similar (that is, the assay may not be sufficiently sensitive to distinguish 4 versus 8 epitopes, 1 epitope per ribosome), yet these transcripts would be expected to produce different amounts of protein. An alternative high-resolution method is ribosome profiling[57] in which tissues or cells are incubated with cycloheximide to 'freeze' ribosomes that are translating mRNA, followed by RNase digestion, collection of the small (~29 base) ribosome-protected mRNA fragments, and sequencing. Input mRNA is sequenced as well, thereby yielding the number and positions of ribosome footprints relative to mRNA transcriptome-wide (Figure 3). This ratio, number of ribosome footprints relative to amount of input mRNA is referred to as translational efficiency (TE), a useful proxy for protein output. However, TE does not allow for the assessment of mRNAs associated with stalled ribosomes (that is, ribosomes that have initiated translation but that become stalled during the process of polypeptide elongation) (see below). Several studies have used ribosome profiling to investigate translational control

by FMRP in various tissues and cells[36,58,59,60,61,62,63]. A case in point is adult mouse neural stem cells (aNSCs); they give rise to neurons and glia but when FMRP is absent, the cells over-proliferate and primarily differentiate into more glia at the expense of neurons[64,65]. Ribosome profiling of these cells demonstrated that *Fmr1* deficiency resulted in increased or decreased ribosomes on specific transcripts, indicating that FMRP can be both an activator and repressor of translation[58]. This study also showed a remarkable degree of translational "buffering" in FMRP deficient aNSCs and mitochondrial ribosome mRNAs are enriched in this category. Buffering is a phenomenon where, for example, a reduction in the amount of an RNA is accompanied by an increased number of ribosomes such that the (predicted) protein output would be unaffected. In a similar vein, an increase in an RNA may be compensated by reduced ribosome number – again the predicted protein output would be unaffected. How translational buffering in the absence of FMRP occurs is unknown, but genetic and dosage compensatory mechanisms in general occur in probably most cell and animal models[66]. Liu et al[58] also revealed remarkable biology through ribosome profiling; their studies demonstrated that the transcription factor Necdin was responsible for aNSC over-proliferation and improper differentiation into glia.

FMRP regulation of ribosome stalling

How does FMRP regulate translation? There are several clues: it binds primarily to CDS[9], it represses general translation[54] and it co-sediments with polyribosomes in sucrose gradients [502] These connections suggest that FMRP slows or stalls ribosome translocation, thereby reducing the rate of polypeptide elongation. Indirect assessments of ribosome translocation in brain extracts[9,10] support this idea but the clearest evidence comes from dynamic ribosome profiling of the mouse hippocampus (Figure 3)[62]. Hippocampal slices of WT mice were treated with homoherringtonine (HHT), which freezes initiating ribosomes on translation start codons (usually AUG), but allows translocating ribosomes to continue to elongate polypeptides. Stopping ribosome translocation at various times by treatment with cycloheximide followed by ribosome profiling revealed ribosome transit rates on all mRNAs. This showed that although the ribosomes on most mRNAs wild type mice translocate quickly, other RNAs are associated with stalled or slowly moving ribosomes including many of those involved in neural and synaptic function Treatment of slices with HHT for 30 minutes – most of the ribosomes that can run off (that is, translocate on mRNAs until they terminate at a stop codon) have done so by this time – resulted in FMRP co-sedimenting in sucrose gradients with the few ribosomes that remained on mRNA[62]. RNA-seq from this 6–8 ribosome region of the gradient showed that about 50 mRNAs were reduced in amount in *Fmr1*-deficient slices relative to wild type slices. Because the input amounts of these RNAs were unaffected by genotype, one may infer that the reduction of RNAs in the polysome gradient was caused by the lack of FMRP to stall ribosomes. These RNAs encode many proteins with neural and synaptic functions as well as several that establish epigenetic chromatin marks (see FMRP and Chromatin).

By what mechanism could FMRP slow or stall ribosome translocation and how could it do so on specific mRNAs? FMRP binds RNA, so it could potentially act as a simple roadblock. Translocating ribosomes generate 13 picoNewtons of force, which is sufficient to unwind most stable secondary structures in RNA[67] and perhaps not be impeded by RNA binding

proteins such as FMRP (Figure 4). However, if FMRP binding to RNA is adequate to stop or slow a ribosome, then specific sequences identified by CLIP would be expected to strongly overlap with those showing elevated ribosome occupancy, which is not necessarily the case[58,59,60,62]. These observations suggest that FMRP may not merely act as roadblock to ribosome translocation. Thus, alternative mechanisms of ribosome stalling may underlie the effects of FMRP, including codon bias and inhibition of the association of tRNA and elongation factors with the ribosome.

Codon bias in mRNAs affects ribosome translocation as well as RNA stability[68,69]. That is, mRNAs may have optimal codons whose cognate tRNAs are abundant, or nonoptimal codons whose cognate tRNAs are relatively rare. Ribosome stalling has been shown to occur on RNAs with nonoptimal codons[68,69]. In the brain, FMRP modulates the relationship between ribosome stalling and codon optimality[63]. A current model is depicted in Figure 4. In the wild type mouse cortex, FMRP directly or indirectly associates with the translational machinery on mRNAs with optimal codons and stalls ribosomes[63]. FMRP also blocks an as yet unidentified nuclease(s) to prevent RNA degradation. FMRP does not associate with the translational machinery on RNAs with nonoptimal codons[63]. On these RNAs, ribosome translocation is normal but the mRNAs tend to be unstable because there is no FMRP to block nuclease attack. In FMRP-deficient cortex, RNAs with nonoptimal codons behave similarly in FMRP-deficient cortex as they do in wild type cortex[63]. However, RNAs with optimal codons are associated with normally translocating ribosomes, but the RNAs are unstable because there is no FMRP to block nucleases. Therefore, FMRP deficiency can lead to either increased or decreased RNA stabilities depending on their codon optimality status, which impacts the gene network controlling cellular functions[62].

