FROM mRNP TRAFFICKING TO SPINE DYSMORPHOGENESIS: THE ROOTS OF FRAGILE X SYNDROME

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Abstract | The mental retardation protein FMRP is involved in the transport of mRNAs and their translation at synapses. Patients with fragile X syndrome, in whom FMRP is absent or mutated, show deficits in learning and memory that might reflect impairments in the translational regulation of a subset of neuronal mRNAs. The study of FMRP provides important insights into the regulation and functions of local protein synthesis in the neuronal periphery, and increases our understanding of how these functions can produce specific effects at individual synapses.

CpG ISLANDS
Sequences of at least 200 bp with greater than 50% G+C content and high CpG frequency.

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doi:10.1038/nrn1667

Fragile X syndrome (FXS) is the most common cause of inherited mental retardation, and can be attributed to mutations in the *FMR1* gene on the X chromosome. In most cases, fragile X syndrome is caused by the expansion of a polymorphic CGG repeat in the 5′ untranslated region (UTR) of the gene. Hypermethylation of the CGG repeats and the upstream CpG ISLANDS usually leads to transcriptional silencing of the gene[1]. A different phenotype, fragile X tremor ataxia syndrome (FXTAS), has recently been described in individuals who carry a 'premutation' in the *FMR1* gene[2]. The clinical presentation includes either gait ataxia or intention tremor at onset, associated with a wide range of neurological symptoms and signs that include cognitive impairment, parkinsonism, peripheral neuropathy and autonomic dysfunction.

One of the key findings in patients with fragile X syndrome is that they have more dendritic spines than control subjects, and that the spines are longer and thinner. This morphology is also seen in the mouse model of the syndrome[3–7]. Spines are dynamic structures that can regulate many neurochemical events related to synaptic transmission and modulate synaptic efficacy[8,9].

The development and modification of synaptic connections involves the integration of intrinsic cellular mechanisms and extrinsic information. Synaptic connections are dynamically regulated by many protein–protein interactions and protein modifications, such as phosphorylation and ubiquitylation. Another important aspect of synaptic regulation is protein synthesis and degradation — both locally, in dendrites and growth cones, and in the cell body[10,11]. Therefore, it is of interest to understand how the mRNAs that are required at synapses are selected from the pool of mRNAs in the nucleus, how they are transported along neuronal processes and how they are finally translated (apparently largely postsynaptically in spines and dendrites) in a regulated manner in response to presynaptic and other inputs.

Fragile X mental retardation protein (FMRP), which is encoded by the *FMR1* gene, is one of a family of RNA-binding proteins known as heterogeneous nuclear ribonucleoproteins (hnRNPs) that are involved in many aspects of mRNA metabolism and biology. Recently, it has been shown that some of these are required for the export of mRNAs from the nucleus and their subcellular localization in the cytoplasm. These two processes seem to be connected[12]: when RNA processing is complete, the RNA is exported through the nuclear pores. At this stage, some of the RNA-binding proteins are released, whereas others remain attached[13]. Splicing and other forms of nuclear processing can specify aspects of targeting. One example of an hnRNP that functions in this way is the zipcode binding protein 2 (ZBP2).

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Box 1 | **FMRP RNA-binding modules**

Fragile X mental retardation protein (FMRP) has two K homology (KH) domains, one RGG box (a cluster of arginine and glycine residues) and an amino (N) terminus that has a strong affinity for RNA. The KH module — an evolutionarily conserved sequence motif that was originally identified as three repeats in the human heterogeneous nuclear ribonucleoprotein (hnRNP) K protein[129,130] — is present in proteins from bacteria through to humans. KH-containing domains bind preferentially to specific RNAs[131,132], although they can also bind to DNA[133,134]. Specific RNA targets for this domain have not yet been isolated.

The RGG box is an RNA-binding domain consisting of Arg-Gly-Gly repeats. It is found in hnRNP proteins, nucleolar proteins involved in RNA metabolism and several viral proteins[135–137]. The RGG box is considered to have an accessory role in RNA binding that promotes the unfolding of RNA secondary structure. This role has been shown for RNA-binding proteins that are involved in ribosome biogenesis, such as nucleolin[138,139], fibrillarin[140], glycine arginine rich protein 1 (GAR1) in yeast[141] and nuclear signal recognition protein 1 (NSR1)[142]. Moreover, in hnRNPA2, RGG repeats are crucial for the cellular localization of the protein[143]. Specific binding of FMRP to G-QUARTETS has been mapped to the RGG box[79]. The conformation of RNA targets and their mode of binding to RGG repeats are heterogenous[84], which indicates that other *cis*-acting sequences in target mRNAs are also involved in their association with FMRP. The N-terminal domain of FMRP also binds to RNA[63,77], but is not homologous to any known RNA-binding motif. However, homology has recently been shown with proteins belonging to the 'Royal family', including the tudor proteins[144].

The RNA-binding motifs could also serve other roles. A particularly severe form of fragile X syndrome involves an asparagine for isoleucine missense mutation at amino acid position 304 in the second KH module of the fragile X mental retardation 1 gene (*FMR1*)[36]. The mutation alters the RNA-binding activity of FMRP[132,145–147]. The mutated FMRP cannot form homodimers[28], inhibit the translation of mRNA *in vitro* or inhibit the formation of the initiation complex 80S[148,149], and shuttles more rapidly between the nucleus and the cytoplasm[37].

Although this is predominantly a nuclear protein, it shuttles and contributes to the cytoplasmic localization of β-actin mRNA in fibroblasts and neurons[14].

The best characterized examples of hnRNPs that are involved in mRNA localization include hnRNPA2, which is required for localization of the myelin basic protein (MBP) mRNA in oligodendrocytes[15,16], and the ZBP family, which also includes Vera (hnRNP1) in *Xenopus laevis*[17] and ZBP1/2 in chickens[18] and mammals[19]. These last examples were initially identified because they bind to a 54-nucleotide sequence (zipcode) in the 3′ UTR of the β-actin mRNA[20]. Another important hnRNP that influences the cytoplasmic fate of mRNA is the exon–junction complex, which is deposited onto each exon–exon junction by the pre-mRNA splicing machinery in the nucleus[21]. Eukaryotic translation-initiation factor 4AIII (eIF4AIII) forms part of the exon–junction complex, and is involved in both the localization of oskar mRNA, which is responsible for the *Drosophila melanogaster* pole plasm, and NONSENSE-MEDIATED DECAY[21–23]. Further functions of hnRNP proteins include splice site selection during nuclear pre-mRNA splicing, regulation of translation efficiency, and regulation of mRNA stability and, therefore, abundance.

