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Regulating a translational regulator: mechanisms cells use to control the activity of the fragile X mental retardation protein

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Abstract. Fragile X syndrome results from the loss of a normal cellular protein, FMRP. FMRP is an RNA binding protein, and it is likely that altering the way FMRP's messenger RNA (mRNA) targets are processed results in the clinical features associated with the disease. Using complementary DNA microarray screening, a number of brain-derived mRNAs that interact directly with FMRP in vitro and associate with FMRP-containing mRNPs in vivo have been identified. These target messages encode RNA-binding proteins, transcription factors, neuronal receptors, cytoskeletal proteins, a few enzymes as well as several unknown proteins. For a subset of these mRNAs it has been shown that modulating FMRP levels in cultured cells correspondingly affects their expression. In addition, several modes by which cells modulate FMRP activity have been described; these include posttranscriptional processing and posttranslational modification. Here, the most recent results concerning the biochemical activities of FMRP and how they are affected by various modifications are reviewed. The data lead to a model signaling mechanism by which FMRP normally regulates the expression of its target mRNAs.

Key words. Fragile X syndrome; alternative splicing; translational control; posttranslational modification; asymmetric dimethylarginine; phosphorylation.

Introduction

Fragile X syndrome (FXS) is one of a growing number of triplet-repeat disorders [1]. FXS results from the loss of the fragile X mental retardation protein (FMRP) through CGG- repeat expansion in exon 1 of the *FMR1* gene, which causes transcriptional silencing [2]. Less commonly, *FMR1* gene deletions or coding sequence mutations produce the phenotypic features of the syndrome [3–5]. The hallmarks of this disease are mild to severe cognitive impairment and macroorchidism. In addition, other abnormalities, including autism, attention deficit disorder and increased susceptibility to seizures are also linked to the disease [6–9].

From the initial in silico translation of its mRNA, to a variety of subsequent empirical studies, FMRP has been shown to be an RNA-binding protein [10, 11]. Structural analysis has shown that the protein is a member of a unique family of RNA-binding proteins that include its paralogs, FXR1P and FXR2P [12]. All three proteins bear two hnRNP K-homology (KH) domains and a C-terminal arginine-glycine-rich region that has been called an RGG box. Because of these features it has been hypothesized that changes in cellular RNA metabolism i.e. in altered (i) messenger RNA (mRNA) stability, (ii) mRNA trans-

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port, (iii) mRNA localization or (iv) mRNA translation, resulting from the loss of FMRP produces the phenotypic features of the disease [13–15].

To assess whether this hypothesis is valid, one needs to know, first, which mRNAs FMRP interacts with and, second, what role the interaction plays in their normal cellular metabolism. Several groups, using variations on a classic binding-amplification-selection strategy, have published lists of putative FMRP targets [16–22]. For a few of these putative targets, it has been demonstrated that FMRP actually binds to them in vivo and regulates their activity. These include EF-1A mRNA [23], Tip60a mRNA and NF-κBp65 mRNA [24], FXR1P$_{84}$ mRNA and SIX3 mRNA [25] and MBP mRNA [26]. Others have a more tenuous association with FMRP, being able to bind in biochemical assays, but with no apparent effect when FMRP levels are perturbed in vivo. Such messages include the GABA$_A$ receptor δ subunit [20], and RhoA mRNA [21]. For still others only in vitro binding data exist [20, 22].

Although a host of phenotypic features result from the loss of the fragile X mental retardation protein (FMRP), as its name suggests, it is the mental retardation (MR) associated with the disease that is its hallmark. Strikingly, early neuropathological studies of fragile X patients found no specific brain deformations or neurodegeneration; however, subtle anatomical abnormalities such as alterations in the size, shape and density of dendritic spines were reported [27, 28]. Such changes are a common feature of MR [29, 30]; however, in fragile X syndrome unlike other MRs the density of dendritic spines is apparently increased. Studies using fragile X knockout mice have, to a large degree, recapitulated these results [31–35]. As dendritic spine development and maturation are required for proper brain connectivity [36], the data suggest that FMRP may play a role in the development of mature dendritic spines and, conversely, that its absence delays their development. Finally, results from organotypic cultures of hippocampal or pyramidal neurons further suggest that synaptic stimulation plays an important role in this process [33, 34].

There have been several recent reviews of FMRP [22, 37–46]. Most have focused on (i) the identification and characterization of FMRP's mRNA target messenger RNAs, (ii) the components of FMRP-messenger ribonucleoprotein particles (mRNPs), (iii) FMRP's potential links to synaptic plasticity and (iv) fragile X animal models and various aspects of FMR1 triplet repeat pathobiology. Here we examine some less-discussed aspects FMRP's workings, namely its posttranscriptional regulation, its role in translational regulation and posttranslational modifications that affect various biochemical properties of the protein, particularly those in its arginine-glycine-rich (RG-rich) domain, that may play important roles in its cellular functions.

Results

Posttranscriptional processing of FMR1 mRNA

The fmr1 gene contains 17 exons [47] that are alternatively spliced into variant mRNAs. Two types of FMR1 pre-mRNA alternative splicing occur, exon skipping and alternative splice site selection. FMR1 exons 12 and 14 can be skipped [48]. The loss of exon 12 results in an FMRP variant with 21 fewer amino acids and is less hydrophobic than the full-length form of the protein. The residues encoded by exon 12 alter the variable region of the KH$_2$ domain linking the β2 and β3 sheets. Recent x-ray structural studies of both mammalian [49] and bacterial KH domains [50] revealed that the variable loop region makes RNA contact points and serves as part of a molecular vise, clamping the RNA in place. Excluding exon 14-encoded amino acids results in the loss of FMRP's nuclear translocation signal and also in the production of +1 frameshifted proteins with novel C-termini.

Alternative splice site selection also occurs in FMR1 pre-mRNA. In humans, alternative splice site selection at exon 10 results in two variant proteins that differ by two amino acids, which lie in the α_1 helix of the KH$_2$ domain. Similarly, alternative splice site selection at exon 15 produces three FMRP isoforms, each of which differ in the predicted α-helix content of the region preceding the RG-rich domain [51] (fig. 1). Conservation of this type of splicing in both mice and human and in FXR1 pre-mRNA [52] may indicate that these proteins share an important common cellular function. Finally, alternative splice site selection at exon 17 produces two isoforms that differ by 17 amino acids near the C-terminus of the protein [53].