In an intriguing study, recombinant, but not full-length, *Drosophila* FMRP was reconstituted with mammalian ribosomes *in vitro* and its structure analyzed by cryo-electron microscopy (cryo-EM)[70]. FMRP bound rpL5 with a stereo-configuration that would be predicted to inhibit association of tRNA and elongation factors to the ribosome and thereby induce stalling (Figure 4). Because these reconstitution experiments did not include mRNA or other translation factors, their biological relevance as yet is unclear. However, if similar results using mammalian FMRP and including mRNA and translation factors can be obtained, then this would offer a window into the mechanism of ribosome stalling. Even so, it is unclear how mRNA specificity could be achieved.

FMRP regulation of translation initiation

Studies have shown that FMRP may also influence the initiation of translation. CYFIP1/2 (Cytoplasmic FMRP Interacting Proteins 1 and 2), identified by a yeast two-hybrid screen[71], are the most studied FMRP-associated proteins. Although CYFIP1 interacts only with FMRP, CYFIP2 interacts with FMRP and the paralogs FXR1 and 2 as well. The CYFIP proteins are components of the five subunit WAVE regulatory complex (WRC) that remodels the actin cytoskeleton[72], which may indirectly influence translation. The authors of this study show that CYFIP bridges FMRP and the translation initiation factor eIF4E; by doing so, CYFIP prevents the scaffold protein eIF4G from binding eIF4E, which inhibits cap-dependent[72] translation. It is possible that the conglomeration of FMRP/CYFIP/WRC-

regulated translation of actin cytoskeleton dynamics controls dendritic spine morphogenesis, which is impaired in FXS[72].

FMRP and RNA Modifications

In addition to its role as a translational repressor, recent studies indicate that FMRP contributes to other aspects of gene expression, including its regulation of RNA modifications.

FMRP Interactions with m^{6}A

N^6-methyladenosine, commonly referred to as m^{6}A, is an RNA modification that has received considerable attention in the last several years as a multi-faceted regulator of posttranscriptional gene expression[73]. FMRP influences RNA stability[74], translation[75], as well as nucleus-to-cytoplasm export through interactions with m^{6}A[76]. Mechanistically, it is not clear how FMRP affects these molecular processes that depend on this RNA modification, but it is noteworthy that there appears to be some overlap between FMRP CLIP targets and those harboring m^{6}A[77]. This suggests that RNA modifications may assist in the selection of FMRP targets. However, how such selection is mediated and how FMRP recognize m^{6}A marked transcripts remain unknown.

FMRP and RNA Editing

Adenosine deaminase acting on RNA (ADAR) is an editing enzyme that converts adenosine to inosine in double stranded RNA, which the cell recognizes as a guanosine. Consequently, translation of edited RNAs can result in amino acid substitutions, thereby increasing protein diversity[78]. FMRP interacts with ADAR in mice[79], *Drosophila*,[80] and zebrafish[81], indicating an important function conserved through evolution. In human postmortem FXS brain, RNAs are generally hypo-edited, many of which are also hypo-edited in human postmortem autistic brain[43]. RNA editing, therefore, is another remarkable point of convergence between FXS and autism. Hints of how FMRP affects RNA editing come from experiments in *Drosophila*, where expression of an FMRP-ADAR transgene causes editing within RNA CDS[82]. These data indicate that ADAR catalytic activity is directed to certain sequences through FMRP-mediated RNA binding.

FMRP and Chromatin

It has recently become apparent that FMRP also has profound effects on nuclear events such as DNA damage response, transcription, and splicing. Several studies have shown that FMRP controls RNA synthesis through regulated translation of critical transcriptional factors or chromatin modulators[62, 83]. In a remarkable series of experiments, re-analysis of the original FMRP CLIP data discussed above (cf ref[9]) revealed a preponderance of RNAs encoding proteins with nuclear functions (110 out of 842), 44 of which modify chromatin, particularly histone acetylation and methylation[83]. In line with the identity of these enzymes as chromatin modifiers, large increases in H3K4me3, H4K8ac, and H4K19ac levels were observed in *Fmr1* deficient neurons, which were accompanied by commensurate changes in the epigenetic chromatin landscapes. It was shown that Brd4, a bromodomain-containing

transcription factor elevated in *Fmr1* deficient mice could be targeted by JQ1, a small molecule inhibitor of this class of proteins. JQ1 mitigated aberrant dendritic spine density and several FXS-associated behaviors, suggesting a possible therapy to treat the disorder in humans.

Another chromatin modifier that is regulated by FMRP is SETD2, which catalyzes trimethylation of lysine 36 on histone H3 (H3K36me3). SETD2 mRNA is associated with FMRP-stalled ribosomes, but when FMRP is absent, translation of this transcript is elevated and results in an increase of SETD2 protein by ~2.5 fold[62]. A chromatin immunoprecipitation experiment followed by DNA sequencing (ChIP-seq) showed that the H3K36me3 landscape was altered in *Fmr1* KO hippocampus. H3K36me3 predominantly resides within the body of genes and mediates alternative splicing. Not surprisingly then, there is widespread dysregulation of alternative splicing, particularly exon skipping, in *Fmr1* deficient hippocampus. Interestingly, a number of these mis-splicing events also occur in human postmortem autistic brain, indicating a molecular convergence between FXS and other ASDs[62].