FMRP forms part of a large MESSENGER RIBONUCLEOPROTEIN (mRNP) complex that is involved in the transport and translation of mRNA in neurons (see below). The domain structure of FMRP — which includes two ribonucleoprotein K HOMOLOGY DOMAINS (KH domains) and a cluster of arginine and glycine residues (RGG BOX) that supports RNA binding — bears the hallmarks of a typical hnRNP protein (BOX 1) and is probably involved in many pathways that relate to RNA biogenesis and metabolism. In addition, there is evidence that the neuronal alterations associated with fragile X syndrome, such as abnormal spines[3–7], can be ascribed to impairments in mechanisms that are involved in neural plasticity. Therefore, it is tempting to speculate that FMRP participates in mRNA transport to the synapse and/or in localized protein synthesis in dendrites. To understand its functions at the molecular level, it is important to characterize which mRNAs are targeted by FMRP, and which other proteins bind directly or indirectly to the protein, as their identities can reveal mechanisms that underlie the function of FMRP. It is also important to look at the sub-cellular distribution of 'cargo mRNAs' and proteins that bind to FMRP to understand where and how FMRP exerts its effects. *FMR1* is one such cargo mRNA, and is translated at synapses in response to the activation of group I metabotropic glutamate receptors (mGluR1s)[24,25], so FMRP might have functions other than transporting and activating or inhibiting the translation of the transported mRNA. In this review, we summarize recent progress that has been made in answering these questions and describe a possible role for FMRP in translational control. Finally, we discuss how the molecular functions of FMRP might lead to the defects in spine morphology that are seen in fragile X syndrome.

G-QUARTET
Intramolecular, bimolecular or tetramolecular structures. Guanine is arranged in series to form a planar layer, which is cation dependent.

NONSENSE-MEDIATED DECAY
(NMD). A mechanism by which cells recognize and degrade mRNAs that prematurely terminate translation.

MESSENGER RIBONUCLEOPROTEIN
(mRNP). An mRNA, associated with proteins, that is translationally inactive.

K HOMOLOGY DOMAIN
(KH domain). A sequence motif that was originally identified as three repeats in the human hnRNP K protein and that is present in proteins from bacteria through to humans. The motif expands around a conserved VIGXXGXXI core (where X is any amino acid, with a preference for positive residues).

Localization of FMRP

FMRP in the nucleus. Although conventional methods do not usually show FMRP in the nucleus, it has been detected there using sensitive techniques. A small amount of FMRP immunoreactivity has been seen in the nucleus with both light and electron microscopy[26–28], although the protein was detected using an antibody, 1C3, which also reacts slightly with the FMRP-homologous protein FXR1P[29]. FMRP can interact with a distinct set of nuclear proteins including nucleolin, the nuclear FMRP interacting protein (NUFIP), FXR1P and FXR2P[30,31]. Interestingly, a particle that contains FMRP and the nuclear/cytoplasmic Y-box-binding protein 1 (YB1, also called p50) has also been identified[32]. YB1/p50 participates in several steps of mRNA biogenesis, including mRNA transcription, processing and transport from the nucleus, and is also involved in the regulation of mRNA localization, translation and stability in the cytoplasm[33,34].

In the nucleus, one role of FMRP could be to associate with mRNAs and escort them out of the nucleus (FIG. 1). This hypothesis is supported by the fact that FMRP contains both a functional nuclear localization signal (NLS) and a nuclear export signal (NES), which indicates that it can shuttle between the nucleus and the cytoplasm[27]. However, not all isoforms shuttle between the nucleus and cytoplasm — the alternatively spliced exon 14 of FMRP encodes the NES, and this FMRP isoform remains in the nucleus and cannot be exported to

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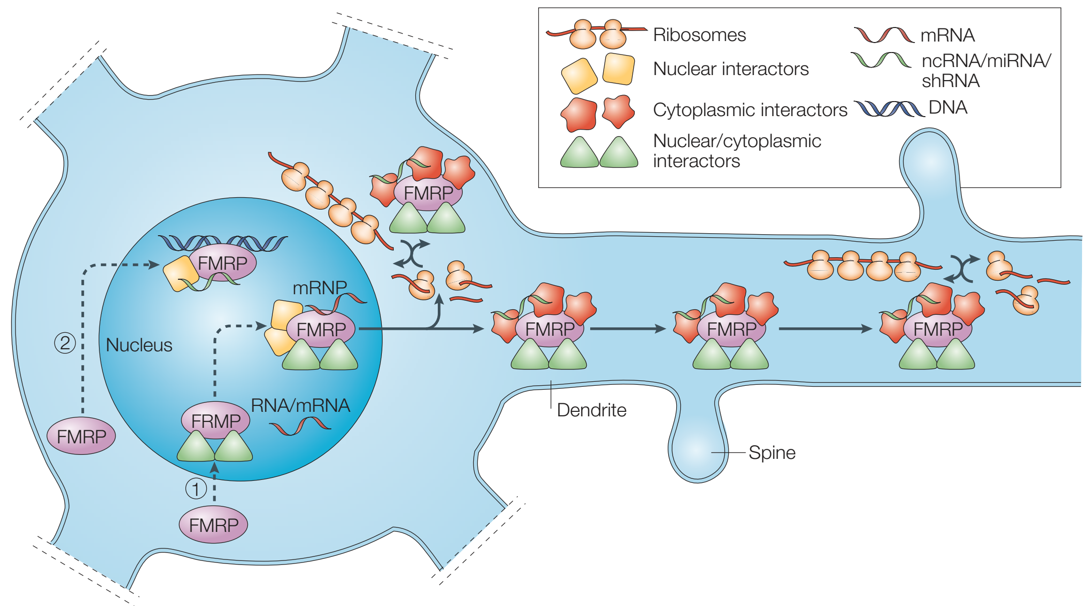

Figure 1 | **Speculative model for FMRP shuttling between the nucleus and cytoplasm.** Fragile X mental retardation protein (FMRP) enters the nucleus and could function through two possible mechanisms. In the first (1), FMRP could interact with other proteins, with itself (for example, the FMRP-homologous proteins FXR1P and FXR2P), and with RNA/mRNA to form a ribonucleocomplex that is probably involved in mRNA export from the nucleus to the cytoplasm. Once in the cytoplasm, a 'core' complex, containing FMRP and some of its nuclear partners, would interact with cytoplasm-specific proteins (such as cytoplasmic FMRP-interacting protein 1 (CYFIP1), CYFIP2 and Staufen) and move along dendrites to the synapses, transporting RNA/mRNA and, later, regulating synaptic protein synthesis. In the second mechanism (2), FMRP could be involved in the nuclear RNA interference pathway that is associated with small, non-coding RNAs (short hairpin RNAs or shRNAs) and specific nuclear partners (that is, nucleolin and Y-box binding protein 1 (YB1)). miRNA, microRNA; ncRNA, non-coding RNA.