Alternative splicing of a pre-mRNA is a common mechanism for introducing diversity in the population of proteins expressed in cells and tissues. Because of this, Ashley et al. posited that the preceding variations might alter FMRP's function [47]. Indeed, effects resulting from alternative splicing have been found. For example, elegant work by a number of groups has conclusively demonstrated that FMRP isoforms lacking sequences encoded by exon 14 are predominantly nuclear due to the loss of a nuclear export signal [54–56]. In addition, the variant proteins also lack the RG-rich domain found in proteins in which exon 14 is not skipped. Hence, the RNA repertoire bound to these two sets of proteins is likely to be quite different. Similarly, isoforms that have exon 12 sequences excluded also should bind RNA differently.

The preponderance of FMR1 pre-mRNA alternative splicing occurs in exons encoding the RNA binding domains. This, coupled with the fact that alternative splicing in other RNA-binding proteins gives rise to variants with different nucleic acid specificities [57–61], led us to investigate whether particular FMRP isoforms have different RNA-binding properties. Using homoribopolymer mimetics and FMR1 mRNA (the only known target of

Figure 1. Known and potential posttranslational modification sites in FMRP. Pro-Site (http://us.expasy.org80/prosite/) and PepTool (BioTools) scans of human FMRP identified posttranslational modification sites for N-myristoylation (Myr), N-linked glycosylation (Asn), amidation (Am) and phosphorylation by PKC, CK1, CK2, cGK and TYR (underlined). Arginine residues that may be modified by protein arginine methyltransferases (red R) as well as those in contexts that are modified in other proteins (green R) are also shown. Residues encoded by FMR1 exon 15, which may serve as a functional regulatory switch, are bracketed. The sequence in this region contains 3 CK2 sites, including S_{499} (blue), which is known to be phosphorylated [80], 2 CK1 sites, 10 R residues, of which R_{544} and R_{546} (purple) are monomethylated by rat brain PRMT in vitro, an N-glycosylation site and a conformational switch (black highlight). The three alternatively spliced FMRP exon 15 isoforms (Ex15a, Ex15b and Ex15c) are also shown (dashed over line).

FMRP at the time of these studies), we demonstrated that two FMRP isoforms that differed in their KH_2 domains by including or excluding exon 12 residues had distinctive in vitro RNA binding profiles. Furthermore, two other isoforms that arose by alternative splice site selection at exon 15, leading to distinct sequences preceding the RG-rich region, also bound RNA uniquely [62].

Based on this we proposed that FMRP uses unique sets of protein determinants to bind its different target mRNAs. Recent independent biophysical studies examining the interaction of a purified bacterial recombinant FMRP peptide with short RNA transcripts that mimic G-quartet elements that have been shown, or hypothesized to interact with FMRP, have led to essentially the same conclusion [63].

These data strongly suggest that the diversity of FMRP variants produced by cells occur for specific, albeit unknown reasons. To explore this, it will be necessary to produce tissue- and cell-specific expression maps of each of the FMR1 alternatively spliced variants. In situ hybridization and Northern-blotting studies with variant-specific probes akin to those showing differences in FMR1 homolog expression [64, 65] should be performed first to give an indication of brain regions with unique FMR1 expression. Ultimately, however, it will be necessary to examine the expression of the various FMRP variants. To this end it will be necessary to develop antibodies that specifically recognize particular FMRP isoforms, as has been done with FXR1P [66]. In addition, both the mRNA and protein studies should have a developmental component to examine time-dependent changes in alternatively spliced variant expression.

Translational regulation of FMRP

From the initial findings that (i) FMRP associates with polyribosomes as a poly (A^+) RNA-containing mRNP complex [67] and (ii) that FMRP itself is locally synthe-

sized in response to metabotropic glutamate stimulation, an important role in translational regulation, especially that associated with synaptic plasticity, has been implied. Indeed, studies conducted by Li et al., Laggerbauer et al. and by Schaeffer et al. demonstrating that recombinant FMRP inhibits both poly (A^+) mRNA translation and select mRNA translation in vitro [68–70] support this hypothesis. In addition, the altered distribution of mRNAs on fragile X and control lymphoblastoid polyribosomes has been interpreted to indicate that the loss of FMRP alters a particular subset of mRNPs, resulting in their 'abnormal' translation [18]. In keeping with this hypothesis, Feng et al. found that a naturally occurring KH_2 domain missense mutation, $I_{304}N$, associated with a severe FXS phenotype is incorporated into abnormal mRNP particles that are not associated with polyribosomes [71].

Recently, Sung et al. and Dolzhanskaya et al. have provided the first evidence that FMRP negatively regulates mRNA translation in vivo [23, 24]. Specifically, they found that the expression of mRNAs encoding the translation elongation factor, EF-1A, the transcription factor, NF-κBp65, and the nuclear hormone co-activator, Tip60a, are elevated ca. three-fold in fragile X lymphoblastoid cells, while their corresponding mRNA levels remained the same. Furthermore, NF-κBp65 elevation, coupled with the constitutive activation of an upstream signaling pathway that phosphorylates inhibitor of $\kappa B\alpha$, (I-$\kappa B\alpha$), results in elevated levels of transcriptionally competent nuclear NF-κBp65. FMRP binds to each of these mRNAs in vitro. More important, each mRNA associates with FMRP mRNPs in PC12 cells that express human FMRP, and this association represses its translation. Subsequent work by Mazroui et al. and Wang et al. has demonstrated that FMRP also negatively regulates the expression of several other mRNAs [25, 26]. In the case of Wang et al. they demonstrated that in oligodendrocytes, FMRP negatively regulates the expression of myelin basic protein (MBP) by binding to the 3′UTR (3′ untranslated region) of MBP mRNA. Furthermore, the observed regulation occurs temporally during early brain development via changes in the local concentration of FMRP. Interestingly, and a point not mentioned by Wang et al., the region in MBP mRNA they delimited for efficient FMRP binding (Ac# BC008749, bases 947–1126) contains two 20-base U-rich elements [21] with significant homology to part of the U-rich region in the FMR1 mRNA 3′UTR, (Ac#, bases 2796–2872). Previous work has shown that FMRP binds to the U-rich region in the FMR1 mRNA 3′UTR in vitro [17], and to U-rich elements in other mRNAs in vivo [72]. Thus, these data underscore the importance of this novel binding element in the biology of FMRP.