A complementary study observed elevated levels of the histone acetyltransferase EP300 and ubiquitin-mediated destruction of the histone deacetylase HDAC1 in *Fmr1*-depleted adult mouse hippocampal neural stem cells[84]. Treating the cells with nutlin-3, a compound that inhibits the interaction between the ubiquitin ligase MDM2 and HDAC1 restored histone acetylation to normal levels in *Fmr1*-deficient NPCs, which not only rescued characteristic over-proliferation and differentiation defects of NSCs but also several behavioral deficits of *Fmr1* deficient mice.

Not only does FMRP modify chromatin indirectly by regulating the translation of mRNAs encoding epigenetic and transcription factors[62,83], in non-neuronal cells, it also acts directly in the nucleus to bind and alter chromatin structure[85]. By way of its Agenet domain, FMRP regulates the levels and positioning of gammaH2A.x, a histone H2 subtype associated with cell death, in response to replicative stress in mouse embryo fibroblasts and mammalian spermatocytes. Without FMRP, spermatocytes are unable to undergo DNA repair and resolve single stranded chromatin intermediates at the pachytene stage, a necessary event for meiotic progression. Whether FMRP directly regulates chromatin in neurons and if so whether it plays a role in activity and/or stress-induced cellular responses has not been assessed.

Linking FMRP molecular biology to FXS

By highlighting recent advances of the molecular biology of FMRP, we can now ask what new mechanisms need to be explored, what FMRP activity tells us about FXS, and whether this information can form the basis new therapeutic development to treat the disorder. One clear message is that FMRP acts at multiple levels of gene expression, and that an original driving force for investigating this protein – that it is a translational repressor – is certainly true but is far too simplistic. It is also too simplistic to think that FMRP performs the same molecular tasks in all cells. The fact that FMRP interacts with many different proteins and RNAs and in certain combinations in particular cells as noted in this essay makes it self-evident that molecular readouts of the protein will have cell type specificity (Figure 5). Even

so, there are several general themes of FMRP molecular biology that are useful to consider, particularly where new avenues of investigation are needed.

FMRP target selection

The observation that FMRP binds mainly coding sequences of mRNA has now been made several times in several cell types, but what remains unclear is how this occurs. Candidate cis recognition elements in RNA have been proposed, but they are far from absolute requirements[46, 47]. The G4 structure identified by FMRP in *in vitro* binding assays appears clear, yet the structure generally is not detected in CLIP studies and has been questioned as to whether it even exists *in vivo*[86]. What distinguishes CDS from UTRs are, of course, ribosomes, so perhaps ribosomes are involved in positioning where FMRP binds[9]. In any event, the mechanism by which FMRP binds CDS remains to be determined. Recent identification of cell type specific targets for FMRP strongly suggests that FMRP has distinct roles in different cell types in different brain regions. Therefore, studies of cell type-specific FMRP targets may point to certain brain circuitry that goes awry in FXS, which in turn could suggest more targeted therapies to mitigate pathophysiology of the syndrome.

FMRP regulates translation

This is certainly true, but it can repress or activate specific RNAs[58]. Many studies have now shown that FMRP regulates ribosome translocation, but how it does so is unknown. A key extant question is whether FMRP acts directly on the ribosome as suggested by *in vitro* reconstitution studies[70]. Even if FMRP does interact with the ribosome, it is not clear how mRNA specificity would simultaneously be achieved. The importance of understanding how FMRP controls translation is evidenced by a number of FXS rescue paradigms in mice that focus on the translational machinery or signaling pathways leading to it[11]. Specific molecules targeting translation factors or the ribosome could lead to new therapeutic development.

FMRP regulates nuclear activities including transcription and splicing

These are newly emerging functions of FMRP requiring further investigation. Two studies[62,83] indicate that transcription and splicing are likely to be downstream activities of FMRP-regulated translation. On the other hand, another study demonstrates that FMRP acts directly on chromatin[85]. FMRP has an NLS and NES and although it is predominantly cytoplasmic at steady state, shuttling in and out of the nucleus may occur rapidly, which could be why potential nuclear activities of FMRP have been overlooked. Indeed, the etiology of autism is associated with changes of chromatin-modifying factors in early development and synaptic proteins primarily during postnatal periods. Because FMRP directly binds and regulates many mRNAs related to autism genes, one avenue for FXS treatment at early developmental stages could target epigenetic chromatin modulators.

FMRP and RNA modifications

Here, we refer to m[6]A and RNA editing by ADAR. These fields are rapidly evolving and are likely to expand the FMRP molecular biology universe in substantial ways. Key questions include: to what extent does FMRP recognize m[6]A and does it influence translation as well

as RNA stability? how widespread is RNA editing by the FMRP-ADAR complex? and does RNA editing drive FXS pathophysiology? Although this field is still in its infancy, FMRP-ADAR and RNA editing pathways may provide a rich source of targets for therapeutic development.