the cytoplasm, as shown by the transfection of a cDNA that lacks exon 14 (REF. 35). A mutated FMRP that contains an asparagine for isoleucine substitution at amino acid position 304 in the second KH domain[36] (see also below) shuttles more frequently between the nucleus and cytoplasm, which indicates either that many domains are involved in nuclear entrance and export[37] or, more probably, that bound mRNA slows the transport/shuttling process or makes re-entry to the nucleus unlikely. Moreover, cells that have been treated with leptomycin B, which blocks mRNA export, partially retain FMRP in the nucleoplasm[35]. Further support for a putative nuclear 'shuttle' function comes from the finding that the FMRP carboxyl (C) terminus interacts with the Ran-binding protein RanBPM[38,39]. RanBPM was originally identified as a protein that interacts with Ran, which comes in two conformationally distinct states (RanGTP or RanGDP) and which governs the assembly and disassembly of cargo complexes for nuclear trafficking[40]. As it is involved in nuclear trafficking[41], RanBPM might help FMRP to shuttle.

A second possible role for FMRP in the nucleus could be CHROMATIN REMODELLING (FIG. 1). *In vitro*, FMRP binds strongly to single-stranded DNA and, to a lesser extent, to double-stranded DNA[42]. In addition, human FMRP associates with non-coding RNAs[43] and MICRORNAS (miRNAs)[44]. miRNAs regulate mRNA expression[45] and participate in chromosome methylation[46,47]. Mammalian FMRP has been shown to interact with a mammalian Argonaute protein (eIF2C2)[44] and the three components (FMRP, Argonaute and miRNAs) have also been detected in the nucleus, where RNA interference (RNAi)-mediated pathways operate[48]. It is, therefore, tempting to suggest that FMRP could contribute to chromatin remodelling through the RNAi pathway in the nucleus. Although it is clear that FMRP interacts with non-coding RNA[43,44], the sequences of the miRNAs that might associate with human FMRP and the nuclear functions of FMRP require further investigation.

FMRP in dendrites and mRNP transport. Gene expression in neurons also involves the transport of some mRNAs away from the cell body and local protein synthesis in dendrites. These two steps are required for establishing and maintaining synaptic plasticity[11] and are widely thought to be involved in learning and memory.

Several lines of evidence suggest that FMRP has an active role in mRNA transport, although there is no direct evidence that it transports mRNA. FMRP and its mRNA are found in both the soma and dendritic processes, including dendritic spines[49]. In dendrites and spines, FMRP and *FMR1* mRNA co-localize in granules, and the movement of these granules into dendrites is enhanced by neuronal activation through mGluRs[49]. The presence of FMRP along the length of the dendrites depends not on protein synthesis but on transport[49]. As *FMR1* is translated in unstimulated SYNAPTONEUROSOMES[43] as well as in response to mGluR

RGG BOX
A protein region that is rich in arginine and glycine residues, is positively charged and has a high affinity for RNA molecules.

CHROMATIN REMODELLING
Epigenetic DNA modifications that silence genes at a transcriptional level without altering their structure. Alterations in chromatin remodelling cause various multi-system disorders and neoplasias.

MICRORNA
A non-coding RNA molecule of 21–24 nucleotides that inhibits mRNA expression.

SYNAPTONEUROSOMES
Purified synapses containing the pre- and postsynaptic termini. The presynaptic compartment contains the synaptic vesicles and the postsynaptic compartment contains the translational machinery.

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stimulation[24], it is possible that *FMR1* is translated only at the synapse and not during transport. In any case, both *FMR1* and FMRP are transported in granules to locations throughout the dendrite, where translation of the accompanying mRNA, and possibly mRNA from other sources, is regulated by synaptic activation. In the absence of FMRP, mGluR activation does not trigger increased protein synthesis in synaptoneurosomes[25].

Further support for the idea that FMRP has a role in transport involves the regulator of G-protein signalling 5 (RGS5) and dystroglycan-associated protein 1 (DAG1) mRNAs, both of which are confirmed FMRP cargoes. Their expression is much less extensive in the dendritic region of *FMR1*-knockout mice than in wild-type mice[50], and this is not a general effect of the knockout, because only some of the cargo mRNAs show this pattern. Moreover, the presence of microtubule-associated protein 2 (MAP2), which, in this study, was not found to be a cargo mRNA, did not change in dendrites as previously reported[51]. So, only some of the mRNAs that bind FMRP seem to need it for transport into dendrites. It might be that more than one complex is involved in the transport/localization of some mRNAs.

In a recent paper, a large RNAase-sensitive granule, binding partner of kinesin, was characterized[52]. The authors used mass spectrometry to identify 42 proteins, and after stringent conditions they could still detect a 'core' composed of α-calcium/calmodulin-dependent protein kinase II (α-CaMKII) and activity-regulated cytoskeletal-associated protein (ARC, also called ARG3.1) mRNAs, as well as the purine-rich single stranded DNA-binding proteins α and β (PURα and PURβ), Staufen, FMRP, FXR1P, FXR2P, elongation factor 1α (EF1α) and kinesin 5 (KIF5). Interestingly, the α-CaMKII and ARC mRNAs were both found to be associated with FMRP in another study[43]. Collectively, these data strengthen the argument that there is at least one RNA-transporting complex that contains FMRP in the mouse brain.

Moreover, FMRP and ZBP1/insulin-like growth factor II mRNA-binding protein1 (IMP1), a protein that has a defined role in mRNA transport in fibroblasts and neurons, can recruit each other into RNP granules *in vivo*[53]. Finally, using *D. melanogaster* S2 cells, Ling *et al.*[54] make a case for the combined involvement of both kinesin heavy chain and dynein in the bidirectional transport of green fluorescent protein (GFP)-tagged FMRP-containing granules on microtubules. Although there is some evidence to indicate that a particular FMRP domain is involved in granule formation in PC12 cells[55], the mechanism by which *FMR1* mRNA is localized to synapses is unknown.