While there is compelling evidence that FMRP negatively regulates the expression of some mRNAs, the question of whether it can also positively regulate the expression of other mRNAs remains open. The microarray studies of Brown et al., which found that the distribution of certain mRNAs on fragile X lymphoblastoid polyribosomes increased compared with controls, while others decreased led them to conclude that depending on its context, FMRP may act either as a positive or negative regulator of translation [18]. Interestingly, Brown et al. found that the mRNA-encoding microtubule-associated protein 1B (MAP-1B) was increased on fragile X lymphoblastoid polyribosomes. This finding suggests that FMRP negatively regulates its translation. In an effort to find a common structural feature shared by messages that bind to FMRP, Darnell et al. proposed that nucleic acid tertiary structure elements called a G-quartets are disproportionately represented in the list of putative FMRP targets isolated by Brown et al. They found that MAP-1B mRNA contains a sequence in its 5′UTR that partially matches a search string they developed to find G-quartet-containing mRNAs [19]. Based on these data, Zhang et al. working with the *Drosophila* ortholog of FMRP demonstrated that futsch mRNA, a MAP-1B homolog, associated with dFMR1-containing mRNPs and its expression increased in dFMR1 null flies [73]. However, futsch's 5′UTR has very limited homology to its MAP-1B counterpart, and again, the sequence contains imperfect G-quartet string candidates [J. R. Currie, unpublished observation]. In contrast to these results Chen et al. showed that MAP-1B protein levels were decreased in specific brain regions of fragile X knockout mice, consistent with FMRP acting as a positive regulator of its translation. This apparent anomaly may be explained by the misidentification of the MAP-1B clone by Brown et al. Based on their failure to pick up MAP-1B mRNA using antibody positioned RNA amplification (ARPA), Miyashiro et al. reexamined the Brown et al. dataset and found that the clone reported as MAP-1B was 98.74% similar to MAP-1A. Additionally, a true MAP-1B clone on the chip was not reported as being an mRNA with which FMRP interacted [20]. Thus, the discrepancy in the action of FMRP proposed by Brown et al. and Chen et al. can be explained by the fact that they were examining two similar but nonidentical messages, MAP-1A, which may be negatively regulated by FMRP, and MAP-1B, which may be positively regulated by FMRP. Nevertheless, this should provide a cautionary lesson concerning the minimal requirements for reporting whether FMRP regulates translation of a particular mRNA. First, FMRP should be able to bind the full-length mRNA in vitro. Second, the mRNA should associate with FMRP-containing mRNPs in vivo. Third, the putative target protein levels, but not mRNA levels, should change when FMRP levels change. For MAP-1A and futsch mRNA direct binding to FMRP has not been demonstrated. In the case of MAP-1B mRNA, a small RNA corresponding to the putative G-quartet in the 5′UTR of the message interacts with a bacterial recombinant FMRP peptide encoding the RG-rich region in vitro

[63], but whether an interaction occurs with the full-length message is unknown. Additionally, MAP-1A and MAP-1B protein and mRNA levels have yet to be assessed when FMRP levels are perturbed.

Recently, it was proposed that FMRP directly associates with the dendritic, nontranslatable RNA, BC1, and that this interaction forms the basis for regulating the binding and translation of FMRP target mRNAs [74]. In support of this hypothesis, Zalfa et al. showed that (i) BC1 RNA and FMRP cofractionated with mRNPs on sucrose gradients, (ii) in electrophoretic mobility shift assay (EMSA) experiments recombinant FMRP bound BC1 RNA, (iii) certain FMRP target mRNAs have a weak inverse homology with the 5′ domain of BC1 RNA and (iv) artificially hybridizing blocking oligonucleotides to the 5′ end of BC1 RNA disrupted the binding of these messages. However, these data conflict with the data of Sung et al. who found that FMRP associated very weakly with BC1 RNA in vitro [17, 22]. How can this be?

There are several anomalies in the Zalfa data that are readily apparent. First, the initial impetus for examining the possibility of a BC1 RNA/FMRP connection rested on the finding that BC1 RNA cofractionated with FMRP in mRNPs on sucrose gradients. This, however, is inconsistent with the finding of many other laboratories that FMRP sediments with polyribosomes [23, 25, 67, 71, 75–81]. In addition, the EMSA experiment, which used an ~1000-fold excess of baculovirus-produced recombinant FMRP/RNA, showed a weak association between the components that could well be interpreted as marginal and may in fact be related to differences in its posttranslational arginine methylation state (see below). Furthermore, it should be pointed out that an interaction, if it did occur, would depend on binding to an as yet unrecognized FMRP RNA binding motif, since BC1 RNA harbors neither a G-quartet [70] nor a U-rich sequence element [21, 72]. Finally, not all FMRP target mRNAs have recognizable homology to the 5′ end of BC1 RNA [R. B. Denman, unpublished observation].

Recent experiments by Wang et al. have shed some light on the role that BC1 RNA plays in translational regulation [82]. Specifically, these authors demonstrated that BC1 RNA forms complexes with both the polyadenylation binding protein (PABP) and with eucaryotic initiation factor 4a (eIF-4a), and in doing so prevents the formation of a stable 48S pre-initiation complex from eIF-4a/PABP-bound mRNA and a 43S pre-initiation complex. In contrast to the results presented by Zalfa et al., these interactions are robust, implying that even if FMRP bound to BC1 RNA, it would rapidly be displaced by these proteins. In addition, the results of a newer study by this group aimed at dissecting the mechanism by which BC1-mediated repression occurs revealed that the 3′ end of BC1 RNA and not the 5′ end contains the necessary determinants for repression (Wang et al, unpublished observation). By co-injecting either BC1 RNA or the 3′ end of BC1 RNA with a luciferase reporter gene into *Xenopus* oocytes, they found that they could significantly inhibit luciferase activity. In contrast, the BC1 5′ end had no effect on luciferase activity. Similarly, the 3′ end of BC1 RNA, but not the 5′ end prevented α-tubulin mRNA, a G-quartet-containing message found associated with FMRP in hippocampal neurons [20], from forming 48S pre-initiation complexes. These data convincingly demonstrate the BC1 RNA does not require FMRP to function as a generalized translational repressor. However, this does not preclude that an indirect or parallel relationship exists between the two molecules. In terms of spatio-temporal distribution for example, Ohashi et al. have found that the DNA/RNA-binding protein, pur-α, coimmunoprecipitates with FMRP-containing mRNPs [83], and pur-α may be involved in transporting BC1 RNA into dendrites [84]. Furthermore, staufen-containing mRNPs, in which one of the major associated RNAs is BC1 RNA [85], also contain FMRP [83]. Therefore, one can assume that BC1 RNA and FMRP can under some circumstances exist within a common mRNP. Furthermore, these new data suggest that translational repression in dendrites may be multilayered such that in order to translate certain mRNAs, one must lift both the general repression mediated by BC1 RNA and that of a more specific repressor such as FMRP.