FMRP does not act in isolation

FMRP likely forms unique protein complexes in different cell types and cellular compartments, and in response to certain stimuli[87]. We described some of them in this review (e.g., FMRP-CYFIP1, FMRP-ADAR), but a comprehensive identification of FMRP interacting proteins at a resolution that mimics that of CLIP-seq has not been performed in neural cell types. Even so, it is already evident that such proteins help define FMRP functions including mRNA target selection. A key extant question is whether differing patterns of expression of FMRP-interacting proteins help promote phenotypic diversity in FXS full mutation populations. The identification of FMRP-interacting proteins in specific neural cell types, while technically challenging, may provide a gateway to understanding how FXS is a spectrum of deficits.

Translation to therapy

A number of therapies to treat FXS are based on the neural activities that go awry when FMRP is absent, and for the most part do not take into account, nor do they need to account, just what FMRP does. For example, agonists or antagonists for certain synaptic receptors that modulate the hyper-excitability of neurons in FXS have been used frequently as therapies, albeit with mixed results[5]. Agents that regulate other signaling pathways to or from FMRP have also been used, for example the diabetes drug metformin, which shows some promise[88]. One possible new therapy could be based on the observation that ribosome translocation is excessive in FXS. Certain antibiotics slow or stop ribosomes, so perhaps these may useful therapeutically[9]. Going further, JQ1, the inhibitor of bromodomain-containing transcription factors that are over-synthesized in FXS ameliorates some pathophysiological symptoms of the disorder in mice[83], and perhaps it may be extendable to humans.

The vast majority of successful therapeutic studies of FXS are based on highly in-bred mouse models but when limited tests are extended to human FXS populations the results have been mixed. Although general inter-species differences can certainly account for the mouse-human disparity, the genetic and epigenetic diversity among FXS individuals may limit the utility of mouse models. A deep molecular understanding of FMRP, its interactors and modulators, will be key elements in future clinical trials and therapeutic applications.