We believe that work towards the involvement of FRMP in mRNP transport will make a significant contribution to the future understanding of fragile X syndrome. There are some patients with fragile X syndrome who cannot be diagnosed using conventional methods because they do not have the expansion in the 5′ UTR as well as mutations in the coding region[56]. In these patients, it is possible that the delivery of FMRP and/or of *FMR1* mRNA to synapses is disrupted by mutations that affect the localization process.

Binding partners for FMRP

Proteins that interact with FMRP. Although fragile X syndrome is a monogenic disease, other factors might involved in the same biochemical pathway as FMRP, which could explain why the severity of the syndrome varies between patients that lack the same protein. Using immunoprecipitation, two-hybrid screens or large mass spectrometry analysis, several groups have identified proteins that interact with FMRP. Many putative FMRP-interacting proteins have been isolated and characterized[30,32,52,57–61], most of which interact with the amino (N)-terminal portion of FMRP[59,62,63]. The only protein that has so far been found to interact with the C terminus of FMRP is RanBPM[39]. A list of FMRP-interacting proteins can be found in TABLE 1. We have also listed homologues in other species, as this allows us to infer whether the function is evolutionarily conserved. In this review, we focus on FMRP-interacting proteins that might explain the fragile X phenotype.

First, FMRP can interact with itself, as the N terminus contains a homodimerization domain. It also interacts with the two FMRP-homologous proteins FXR1P and FXR2P, in both the nucleus and in the cytoplasm[57]. A low level of FMRP expression has been detected in a human prenatal heart[64], which might explain why patients with fragile X syndrome have cardiac abnormalities[65]. A recent study of *FXR1*-knockout mice revealed that this gene is essential for heart development and function[66]. In the cytoplasm, FMRP also interacts with the cytoplasmic FMRP-interacting proteins 1 and 2 (CYFIP1 and CYFIP2), which are highly homologous to each other. CYFIP2 interacts with all members of the FXR family, whereas CYFIP1 is specific to FMRP. CYFIP1 and 2 are localized at synapses[58] and CYFIP1 also interacts with the small GTPase Rac1 (REF. 67). The *D. melanogaster* homologue of FMRP, also known as DFXR, has been linked with the Rho-GTPase pathway and synapse formation[68].

The 82-kDa FMRP-interacting protein (82-FIP) seems to be found in both the nucleus and the cytoplasm. It shows no homology to proteins of known function or to any known functional domain and, like NUFIP1 and CYFIP1, interacts with FMRP but not with FXR1P or FXR2P. 82-FIP is found in most neurons, and its subcellular distribution is cell-cycle dependent in COS cells, which indicates that the composition of some FMRP-containing RNP complexes might be cell-cycle modulated[69]. Immunoprecipitation experiments identified two more putative nuclear FMRP-interacting proteins: nucleolin and YB1/p50 (REFS 30,32). Furthermore, immunoprecipitation of PURα co-precipitated FMRP, Staufen and myosin VA, among others. The immunoprecipitation is sensitive to treatment with RNAase, which indicates that these proteins are part of an (m)RNP complex. PUR proteins and Staufen have been implicated in mRNA

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Table 1 | **Summary of the FMRP interactors**

FMRP interactor	Cellular localization	Similar genes in other organisms	Isolation method
FXR1P	Nucleus and cytoplasm	Human, mouse, rat, *X. laevis*, zebrafish	Yeast two-hybrid system[57]
FXR2P	Nucleus and cytoplasm	Human, mouse, rat, *X. laevis*, zebrafish	Yeast two-hybrid system[57]
NUFIP1	Nucleus	Human, mouse, rat, *D. melanogaster*	Yeast two-hybrid system[151]
CYFIP1	Cytoplasm	Human, mouse, rat, *X. laevis*, zebrafish, *D. melanogaster, C. elegans*	Yeast two-hybrid system[58]
CYFIP2	Cytoplasm	Human, mouse, rat, *X. laevis*	High sequence homology with CYFIP1 (REF. 58)
82-FIP	Nucleus and cytoplasm	Human, mouse, *X. laevis*	Yeast two-hybrid system[69]
Nucleolin	Nucleus	Human, mouse, rat, *G. gallus*, *X. laevis*, *D. melanogaster*, *C. elegans*	Co-immunoprecipitation[30]
YB1/p50	Nucleus	Human, mouse, rat, *X. laevis*, zebrafish, *D. melanogaster*, *C. elegans*	Co-immunoprecipitation[32]
Staufen	Cytoplasm	Human, mouse, rat, *X. laevis*, zebrafish, *D. melanogaster*, *C. elegans*	Co-immunoprecipitation[60], TAP-technology[61,152]
PURα	Cytoplasm	Human, mouse, rat, *D. melanogaster*, *C. elegans*	Co-immunoprecipitation[60]
PURβ	Cytoplasm	Human, mouse, *X. laevis*, zebrafish, *D. melanogaster*	Co-immunoprecipitation[52]
Myosin VA	Cytoplasm	Human, mouse, rat, *D. melanogaster*, *C. elegans*, *X. laevis*	Co-immunoprecipitation[60]
RanBPM	Nucleus and cytoplasm	Human, mouse, rat, *X. laevis*, *D. melanogaster*	Yeast two-hybrid system[39]
eIF2C2/AGO1	Nucleus and cytoplasm	Human, mouse, rat, *D. melanogaster*	Co-immunoprecipitation[44,87]
Dicer	Nucleus and cytoplasm	Human, mouse, rat, *X. laevis*, zebrafish, *D. melanogaster*	Co-immunoprecipitation[88]
PABP1	Cytoplasm	Human, mouse, rat, *X. laevis*, zebrafish, *D. melanogaster, C. elegans*	TAP-technology[61,152], immunoprecipitation[52]
Kinesin heavy chain	Cytoplasm	Human, mouse, rat, *X. laevis*, *D. melanogaster*, *C. elegans*	TAP-technology[60,152], immunoprecipitation[52]
Dynein intermediate chain	Cytoplasm	Human, mouse, rat, *D. melanogaster*	TAP-technology[61,152]

C. elegans, *Caenorhabditis elegans*; CYFIP1/2, cytoplasmic fragile X mental retardation protein (FMRP)-interacting protein 1/2; *D. melanogaster*, *Drosophila melanogaster*; eIF2C2, eukaryotic translation-initiation factor 2C, 2 (also known as Argonaute 1 (AGO1)); FMRP, fragile x mental retardation protein; FXR1P/2P, fragile X mental retardation 1/2; *G. gallus*, *Gallus gallus*; NUFIP1, nuclear FMRP-interacting protein 1; PABP1, poly(A)-binding protein 1; PURα/β, purine-rich single stranded DNA-binding proteins α and β; RanBPM, Ran-binding protein; TAP-technology, tandem affinity purification technology; *X. laevis, Xenopus laevis*; YB1, Y-box-binding protein 1; 82-FIP, 82-kDa FMRP-interacting protein.

transport and the regulation of translation[70–72] through a possible interaction with a kinesin motor[73], and it is, therefore, interesting to find them in the same mRNP complex as FMRP. Moreover, the complex also contains the small dendritic non-coding brain cytoplasmic RNA 1 (BC1)[60,74], which anneals to complementary regions of some mRNA targets[43]. Interestingly, both kinesin 1 and dynein seem to be involved in FMRP and *Fmr1* transport in *D. melanogaster*[54].