Another interesting aspect to the story of FMRP's role in regulating protein synthesis is found in the area of experience-dependent translation. Todd et al., using whisker stimulation as a model of experience-dependent plasticity, found that FMRP increased significantly in both rat synaptosomes and in a subcellular fraction enriched in polyribosomes following unilateral stimulation [86]. More recently, they have extended these findings by showing that the observed increase is due to new protein synthesis and does not require increased synthesis of FMR1 mRNA. Furthermore, the effect was blocked by the NMDA-type glutamate receptor antagonist diclozipine and by the metabotropic glutamate receptor type I (mGluRI) antagonist AIDA [87], indicating there are at least two routes to locally increase FMRP near the synapse. Using a different model for experience-dependent plasticity, Wells et al. have also shown that FMRP increases in response to N-methyl-D-aspartate (NMDA)-type glutamate receptor stimulation [88]. Specifically, they have shown in the visual cortex of dark-reared rats exposed to light for brief periods that Fmr1 mRNA is polyadenylated, and that this leads to a significant, albeit transient increase in its translation near synapses. The mechanism by which this occurs depends on binding of the cytoplasmic polyadenylation element binding protein (CPEB) to a CPE sequence in the 3′UTR of Fmr1 mRNA and can be blocked by treatment with the NMDA receptor antagonist 3-(2-carboxypiperazin-4-yl) propyl-1-phosphonic acid (CPP) prior to light exposure [89].

Although the above studies have extended our knowledge concerning various aspects of FMRP's role in translation, other questions remain. One important question is, What step in translation does FMRP block? Although its association with polyribosomes suggests that the step is downstream of initiation, recent unpublished results from this laboratory indicate that FMRP does not inhibit cap-independent translation, which could indicate a block somewhere in initiation. Experiments using inhibitors that selectively target the various steps in initiation, 80S-ribosome assembly, elongation and termination are one means of resolving this issue, and they are currently being conducted.

Posttranslational modification of FMRP

The role post-translational modifications play in modulating FMRP's ability to (i) bind RNA, (ii) homo/heterodimerize, (iii) associate with polyribosomes or (iv) localize to a particular cellular compartment has received little experimental attention. Analysis of FMRP's sequence by tools such as ProSite [90] and PepTool reveal a host of motifs that could lead to its phosphorylation, amidation, N-myristoylation, N-glycosylation or methylation (fig. 1). As each of these activities might be influenced either positively or negatively by the others and could be developmentally or cell-specifically regulated, or subject to temporal stimulation, a large array of FMRPs with slightly altered properties could result.

A potential 'hotspot' of posttranslational activity is located in the sequences encoded by exon 15, which, as mentioned above, is subject to alternative splicing. The largest and smallest of these variants differ by 25 amino acids preceding the putative RG-rich region. These 25 amino acids contain a potential N-glycosylation site, two casein kinase II phosphorylation sites, a casein kinase I site, a potential protein arginine methylation site and a region of ambivalent secondary structure [91] indicative of a conformational switch [62] that the smaller variant lacks. These differences may well dictate the individual functions of the isoforms (see model below).

What possible reason could there be for such a cluster of potential modifications? The answer may well be to control the diverse functions of the RG-rich domain. While the functional role of FMRP's RG-rich region is not well known, important clues may be derived from the known roles RG-rich regions play in other proteins i.e. RNA binding, protein-protein interactions and protein localization. For example, protein kinase A (PKA) phosphorylation of hnRNP A1 at S_{199}, in a hinge region that lies just upstream of the protein's RG-rich domain, abrogates its ability to promote nucleic acid strand annealing [92]. Similarly, tyrosine phosphorylation of hnRNP A/B in its RG-rich region impairs its ability to bind some RNAs [93]. Furthermore, nucleolin's C-terminal RG-rich domain was found to be the binding site of 10 ribosomal large subunit proteins [94], while the N-terminal RG rich region of the pre-rRNA 2′-O-ribose methyltransferase, fibrillarin, contains binding determinants for splicing factor 2-associated p32 [95]. Finally, arginine methylation of hnRNP A2's RG-rich domain serves as a tag that relocates the protein to the nucleus [96]. If any, or all, of these changes applied to FMRP, they could significantly affect its activity.

FMRP methylation by protein arginine methyltransferases (PRMTs)

Three pieces of evidence highlight the potential importance of protein arginine methylation in the various cellular function(s) of FMRP. First, searching the available protein arginine methylation literature reveals that arginine methylation can have varied effects on the functions of other proteins. Second, anecdotal data exist stating that recombinant FMRP is methylated by a protein arginine methyltransferase found in HeLa cell extracts [97]. Third, it was reported that a peptide that mimics part of FMRP's RG-rich region is monomethylated by a protein arginine methyltransferase (PRMT) isolated from rat brain [98].

Protein arginine methylation commonly occurs in RG-rich domains of RNA-binding proteins [99–105]. To date, seven distinct PRMTs have been cloned from both higher and lower eucaryotes. Structural analysis indicates these enzymes represent a subfamily in the larger superfamily of S-adenosylmethionine-dependent methyltransferases whose members methylate RNA, DNA, protein, lipids and small molecules [106]. The seven PRMTs are further subdivided into two classes based on the type of arginine modification they confer. Class I enzymes produce asymmetric dimethyl-arginine-modified proteins, while class II enzymes produce symmetric dimethyl-arginine-modified proteins. Interestingly, each PRMT appears to have both unique and overlapping targets [107, 108]. The most prevalent PRMT, PRMT1, represents more than 90% of class I PRMT activity [109], and nearly 72% of the total methylating activity in PC12 cells [109]. PRMTs efficiently methylate proteins in vivo [97, 105]. The resulting modified proteins display biochemical properties, i.e. RNA binding, protein-protein interactions and protein localization, that differ from their nonmethylated counterparts [96, 101, 105, 110–115].