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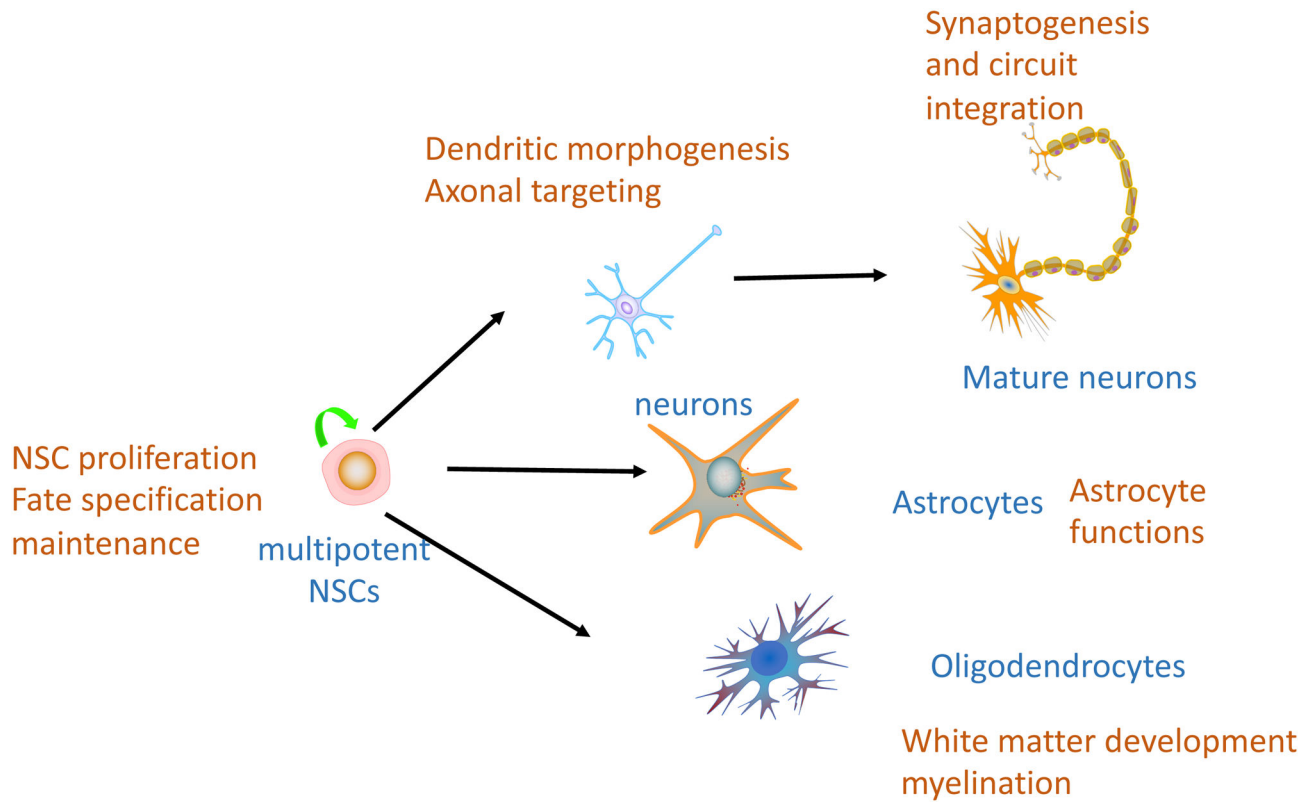
Figure 1. Brain cell types affected in FXS.
FXS is a neurodevelopmental disorder resulting from FMRP deficiency, which adversely affects nearly every aspect brain development and functioning of many brain cell types. FMRP-deficient neural stem (progenitor) cells (NSCs) exhibit altered proliferation and fate specification in both developing brains as well as adult neurogenic zones. FMRP-deficient neurons display stunted dendritic morphogenesis, altered axonal targeting, reduced synaptogenesis, and abnormal circuit integration. FMRP is also expressed in astrocytes where it regulates astrocyte-neuron interactions. FMRP deficiency may lead to transient impairment in white matter development.
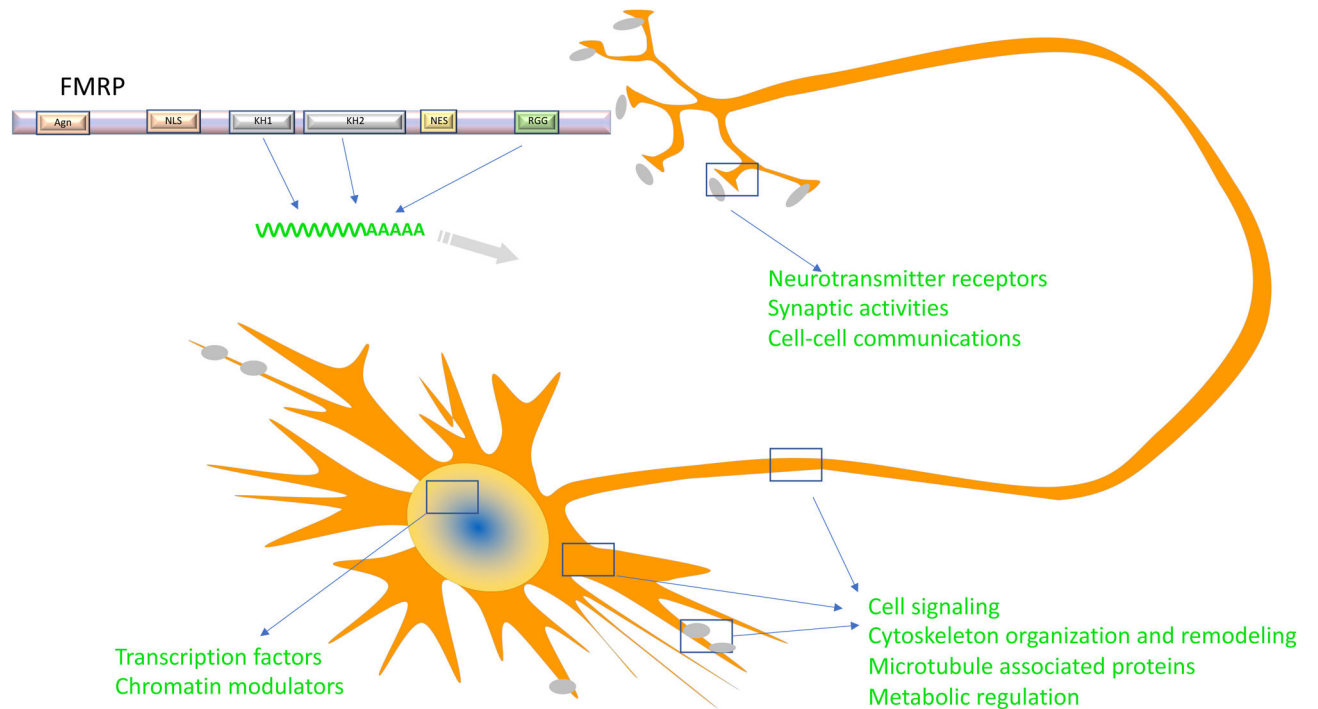
Figure 2. Functions of FMRP targets.
FMRP protein contains multiple domains that are important for its functions, including two Agenet/Tudor domains (Agn) for binding DNA and other proteins, nuclear localization (NLS) and exit (NES) sequences, and several RNA binding domains, KH0, KH1, KH2, and RGG box. FMRP binds >1000 mRNAs in the brain that are involved in neural processes such as synaptogenesis (synaptic proteins), cell-cell communication (neurotransmitter receptors), cytoskeleton and microtubule modulators, and metabolic regulators. FMRP also binds to mRNAs encoding transcription factors and epigenetic chromatin modulators, especially in immature cells (e.g. NSCs and immature neurons), although transcription factors and chromatin modulators are not among the top categories identified in neurons or brain tissue.
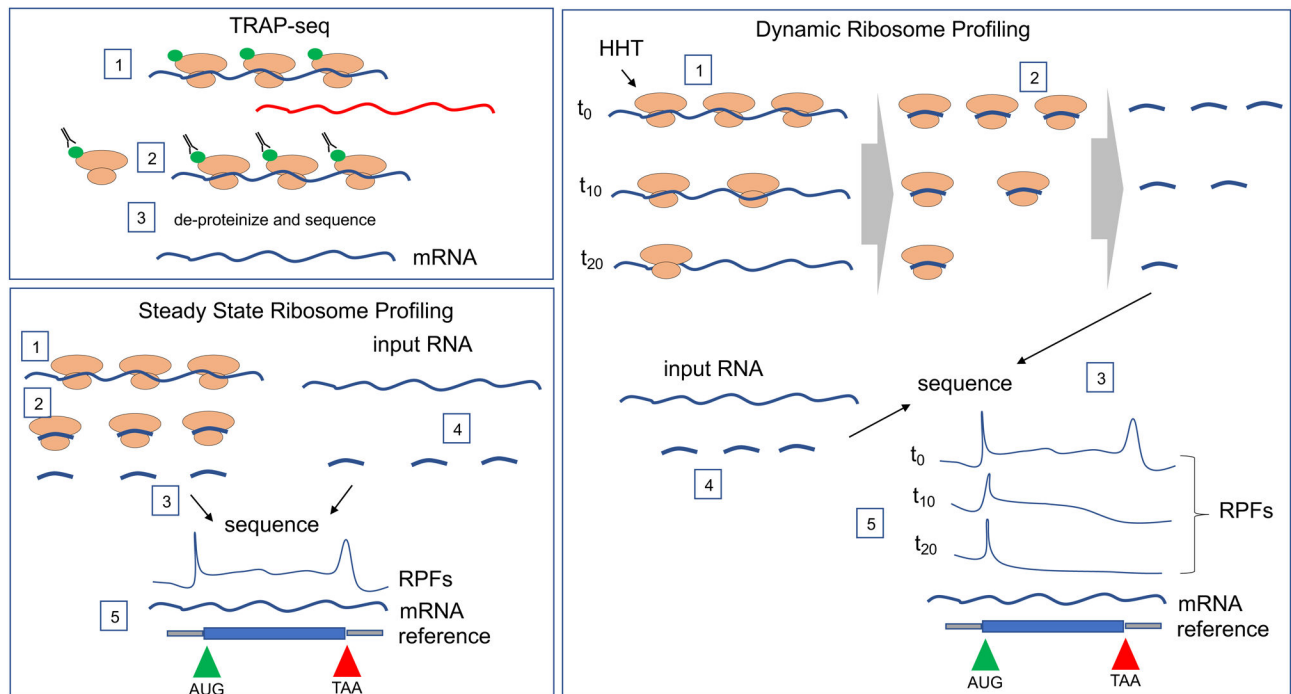
Figure 3. Methods for the analysis of transcriptome-wide translation.
TRAP (translating ribosome affinity purification)-seq. Cells or tissues are transduced
with an epitope-tagged ribosomal protein (green), which can be expressed in specific cells or
tissues (step 1). The cells/tissues are then subjected to immunoprecipitation with antibody
against the epitope (usually HA, FLAG, or GFP) (step 2); the co-precipitating RNA is then
de-proteinized and sequenced (blue line) (step 3). Note that mRNAs associated with few or
no ribosomes are not immunoprecipitted (red line). TRAP-seq has been performed in WT
and *Fmr1*-deficient mouse brain[35, 55] and has identified mRNAs under translational control
by FMRP in specific neuron subtypes.
Steady state ribosome profiling. In cells or tissues, ribosomes are "frozen" on RNA by
treatment with cycloheximide (step 1). The RNA is digested with RNase (step 2) and the
small mRNA fragments protected from hydrolysis by the ribosomes are collected and
sequenced (step 3). Input total RNA, which serves as a measure of all mRNA in the cells is
also sequenced (step 4). The ribosome protected fragments (RPFs) and input mRNA are then
aligned to the reference genome (step 5). RPFs are generally higher of the start (AUG) and
stop (TAA) codons and are not present on 5' or 3' untranslated regions. Steady state
ribosome profiling from WT and *Fmr1*-deficient cells or brain tissue has identified mRNAs
whose translation is up and down regulated by FMRP[58,59,60,61]. **Dynamic ribosome
profiling.** Cells or tissues are treated with homoherringtonine (HHT), which freezes
ribosomes on initiation codons but allows translocating ribosomes to continue to elongate
polypeptides (step 1). The ribosomes are evident at t_{10} and t_{20} when compared to t_0. At
various time points after HHT, the translocating ribosomes are frozen by treatment with
cycloheximide (step 2), which is followed by ribosome profiling, as described above (step
3). Input RNA is also sequenced (step 4). Because there are fewer ribosomes at, for example,
t_{20} relative to t_0, there are fewer ribosome footprints. Ribosome footprints on the AUG start
codon (green) are maintained throughout the time course where as footprints on the TAA