The *FMR1* gene has attracted substantial attention because of its direct correlation with fragile X syndrome, but none of the proteins that interact with FMRP has yet been associated with a disease, except for myosin VA, which is associated with Griscelli syndrome, an autosomal recessive disorder that is characterized by partial albinism with variable immunodeficiency[75]. Moreover, none of the genes that encode FMRP-interacting proteins has, so far, been linked to hereditary mental retardation.

mRNA targets of FMRP. FMRP and the related proteins FXR1P and FXR2P share the same domain structure (BOX 1) and show more than 60% amino acid identity to each other[57,76]. The domain structure includes two KH domains, an RGG box and an RNA-binding domain in the N-terminal region of the protein[63,77] (F. Zalfa and C.B., unpublished observations). Consistent with this domain structure, FMRP binds to RNA homopolymers and to a subset of transcripts that are found in the brain[42,43,50,77–80]. The learning and memory difficulties that are found in patients with fragile X syndrome and in the mouse model of the syndrome are probably due

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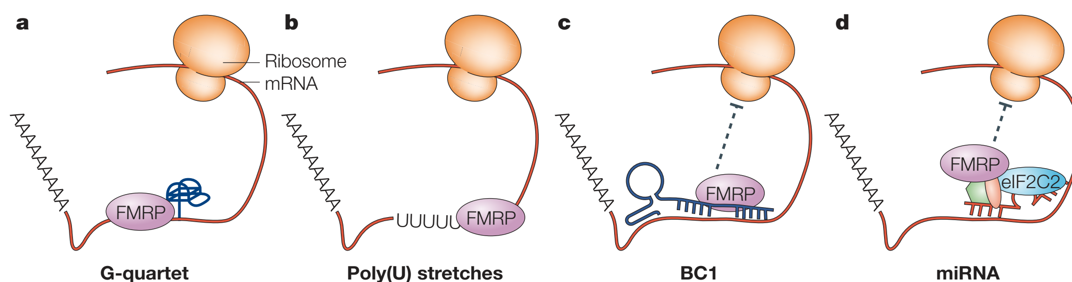

Figure 2 | **FMRP recognizes different RNA sequences.** Fragile X mental retardation protein (FMRP) binds different neuronal mRNAs. Four mechanisms of target recognition have been characterized. FMRP could recognize a G-quartet structure (**a**) or a poly(U) stretch (**b**) in the mRNA. Alternatively, FMRP could bind indirectly to the mRNA through either the small non-coding RNA brain cytoplasmic RNA 1 (BC1) (**c**) or microRNAs (miRNAs) (**d**). eIF2C2, eukaryotic translation initiation factor 2C, 2.

to alterations in the metabolism of mRNAs that are important for synaptic structure and function. As a result, several attempts have been made to characterize the mRNAs that are bound by FMRP in neurons.

In the first study[78], the FMRP–RNP complex was immunoprecipitated and the mRNAs were screened with microarrays. More than 400 mRNAs were identified as being associated with FMRP in the mouse brain. In parallel studies, the same group compared the mRNA polysomal profile of LYMPHOBLAST CELLS from control subjects and patients with fragile X syndrome. This produced another set of 251 mRNAs that showed variations in their distribution on a POLYSOME gradient, which reflects a difference in the translational status of these mRNAs. However, the cytoplasmic abundance of these mRNAs remained unchanged. The two sets have 14 mRNAs in common. These mRNAs encode proteins that are important for neuronal function, synaptic plasticity and neuronal maturation, including the mRNA for MAP1B, which is also translationally dysregulated in *D. melanogaster* mutants for the homologous *FMR* gene[81].

Using *in vitro* selection of random RNA sequences (SELEX, systemic evolution of ligands by exponential enrichment), Darnell and collaborators[79] showed that FMRP binds a class of RNAs with G-quartet motifs (FIG. 2a). Thirty-one candidate mRNAs containing this motif were identified in the database. Twelve of these were assayed for FMRP binding and six bound strongly to FMRP. All six are associated with synaptic function, and they include MAP1B mRNA. They also show altered translational efficiency in fragile X cell lines. *FMR1* mRNA also contains a G-quartet structure[82]. The G-quartet is apparently not the only RNA motif that is recognized by FMRP — as might be expected from its many mRNA-binding domains, FMRP can also bind to mRNAs that contain U-rich stretches[80] (FIG. 2b).

Another study that used immunocytochemistry, antibody-positioned RNA amplification (APRA) and follow-up direct binding assays identified 81 additional mRNAs — perhaps mRNAs that are more likely to be associated with the *in vivo* FMRP mRNP complex[50]. These RNAs and their encoded proteins have diverse physiological functions, including involvement in cytoskeleton structure and function, synaptic transmission, peroxisomal biogenesis, membrane docking and fusion, nuclear transport and molecular chaperone-like activity. Several of the identified genes were near loci that have been linked to autism, a syndrome that is associated with fragile X syndrome, and another encodes the glucocorticoid receptor, which could be involved in the delayed return of glucocorticoids towards baseline after stress that is seen in individuals with fragile X syndrome[83]. These 81 mRNAs show little overlap with those identified in previous studies, in part because there was little overlap in the sequences on the different array platforms; of those that did overlap with the array used by Brown *et al.*[78], about 50% were identified using APRA, and some of them (23%) contained the G-quartet structure[78,79,82,84]. Some of these mRNAs also showed upregulation, downregulation or differential localization of the mRNA or encoded protein in *FMR1*-knockout cells, which supports the idea that FMRP can function as either a translational repressor or activator, depending on the situation.