PRMT1 is developmentally expressed [116, 117], and its activity is positively modulated by interleukin enhancer binding factor 3 [109] and the tumor supressor protein BTG1 [118]. Both effectors modulate PRMT activity through direct interaction. In the latter case, it was shown that blocking the PRMT/BTG interaction with a BTG peptide analog interfered with PC12 cell and embryonic stem cell neuronal differentiation [119]. Similar effects were also observed in PC12 cells using general PRMT inhibitors [120], indicating that protein arginine methyla-

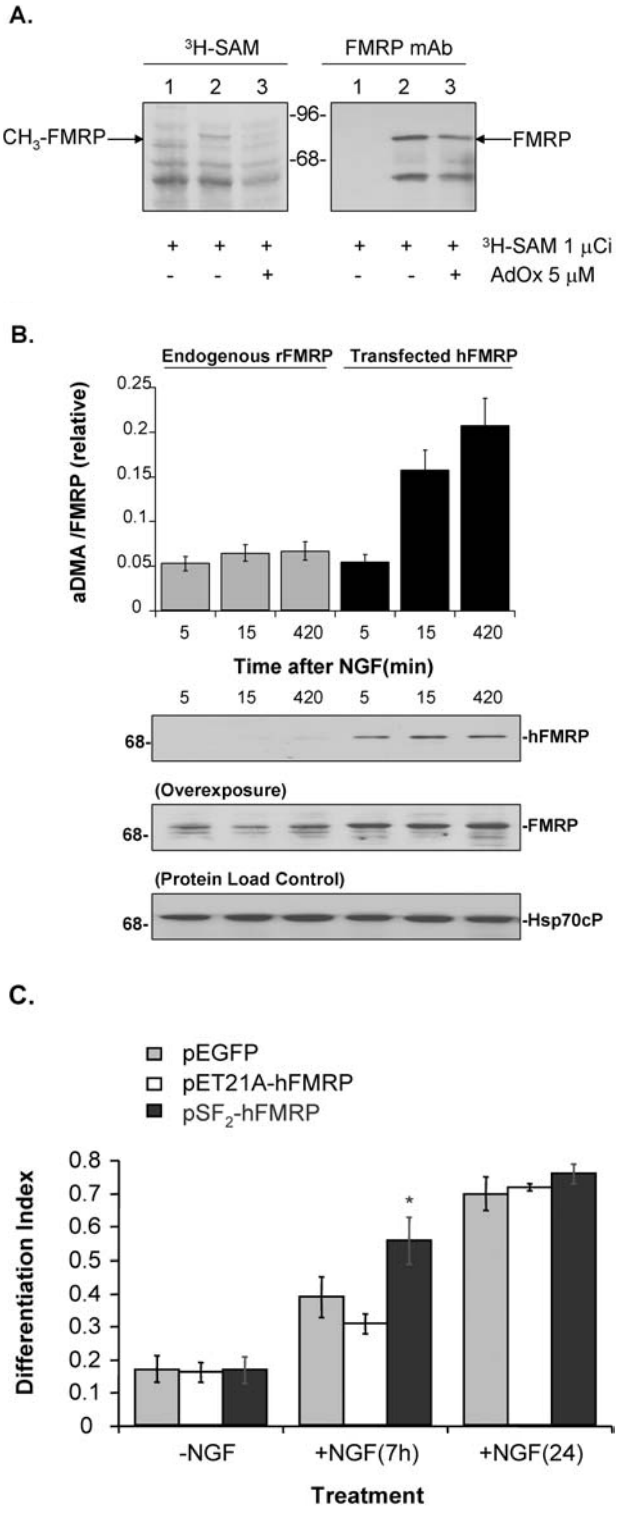

Figure 2. (*A*) FMRP is methylated by an endogenous PRMT in rabbit reticulocyte in vitro translation lysates. In vitro transcription translation of FMRP was carried out in the presence of 1 μCi of ^{3}H-SAM, and in the absence (lane 2) or presence (lane 3) of 5 μM AdOx in 25-μl coupled transcription translation reactions containing 1 μg of FMRP plasmid DNA. Lane 1 is a control reaction containing the lysate sans plasmid DNA. Duplicate 10-μl aliquots of each sample were resolved by SDS-polyacrylamide gel electrophoresis (PAGE). Half of the gel (lefthand panel) was fixed, then soaked in En^{3}Hance (Dupont) for 1 h and processed for fluorography as described [135]. The other half of the gel was blotted and probed with FMRP monoclonal antibody (mAb) 2160 (Chemicon) as described [23]. The resulting fluorogram shows ^{3}H-methyl incorporation into a band that comigrates with full-length FMRP (lane 2) and is absent from the lysate alone (lane 1), excluding the possibility that it arose by methylating another protein in the lysate. Furthermore, the band is reduced more than tenfold in AdOx-treated lysates (lane 3), indicating that it was produced by methylation and not by incorporation of ^{3}H-methionine into FMRP. Note that AdOx (lane 3), inhibits endogenous methylation in the RRL without affecting FMRP translation. (*B*) NGF stimulation of human FMRP-expressing PC12 cells alters FMRP's arginine methylation profile during a period of human FMRP-enhanced NGF-mediated neurite outgrowth. PC12 cells were transfected with a sham vector or pSF_2-hFMRP, which leads to the expression of human FMRP [23]. aDMA incorporation into endogenous PC12 cell FMRP in the sham vector transfected cells and human FMRP was followed after treatment with 200 ng/ml mouse 7S NGF for the indicated times by Western blotting with antibodies to aDMA and FMRP. The relative extent of aDMA incorporation into FMRP is plotted. Over the period examined, endogenous FMRP and human FMRP expression did not vary greatly, as indicated in the blots below the graph. The relatively low expression and detectability of endogenous FMRP makes quantitation difficult. Thus, although NGF treatment slightly increases the amount of aDMA incorporated into endogenous FMRP, it was not significantly different from that in the untreated control. (*C*) Human FMRP accelerates NGF-mediated differentiation in PC12 cells. PC12 cells were transfected with a nonexpressing FMRP vector (pET21A-hFMRP), pSF_2-hFMRP or pEGFP. 24 h later, the cells were treated with NGF. At various times afterward the cells were immunostained for FMRP and phase contrast, and fluorescence images were acquired. A factor which measures deviation from a perfect circle, i.e. the cell's roundness, 4π*Area/[(Perimeter)*0.5], was computed for each cell at each time point using Simple PCI software version 3.6.18 (Compix). Fluorescence images were used to distinguish between transfected and nontransfected cells. 1-{4π*Area/[(Perimeter)*0.5]}, which we call the index of differentiation, was then plotted for each population of cells as function of time. The plot shows results for 25 or more cells of each type for every point following NGF treatment. In the absence of NGF, the oval-shaped PC12 cells have a low differentiation index, regardless of whether they are transfected or express a heterologous protein. NGF treatment causes a time-dependent increase in the differentiation index. After 24 h ~ 80% of all cells are fully committed to differentiation, and the index does not increase further. At intermediate times, cells expressing human FMRP are significantly more differentiated than their non-expressing pET21A-hFMRP counterparts as well as cells expressing enhanced green fluorescence protein (EGFP) ($p < 0.005$ by ANOVA).

tion and PRMT substrates play important roles in neuronal differentiation.