stop codon (red) are generally reduced over time (step 5). The time it takes for ribosomes runoff following HHT treatment has demonstrated that on most mRNAs, ribosomes translocate quickly whereas on other mRNAs, the ribosomes translocate slowly or nearly not at all[62]. Dynamic ribosome profiling performed using WT and *Fmr1*-deficient mouse brain has revealed that FMRP stalls ribosomes on specific mRNAs[62]. Note that dynamic ribosome profiling, but not steady state ribosome profiling, can distinguish between translocating and stalled ribosomes.
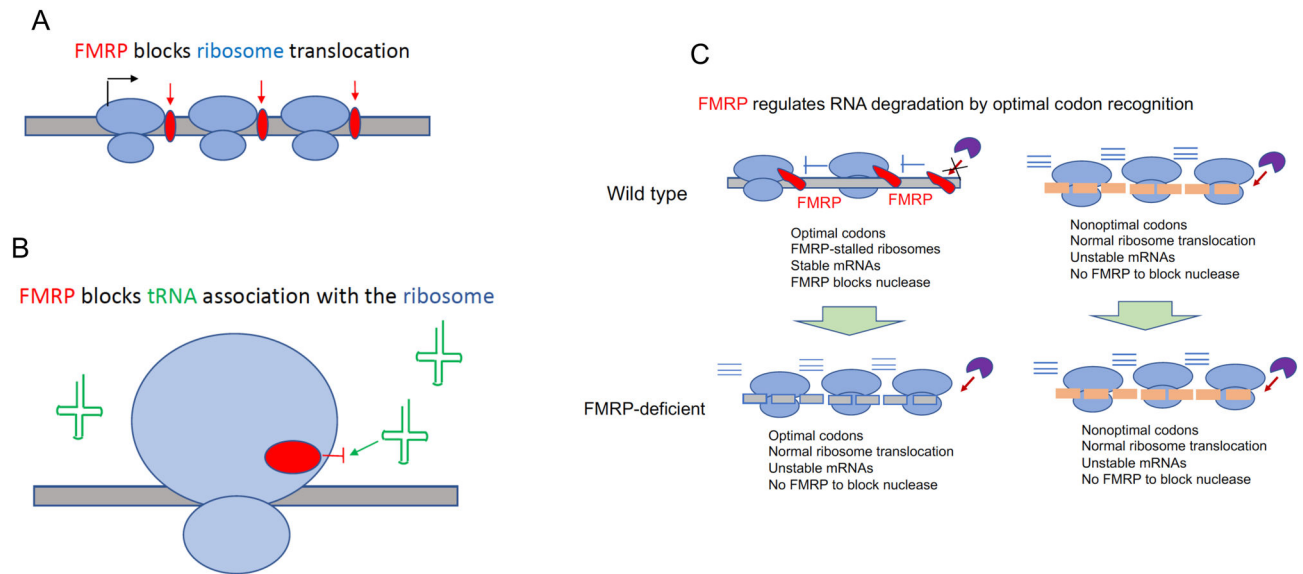
Figure 4. Mechanisms of FMRP-regulated translation.