FMRP can also recognize mRNAs by associating with the small dendritic non-coding RNA BC1, which anneals to complementary regions of some mRNA targets, and could thereby recruit FMRP to specific mRNAs[43] (FIG. 2c). In particular, BC1 RNA is predicted to base-pair to neuronal mRNAs that encode molecules that are important for synaptic structure and function (including MAP1B mRNA). BC1 RNA is predicted to have a stable secondary structure that includes two stem loops[85]. FMRP target mRNAs have sequence complementarity with the longer stem loop of BC1. A recent study showed that FMRP has all the properties of a potent nucleic acid chaperone *in vitro* — it promotes the annealing of nucleic acids with complementary sequences and strand exchange in a duplex nucleic acid structure[86], raising the possibility that FMRP has a direct role in BC1/mRNA annealing. Further *in vivo* studies could elucidate whether BC1, mRNAs and FMRP can form a translational inhibition complex.

Another potential mechanism for FMRP-mediated translational control is based on its interaction with miRNAs. It has recently been shown that human FMRP associates with miRNAs, which inhibit mRNA expression[44]. FMRP could contribute to this regulatory pathway by stabilizing the specific annealing between

LYMPHOBLAST CELLS
Immortalized blood cells (lymphocytes).

POLYSOME
A string of 80S ribosomes bound to an mRNA molecule.

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miRNAs and the complementary region in the 3′ UTR of the target mRNAs (FIG. 2d). miRNAs associated with the *D. melanogaster* homologue of FMRP have been cloned and their mRNA targets have been isolated[87,88], but studies in mammals are still ongoing.

In summary, many candidate FMRP-interacting mRNAs and proteins have been identified, and further studies are now needed to characterize the specificity of these interactions. We believe that it is important to discuss these interactions in the context of our current knowledge of the properties of the RNA-binding domains found in FMRP, because they determine how FMRP binds its RNA targets (BOX 1).

The role of FMRP in translation

It has been proposed that FMRP is involved in the translational regulation of gene expression in neurons, and probably in the testes. mRNA translation can be regulated at various stages. In general, mechanisms that regulate translation include inhibition of translation initiation, blocking translation at the 48S complex stage and inhibition of elongation[89].

mRNA-specific inhibition at the elongation stage, which is also known as 'stalling ribosomes', has been described in mechanistic detail in only one case — in lower eukaryotes[90]. The various stages of translation can be distinguished by the size of the mRNP complex: non-translated mRNPs and translation-initiation complexes are all smaller than a ribosome (80S), whereas translated mRNPs contain at least one ribosome and are, therefore, heavier than 80S. Furthermore, some forms of non-translated mRNP tend to aggregate. The aggregates vary in size, but tend to be bigger still than translating polyribosomes. Examples include stress granules, which contain stalled 48S-initiation complexes[91], and neuronal mRNA transport granules, which contain inactive polysomes[92] or granules that seem to lack ribosomes[93].

Because FMRP is probably involved in translation, the distribution of FMRP-containing complexes on sucrose gradients has attracted considerable interest. Unfortunately, the results of such experiments have differed, depending on important methodological details. Initial studies found that, in mammalian cells, FMRP co-sedimented with actively translating polyribosomes[94], whereas a different study found that FMRP co-fractionates with the mRNP fraction, which contains non-translated mRNPs and translation-initiation complexes[62]. Later, Warren and colleagues showed that FMRP is equally distributed between polysomes and mRNPs[28,78]. In *D. melanogaster*, FMRP associates with the 80S and ribonucleoprotein fractions of the gradient[88]. Finally, Zalfa *et al.* reported that, in brain extracts, a large amount of FMRP co-sedimended with the mRNP fraction[43]. These discrepancies could be caused by differences in the extract preparation procedure or the cell lines used, or by differences in the ages of the animals from which the cells were taken. For example, Stefani *et al.*[95] showed differences in the distribution of FMRP on sucrose gradients between young mice (9 days) and adult mice (5 months).

As putative FMRP target mRNAs are both up- and downregulated[50], FMRP could both activate and repress translation. FMRP could shift between these roles during development and/or in different parts of the brain, or, perhaps more likely, it could serve as a repressor until activated by phosphorylation and/or other signalling mechanisms. Indeed, recent work in Hela cells has shown that, in the presence of sodium azide, FMRP can move between polysomes and stalling ribosomes according to its phoshorylation status[96]. A significant portion of brain FMRP assembles in RNP granules — visible as a peak on sucrose gradients that is even larger than that seen for polysomes[97]. The presence of FMRP in a granular form co-sedimenting with light mRNPs, ribosomes, polysomes and heavy mRNPs might reflect the existence of more than one FMRP complex, possibly associated with different FMRP functions such as transport, inibition and/or activation of translation.

Structural and functional abnormalities

Spine abnormalities. Various types of dendritic spine dysmorphogenesis associated with mental retardation have been detailed since the original descriptions appeared 30 years ago[98,99]. In patients with fragile X syndrome, spines are commonly longer and thinner than in control subjects[3,5,100,101]. Furthermore, patients with fragile X syndrome have more spines per unit length of dendrite[5]. Both the spine shape and spine density phenotypes are seen in *FMR1*-knockout mice[4,102]. So far, a maximum of six brains from patients with fragile X syndrome have been studied for various brain areas, and the detailed family histories are not known for each patient, so other brain pathologies cannot be excluded. Nevertheless, all studies showed an excess of long, thin spines that resembled immature spines[3,5].

FMR1 is highly conserved between humans and mice, with nucleotide and amino acid identities of 95% and 97%, respectively[42]. *FMR1*-knockout mice, strain C57/BL6, were generated by homologous recombination to disrupt exon 5. Normal FMRP protein is absent in this murine model of fragile X syndrome, which shows macroorchidism, deficits in spatial learning, hyperactivity[103] and dendritic spine abnormalities (see below). With these features, FMR1-knockout mice are at least phenotypically a fair model of fragile X syndrome.