Denman et al. have recently begun to investigate whether PRMT-mediated methylation might modify FMRP's biochemical properties. Initially, they focused on FMRP's ability to interact with RNA. They used a product inhibitor of S-adenosyl-homocysteine hydrolase (SAHH), an enzyme that catabolizes S-adenosyl-methionine (SAM), called adenosine-2′, 3′-dialdehyde (AdOx) [96, 109, 121–124] to inhibit endogenous PRMT activity in rabbit reticulocyte lysate (RRL) translation reactions. They then compared the RNA-binding properties of FMRP produced in the AdOx-inhibited RRL with FMRP from uninhibited RRL. The results clearly demonstrated that (i) AdOx inhibition altered the in vitro binding of homoribopolymers and also some of FMRP's target mRNAs, and (ii) each RNA examined was differentially affected [124]. Interestingly, there was a twofold increase in AdOx-inhibited FMRP's ability to bind to poly (rA) compared with non-treated FMRP. This observation might explain the differences in RNA binding to the A-rich BC1 RNA reported by Sung et al., who used in vitro translated FMRP, and Zalfa et al., who used baculovirus-produced recombinant FMRP which may not have the same posttranslational methylation pattern [17, 74].

Subsequent analyses using the rabbit reticulocyte in vitro translation system demonstrated that FMRP is constitutively methylated by a class I PRMT. PRMT1 was detected in RRL by Western blotting and AdOx specifically blocked SAM-dependent ^{3}H-methyl incorporation into FMRP, (fig. 2A). In keeping with these results, asymmetric dimethylarginine (aDMA) was detected in de novo FMRP by Western blotting [125]. Furthermore, using two aDMA antibodies that recognize slightly different epitopes, an aDMA-modified protein band that comigrated with FMRP was observed in various cultured cell lines (Jurkat,

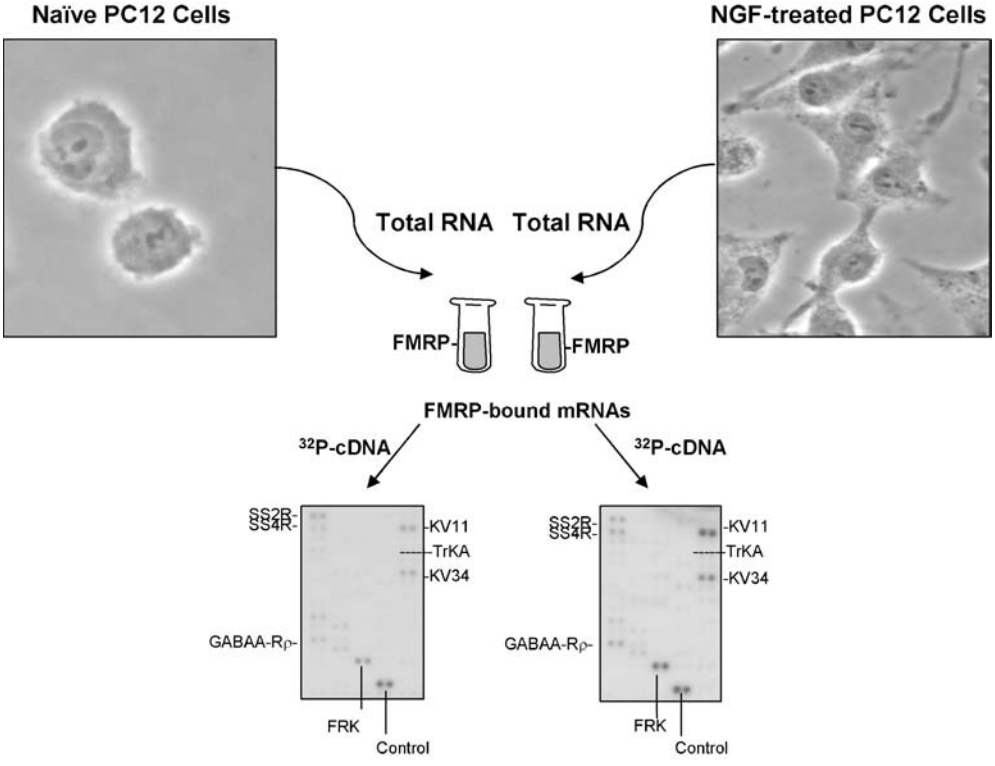

Figure 3. NGF stimulation alters the population of PC12 cell mRNA that binds to FMRP. Equal amounts of total RNA from undifferentiated PC12 cells and PC12 cells treated with 200 ng/ml of mouse 7S NGF for 48 h were bound to biotinylated-FMRP affinity columns, as previously described [16]. The bound mRNA from each column was converted into ^{32}P-labeled complementary DNA (cDNA) and 2×10^{6} cpm was used to probe Atlas microarrays, as described by the manufacturer (Clontech). Messages classified as positive had intensities that were more than four times background intensity. Positive messages identified by the NGF-treated probe were classified as induced or repressed if they had intensities that were two-fold higher or two-fold lower than the intensity of the message in the absence of NGF, respectively. A single grid, comprising 70 arrayed cDNAs, provides examples of mRNAs with enhanced interaction with FMRP following NGF treatment, possibly due to changes in their steady-state expression; these messages include receptors for somatostatin-4 (Ac# D16826), $GABA_A$ ρ subunit (Ac# M62400), voltage-gated K^+ channels KV11 (Ac# L2750) and KV34 (Ac# M64676) as well as FRK tyrosine kinase (Ac# U00803). The average ratio of the intensities from the NGF-treated vs. untreated arrays from two experiments for these messages was 3.1, 2.7, 2.5, 2 and 2.3, respectively. In contrast, those for somatostatin-2 receptor (Ac# M81830) and the hybridization control were 0.9 and 1.15. Note that 90% of the cDNAs in the grid do not interact with FMRP. Among them was the TrkA receptor (Ac#X03541) indicated at the end of the dashed line.