A. FMRP as a roadblock to ribosome translocation. FMRP binding to mRNA might impede ribosome translocation, thereby leading in reduced polypeptide elongation.

B. FMRP as a ribosomal protein that inhibits tRNA association with the ribosome. *In vitro* reconstitution experiments followed by cryo-EM analysis suggest that FMRP binds the 60S ribosomal subunit in such a manner as to prevent tRNA and/or elongation assembly into the ribosome, thereby causing ribosome stalling[70]

C. FMRP and codon optimality. According to one current model, FMRP directly or indirectly associates with the translational machinery on wild type mouse cortex mRNAs that have optimal codons and stalls ribosomes (as indicated by the blue |-). In this model, FMRP also blocks a hypothetical nuclease(s) (purple pac-man) to prevent RNA degradation. Because there is no FMRP association with the translational machinery on RNAs with nonoptimal codons, these RNAs have normal ribosome translocation but the mRNAs are unstable because nuclease attack is not hindered by FMRP. Because of this RNA instability, there is reduced protein production. In FMRP-deficient cortex, RNAs with optimal codons are associated with normally translocating ribosomes, but the RNAs are unstable because there is no FMRP to block prevent nuclease hydrolysis. RNAs with nonoptimal codons behave similarly in FMRP-deficient cortex as they do in wild type cortex[63]. According to this model, FMRP regulates protein synthesis primarily at the level of RNA degradation as opposed to translational control.
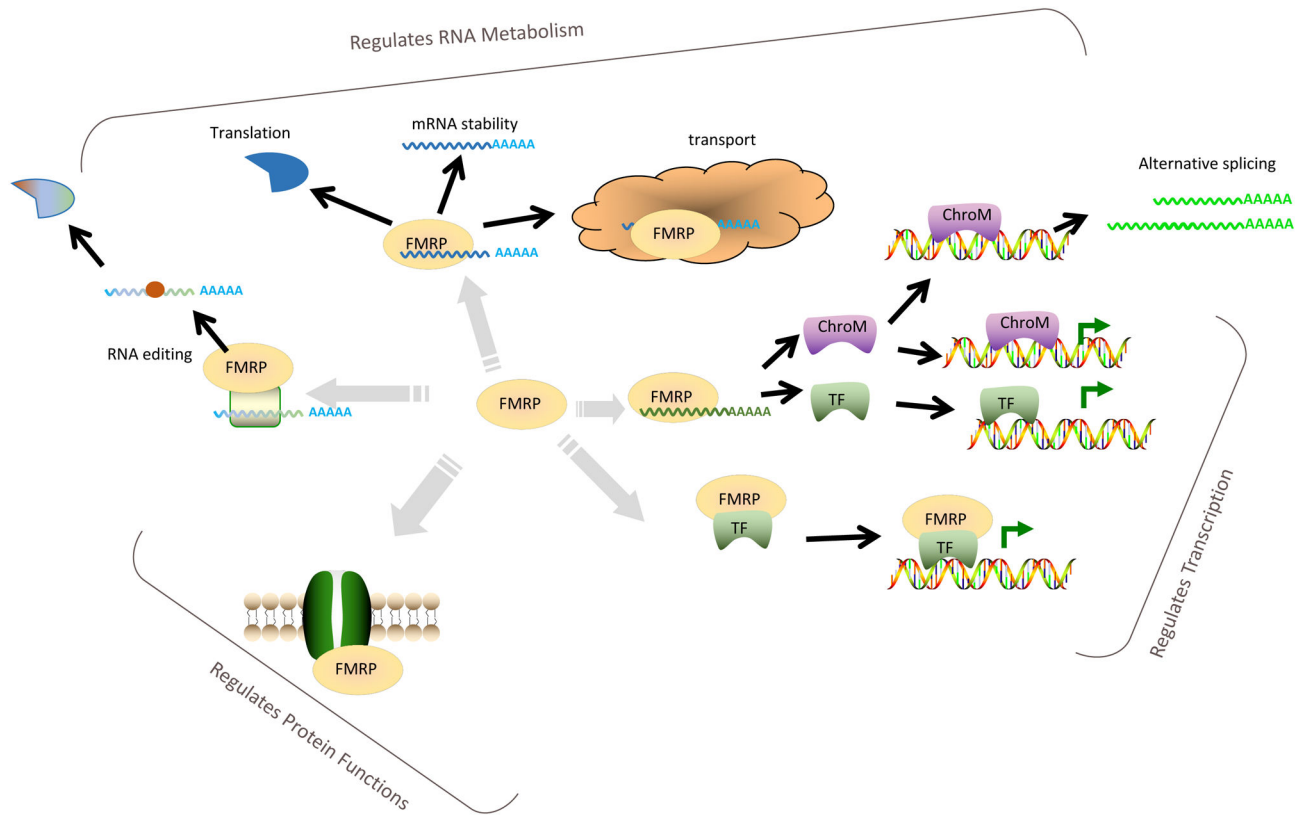
Figure 5. Summary of FMRP activities.