Although results from an animal model must be interpreted with caution, data from knockout mice[103] have corroborated and expanded on the findings from patients. The mice have abnormal spines in the visual cortex, the barrel region of the somatosensory cortex and in cultured hippocampal neurons[4–6,102,104]. These spine abnormalities were reported to diminish in the somatosensory barrel cortex during development[6], but recent research indicates that the abnormalities re-emerge as the mice mature[105] (L. Restivo, M. Ammassari-Teule and C.B., unpublished observations). The excess of long, thin, immature-looking spines indicates that a pruning process that would normally eliminate excess spines during development, and that contributes to the

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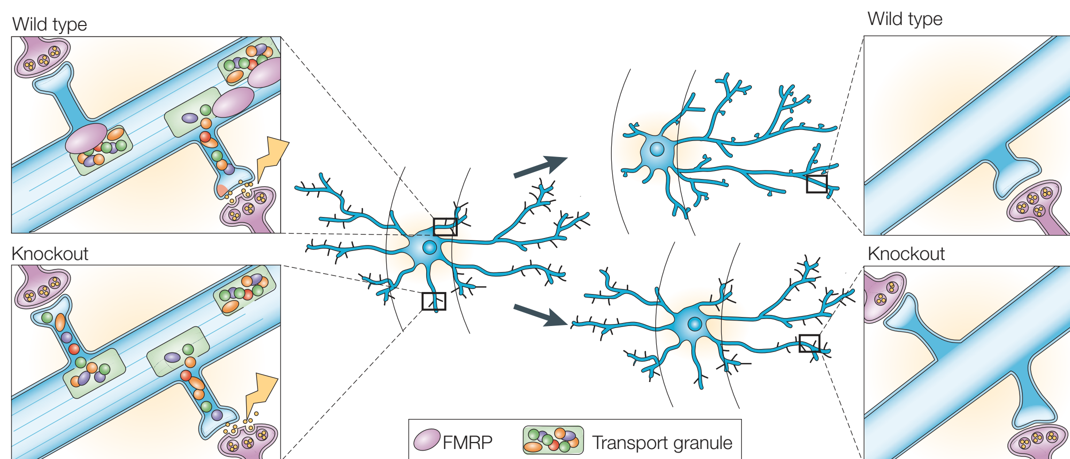

Figure 3 | **How might an absence of FMRP lead to a failure to prune synapses?** The figure shows a hypothetical mechanism through which the absence of fragile X mental retardation protein (FMRP) could lead to failure of synapse pruning and, as a consequence, dendrite pruning, in a typical spiny stellate neuron in a whisker barrel (centre). The model assumes that FMRP regulates the synthesis of structural proteins (for example, postsynaptic density protein 95 (PSD-95)) or signalling proteins that form part of a complex that is important for stabilizing and maturing developing synapses (see FIG. 4 for one possible conceptualization of this process). When FMRP is present, this stabilization complex (carried by the transport granule) is selectively targeted to active synapses (upper left), which results in selective maturation and stabilization of spines (upper right) and pruning of non-stabilized synapses. In the absence of FMRP (lower left), the stabilization complex is equally targeted to active and inactive synapses, which results in a weaker form of maturation and stabilization, and gives rise to greater numbers of synapses and an immature morphology (lower right).

development of brain circuitry[106,107], might fail in these mice[106,108]. In a region of the somatosensory whisker barrel cortex where dendrites are normally withdrawn during development, the improperly located dendrites are not withdrawn in *FMR1*-knockout mice, and diminishing spine density parallels development of the spine shape phenotype from postnatal week 4 to adulthood[105,108]. Combined with additional evidence[102], this supports the view that the neuromorphological abnormalities that are seen in both fragile X syndrome and the mouse model involve, at least in part, a failure to prune synapses and parts of dendrites that would normally be eliminated during development.

If this is the case, then a reduction in FMRP must lead to an increased number of immature-looking synapses[4,5,108]. One way in which this could arise is if, in the absence of FMRP, newly formed synapses are stabilized (protected against loss), as in normal development, but the message or events that destabilize and lead to the removal of inappropriately positioned or inactive synapses do not occur. Of course, the normal destabilizing process could result simply from the absence of stabilizing events. One way in which such a process could yield the morphological results reported for spines and dendrites is illustrated in FIG. 3. If the stabilization of spines, and consequently dendrites, occurs because afferent activity regulates FMRP-dependent, directed transport of crucial molecular elements of the stabilization process — the mRNA for a hypothetical 'stabilization protein' — this could lead to an absence of the stabilization protein at inactive synapses and, consequently, their loss, or pruning, in healthy humans and wild-type mice (FIG. 3, upper left panel). In the absence of FMRP in patients with fragile X syndrome or the mouse model, the stabilization protein would not be directed selectively to active synapses or dendrites and so all synapses in all dendrites would share equally in its effects (FIG. 3, lower left panel). In this case, activity-dependent pruning would not occur, or would not be selectively apparent in a particular region of the dendrite[108] (FIG. 3, lower panels). This line of thinking is supported by reports that the FMRP–mRNA transport granules are actively (and bidirectionally) transported and that their transport from the soma into processes and spines is driven by neuronal activity[49,54].

ENVIRONMENTAL ENRICHMENT
A combination of complex inanimate and social stimulation. Toys, ladders, tunnels and a running wheel are placed in the cage for voluntary physical exercise and are routinely changed during experimental periods.

Environmental effects and plasticity. *FMR1*-knockout mice show abnormalities in behaviour and neuronal morphology that resemble those described in patients with fragile X syndrome[7] (FIG. 4b,c). The importance of environment in the regulation of the brain, behaviour and physiology has long been recognized. ENVIRONMENTAL ENRICHMENT has been used extensively to show behavioural and brain plasticity in response to experience[109,110]. Rearing animals in a complex environment reduces anxiety[111], accelerates habituation[112], enhances learning[113,114] and deeply affects brain morphology. In particular, rodents reared in enriched environments show increases in dendritic length and branching[115], spine density, and the number, size and structure of synapses[116,117].

Recent research indicates that environmental enrichment can largely rescue the behavioural and neuronal abnormalities in *FMR1*-knockout mice (L. Restivo, M. Ammassari-Teule and C.B., unpublished observations). In mice, FMRP is found in dendrites and spines[28,49] and is regulated by activity: FMRP levels in the barrel cortex increase after stimulation, and levels in the cortex and hippocampus also increase when