HeLa and PC12) and in mice, but was absent from fragile X knockout mice. In contrast, the presence of symmetric dimethylarginine (sDMA) in FMRP could not be detected using an antibody that recognizes human and murine sDMA [R. B. Denman, unpublished observation].

Where is FMRP methylated? Class I PRMTs have been shown to methylate a number of proteins in vitro. A variety of arginine methylation studies on peptide substrates, including one that mimics part of FMRP's RG-rich region, have led to the conclusion that class I PRMTs preferentially methylate R residues within GRG contexts [98, 126–128]. Nevertheless, not all GRG sequences are recognized, and recent studies have indicated that the PRMT recognition motif is quite varied [105, 129] and that different PRMTs target different R-residues on the same protein [108]. Full-length FMRP contains 52 R-residues scattered throughout the protein (fig. 1). Ten of these residues have contexts that are methylated by PRMTs in other proteins. Five of these 10 residues are coded for by exon 15 of the FMR1 gene. In vitro methylation studies by Ai et al. using a 9-amino acid peptide substrate that is homologous to FMR1 exon 15 residues (amino acids 542–550) demonstrated that R_{544} and, to a much greater extent, R_{546} were monomethylated by rat brain PRMT preparations [98]. Our studies indicate that full-length FMRP and truncated proteins that express FMR1 exons15a/c-17 contain aDMA residues [R. B. Denman, unpublished observation]. The identities and sites of arginine methylation, how they change as a function of second messenger stimuli, as well as the specific PRMT that methylates the protein in vivo are currently being investigated.

PRMT methylation also affects FMRP's ability to interact with other proteins. Denman et al. have recently found, for example, that arginine methylation was required for FMRP to bind FXR1P in vitro and that in vivo arginine methylation decreased FMRP's association with dense granules, reminiscent of those observed by Mazroui et al. and Funakoshi et al. [25, 81]. These data indicate that changes in FMRP's protein arginine methylation pattern (sites or stoichiometry) might be one avenue by which FMRP activity is regulated. In fact, it has been hypothesized that protein arginine methylation may play a specific role in signal transduction [130]. As with other posttranslational modifications, arginine methylation results in the formation of a covalent bond, yet unlike phosphorylation which is balanced by the relative activities of specific kinases and phosphatases, arginine-demethylating enzymes have not been observed. Nevertheless, it has been shown that NGF-stimulated PC12 cells exhibit transient increases and decreases in arginine-methylated proteins [122]. Indeed, in addition to constitutive FMRP methylation, NGF-dependent FMRP methylation has also been observed (fig. 2B). The increased methylation occurred concomitantly with FMRP-enhanced NGF-mediated neurite outgrowth and a switch in FMRP's RNA binding repertoire (fig. 3).

FMRP phosphorylation studies

In addition to sites for protein arginine methylation FMRP also harbors consensus sites for phosphorylation by a variety of kinases (fig. 1). Modification at these sites could link FMRP to a variety of specific second-messenger pathways and provide multiple avenues for regulating FMRP and its associated mRNAs. In fact, recent studies have shown that a number of these sites are phosphorylated in vitro and in vivo.

FMRP phosphorylation by casein kinase-2 (CK2) has received the most attention to date. The first indication that CK2 phosphorylation might affect FMRP came from studies by Ceman et al. who showed that 5,6-dichloro-1-B-D-ribofuranosyl-benzimidazole (DRB) inhibited the incorporation of ^{32}P-ortho-phosphate into FMRP, and that CK2 coimmunoprecipitated with FMRP [42, 131]. Subsequent studies by Siomi et al. using the *Drosophila* paralog of FMRP, dFMR1, demonstrated directly that endogenous *Drosophila* CK2 phosphorylated dFMR1 [132]. Serine 406 (S_{406}), which is highly conserved among various FXR proteins, was shown to be the site of phosphorylation. CK2 phosphorylation significantly increased dFMR1's ability to homodimerize and bind to poly (rU) and enabled it to distinguish poly (rG) G-quartets from unstructured poly (rG). More recent studies by Ceman et al. have shown that in murine brain and in cultured cells FMRP is constitutively phosphorylated at S_{499}, the equivalent site of CK2 phosphorylation in dFMR1 [80]. Based on their data, they propose that phosphorylated FMRP represses the translation of its target mRNAs, while unphosphorylated FMRP allows their synthesis. Nevertheless, the data do not exclude the possibility that phosphorylation elsewhere within FMRP-mRNPs (particularly other proteins within the mRNP) may stimulate mRNA translation, nor do they demonstrate what effect transient stimulation of other kinases have on the phosphorylation state of FMRP or its ability to translate mRNA.

In addition to CK2 phosphorylation sites, FMRP also harbors sites that might be modified by protein kinase A (PKA) and protein kinase C (PKC), casein kinase-1 (CK1), cGMP-dependent protein kinase (cGK) and tyrosine kinase (TYR) (fig. 1). The roles most of these kinases play in the normal cellular functioning of FMRP have yet to be investigated. PKA and PKC, however, apparently can phosphorylate human FMRP in vitro and in doing so alter its interaction with poly (rG) [133 and R. B. Denman, unpublished observation].

Toward and integrated model of FMRP function

It is clear from the results described above that the expression level of particular FMRP isoforms and their relative state of posttranslational modification play important roles in determining the rate at which FMRP-associated mRNAs are translated and perhaps in their ultimate selec-

tion. Previous reviews have proposed models of FMRP activity that fail to account for these recent discoveries [40, 42, 44, 46]. One of the most detailed of these was recently reported by Billuart et al. in describing the role dFMR1 plays in axonogenesis [41]. Extrapolating the data of Schneck et al., these authors proposed that activating the Rho-GTPase pathway in *Drosophila* results in dFMR1-mRNP remodeling. Specifically, rac-GTP interactions with the fly cytoplasmic FMRP interacting protein (CYFIP) was proposed to dissociate it from dFMR1-mRNPs, allowing the remodeled mRNP to bind to polyribosomes and regulate the local protein synthesis required for cytoskeleton reorganization during axonogenesis [134].