FMRP is a multi-functional protein with diverse mechanisms of action; it binds mRNAs and may regulate their translation, stability, editing, or intracellular transport. FMRP also directly interacts with proteins (such as ion channels) and regulates their function. Finally, FMRP can regulate RNA synthesis by either controlling the expression of or modulating the activities of transcription factors and chromatin modifying enzymes

Table 1.

High throughput identification of FMRP targeted mRNAs

Publication	species	tissue/cell types	Method	numbers of target	binding regions	motif of targets	other features	Top categories of targets
Brown 2001 (32)	mouse	juvenile mouse forebrains	Anti-FMRP antibody (7G1–1 mAb; Brown et al 1998 (89), RIP-microarray	432	unknown	67% (8 out of 12 top ranked targets has G4		no category specified.
Miyashiro 2003 (33)	mouse	primary hippocampal neurons	Anti-FMRP antibody (1C3 mAb; Devys et al 1993 (90); APRA	83	unknown	23% (18 of 83) has G4		Cell signaling, cell-cell communications, cell structure/ mobility, gene/ protein expressions.
Darnell 2011 (9)	mouse	juvenile mouse forebrains	Anti-FMRP antibody (7G1–1 mAb; Brown et al 1998 (89), CLIP-seq	842	mostly coding sequence, also 3'UTR	none (no G4 abundance)	661 are new compared to Brown 2001	synaptic transmission, neuronal activity, membrane, cell signaling, autism
Maurin 2018 (34)	mouse	hippocampus, cortex, cerebellum	Anti-FMRP antibody (Rb#11, pAb; this study), CLIP-seq	4174 total from all 3 brain regions	coding sequence enriched with GAC codon	CTGKA; GCTGYY; GWRGA; CATCRYC and TAY motifs; G4 is enriched.	680 shared with Darnell 2011; 192 new	about 1/4 are distantly transported mRNAs; Top pathways include: cell signaling, neuronal activity, synaptic function, microtubule.
Van Driesche 2019 (35)	mouse	cerebellar Purkinje cells (Pcp2-Cre) and granule cells (NeuroD1-Cre),	Anti-GFP mAbs (HtzGFP19C8 and HtzGFP19F7, Heiman 2008 (91); from FMRP-cTag mice	135 high confidence FMRP CLIP targets in Purkinje cells; 259 in granule cells, they are distinct	Long, coding sequences	none	has ranked targets based on TRAP-seq of same cell types;	Synaptic functions; microtubule, Cell signaling, GTPase signaling in Purkinje cells, GABAergic transmission in cerebellar granule cells
Sawicka 2019 (36)	mouse	CA1 neurons (Camk2a-Cre)	Anti-GFP mAbs (HtzGFP19C8 and HtzGFP19F7, Heiman 2008 (91); from FMRP-cTag mice	327; different from cb granule neurons	Long, coding sequences	none	has ranked targets based on TRAP-seq of same cell types;	Signaling, transcription, synaptic development and function, microtubule organization, axon transport, autism, circadian rhythm
Ascano 2012 (42)	Human	HEK293 cell lines	Transfected HA-tagged FMRP, anti-HA ab (MMS-101P, Convance), PAR-CLIP	>6000	coding sequence and 3'UTR	ACUK, WGGA		many
Tran 2019 (43)	Human	postmortem adult human frontal cortex	Anti-FMRP pAb (MBL,	4895	binds to A-to-I edited site;	ACUG		Synaptic functions, (focus was editing in

Publication	species	tissue/cell types	Method	numbers of target	binding regions	motif of targets	other features	Top categories of targets
			RN016P), CLIP-seq		predominantly 3'UTR			autism, not FMRP targets)
Li 2020 (44)	Human	human iPSC differentiated dorsal and ventral forebrain NPCs and neurons (four cell types)	FLAG tag knocked into human FMR1 locus, FMRP-FLAG, anti-FLAG ab (Sigma Aldrich F1804), CLIP-seq	1653 total (1232 in dNPCs, 1234 in vNPCs, 629 in dNeurons, 721 in vNeurons	coding sequences, long genes	CUAC; UGGA; GAUG, AGGU, CAGC; unclear about G4		Neurogenesis, axon and dendrite morphogenesis, cytoskeleton, microtubule, RNA transport, histone modification (especially in NPCs), autism.