Based on the data reported here, we propose that under normal conditions FMRP is constitutively methylated by PRMT and constitutively phosphorylated by CK2 shortly after its synthesis (fig. 4, step I). Currently, we do not know whether arginine methylation precedes phosphorylation or vice versa. Methylation allows the protein to bind mRNAs and form mRNPs, while the balance between CK2 phosphorylated-FMRP-mRNPs and nonphosphorylated-FMRP-mRNPs defines the basal translation rates of their associated mRNA [25, 80] (fig. 4, step II). Inhibiting arginine methylation (fig. 4, step III), results in decreased affinity between FMRP and its target messages [124]. This could lead to mRNA decay and a corresponding decrease in the expression of the target protein, or if the message also binds to other proteins within the mRNP, result in an altered mRNP that lacks the ability to repress the synthesis of the mRNA.

Extracellular signals that are transduced by TrkA- mGluR- or NMDA-receptors result in a cascade of events that ultimately modifies FMRP-mRNPs (fig. 4, step IV), allowing them to bind a different complement of mRNA and/or protein partners, and altering either positively or negatively the translation of its target messages. As we have shown, nerve growth factor (NGF) stimulation through the TrkA receptor results in a second wave of arginine methylation in FMRP (fig. 2B). This modification remodels FMRP-mRNPs. We found that FMRPs binding to the catalytic α subunit of CK2 mRNA markedly increased in NGF-stimulated PC12 cells [R. B. Denman, unpublished observation]. In the context of the above model this suggests that NGF-mediated arginine methylation of FMRP reduces its CK2 phosphorylation state. Since CK2 phosphorylated-FMRP is associated with stalled polyribosomes [80], reducing its CK2 phosphorylation state would allow the translation of specific mRNAs, presumably those involved in neurite outgrowth. Two important correlates of this aspect of the model are that the particular PRMT that methylates FMRP and CK2 mRNA should be found in dendrites, and we are conducting studies that will address this. Similarly, signals impinging on mGluR receptors could activate phospholipase C, resulting in the production of diacylglycerol, a known PKC activa-

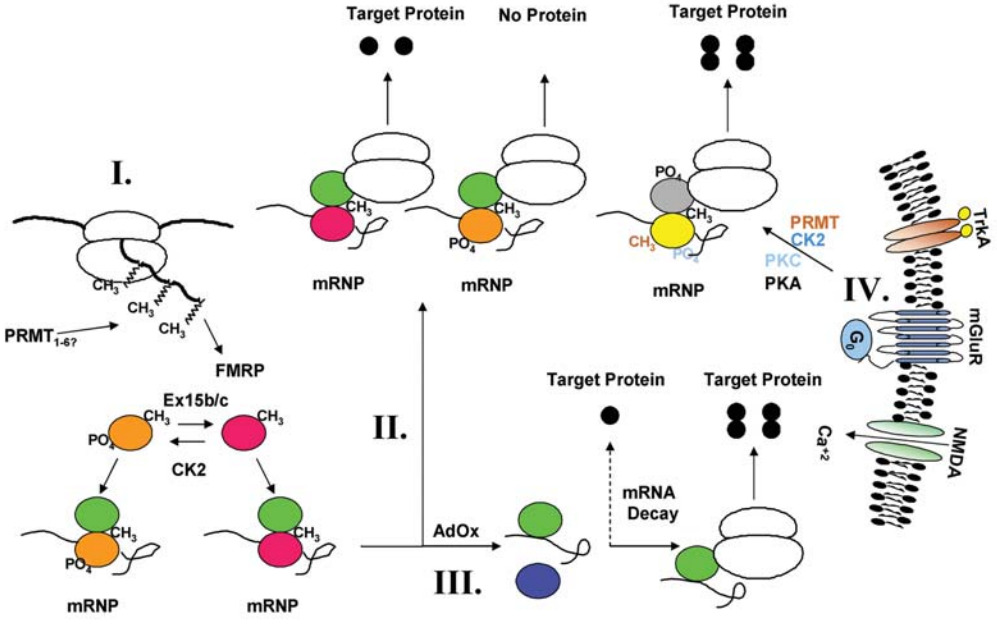

Figure 4. Regulation of FMRP-bound mRNA expression via changes in its posttranslational modification state. Steps I–IV are described in the text. Red, orange, blue and yellow ovals represent FMRP that is methylated, methylated and phosphorylated by CK2, unmethylated and methylated following NGF treatment or other stimuli, respectively. Green and gray ovals represent FMRP interacting proteins. In step I we hypothesize an equilibrium between methylated and methylated and phosphorylated FMRP that is influenced by the level of expression of FMRP exon 15 isoforms. The FMRP exon 15a isoform (Ex15a) contains the conserved CK2 phosphorylation site, while FMRP exon 15b and exon 15c isoforms (Ex15b/c) lack this site.

tor. Activated PKC could then potentially modify FMRP. Finally, as mentioned above, NMDA receptor stimulation leads to increased FMRP synthesis via the CPE/CPEB pathway. The de novo FMRP produced could then act as a translational repressor or enhancer, depending on (i) whether it is phosphorylated and, if it is phosphorylated, by what kinase and (ii) whether it is arginine methylated.

The various alternatively spliced isoforms of FMRP could play important roles in modulating this overall scheme of regulation. For example, the Ex15b and Ex15c isoforms of FMRP lack the site in FMRP that is constitutively phosphorylated by CK2 **(**fig. 1). Thus, the extent to which these isoforms are expressed could influence the balance between translating FMRP-mRNPs and repressed FMRP-mRNPs **(**fig. 4, step II). As this could vary through development and differ in differing brain regions or populations of neurons or in the various spatial compartments of a particular neuron, potentially many different FMRP-mRNPs could arise. Furthermore, if isoform-specific target mRNAs of FMRP truly exist [62], the shorter exon 15 isoforms would be well suited to play the postulated role of translational enhancer [18]. Finally, FMRP nuclear localized isoforms will likely contain a different complement of posttranslational modifications than their cytoplasmic counterparts and thus be subject to differing types of regulation.

Thus, the diversity of FMRP alternatively spliced isoforms and their posttranslational modification state combined with a growing list of protein partners and of synaptic modulating activities could result in a plethora of outputs, depending upon the exact stimulus. Moreover, it should also be stressed that posttranslational modifications of other components of FMRP-mRNPs could also affect the composition and activity of these particles, adding another layer of complexity to this interesting and evolving story.

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