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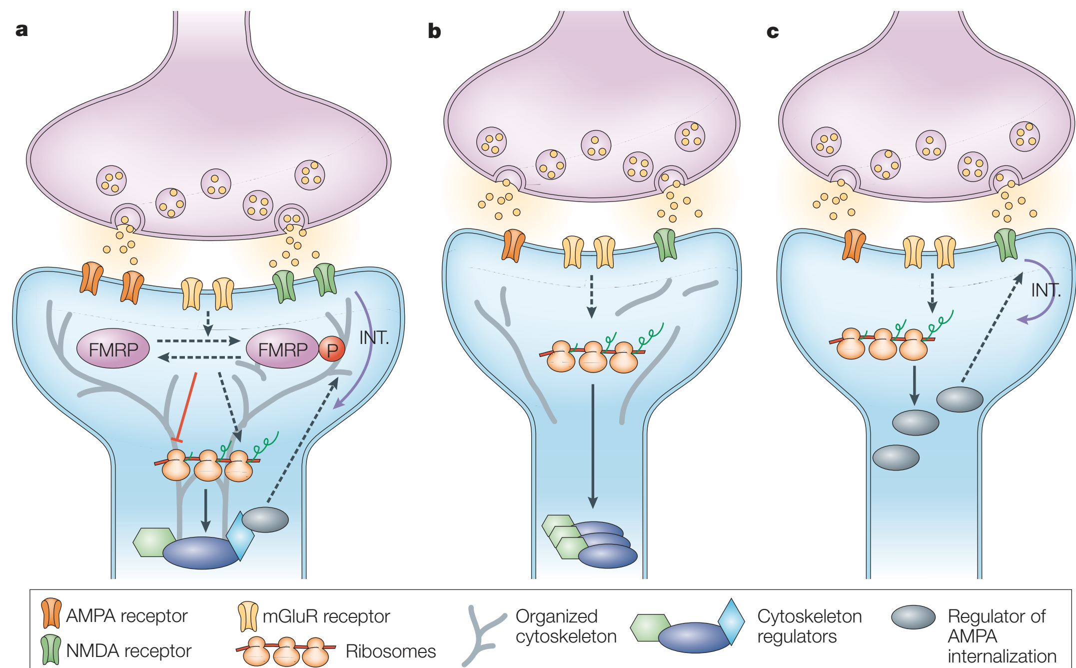

Figure 4 | **A working model of FMRP at synapses.** At synapses, protein synthesis is initiated by different cellular stimuli, and this leads to an independent response of a single synapse that can influence synaptic plasticity. **a** | In a wild-type spine, stimulation of metabotropic glutamate receptors enhances the synthesis of fragile X mental retardation protein (FMRP), which could act to negatively regulate the translation of proteins that are involved in ionotropic receptor internalization during long-term depression and of proteins that regulate the cytoskeleton (such as microtubule-associated protein 1B (MAP1B), activity-regulated cytoskeletal-associated protein (ARC), arginine-binding protein 2 (ARGBP2), postsynaptic density protein 95 (PSD-95) and Rac1). This receptor-coupled signalling pathway might also be responsible for FMRP phosphorylation and the consequent release of mRNAs from translational inhibition and/or the activation of translation of other specific dendritic mRNAs. The correct balance between synthesis and degradation of these proteins would promote and maintain the mature shape of the synapse. **b** | In a spine of a patient with fragile X syndrome, or in the mouse model of the syndrome, the absence of FMRP would lead to an increase and/or decrease in the translation of protein regulators of the cytoskeleton, both of which might have an effect on the lengthening of dendritic spines. **c** | The absence of FMRP could also lead to an increase in the translation of proteins that are involved in ionotropic (AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) and NMDA (*N*-methyl-D-aspartate)) receptor internalization (INT.) during hippocampal long-term depression, which could lead to fewer receptors being present on the postsynaptic membrane and to thinner spines. mGluR, metabotropic glutamate receptor.

mice are reared in complex environments. Both of these areas are important for learning and memory[118,119]. *FMR1* mRNA is present in synaptoneurosomes, and the synaptic level of FMRP increases after the administration of mGluR1-specific agonists[24]. In addition, FMRP regulates local mRNA translation, which could be a regulatory step in long-term plasticity (FIG. 4a), using the same, overlapping or separate mechanisms from those involved in developmental synapse selection and modification. Activity-dependent potentiation of synaptic transmission is expressed through several parallel mechanisms and usually requires the synthesis of new proteins. Protein synthesis is thought to be an important component of many forms of long-term synaptic plasticity, and all of the components that are required for protein synthesis are found at synapses. This might allow neurons to translate new proteins precisely where, and only when, they are needed to modify synapses in response to potentiating stimuli or behavioural learning[11]. In hippocampal slices from developing rats, polyribosomes redistribute from dendritic shafts into spines with enlarged synapses during long-term potentiation (LTP)[120] (BOX 2). Moreover, after stimulation or environmental enrichment, synapses are larger and more commonly contain polyribosomes[25,117,121,122]. This indicates that stimulation of synapses coincides with their enlargement, which accommodates local protein synthesis machinery. So, local protein synthesis seems to be an important component of synaptic plasticity.

Box 2 | **Synaptic plasticity**

Synaptic plasticity — indeed, the ability to change the synaptic structure — is thought to be involved in learning and memory, which are impaired in patients with fragile X syndrome. There are two main *in vitro* models for the study of synaptic plasticity: electrophysiological long-term potentiation (LTP) and long-term depression (LTD). An important contribution to both effects comes from the glutamatergic receptor system. Its receptors fall into two general classes: the ionotropic and the metabotropic receptors. The ionotropic AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) and NMDA (*N*-methyl-D-aspartate) receptors at glutamatergic synapses are heteromeric complexes of homologous subunits (GluR1–4 for AMPA receptors, and NR1 and NR2A–D for NMDA receptors) that differentially combine to form various receptor subtypes[150]. Metabotropic glutamate receptors (mGluRs) consist of eight different members that can be subdivided into three groups on the basis of sequence homologies and their ability to couple to specific enzyme systems.

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Hippocampal and cortical LTP. Possibly reflecting their poor performance in some types of memory and learning, FMR1-knockout mice show altered synaptic plasticity in brain areas involved in learning. In particular, they show reduced LTP in the cortex and enhanced mGluR-dependent long-term depression (LTD) in the hippocampus[123,124] (BOX 2). mGluR-dependent LTD depends on postsynaptic protein synthesis and involves the internalization of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) and NMDA (*N*-methyl-D-aspartate) receptors[125]. It has been proposed that FMRP might negatively regulate mRNAs that encode proteins involved in mGluR-dependent hippocampal LTD[123]. According to this model, FMRP, the glutamatergic system and synaptic plasticity could be linked together, possibly by mGluR-activated signalling pathways or a local increase in calcium from internal stores, which could ultimately alter the structure of dendritic spines[126] (FIG. 4c). Some evidence predicts downregulation of mGluR1s in FMR1-knockout mice[127]. Interestingly, the knockout mice show no impairments in hippocampal LTP[128]. Differential regulation of several mGluRs across age and brain regions could eventually explain this result.

Perspectives and open questions

mRNA export, localization and translation are important mechanisms for neuronal gene modulation. Some of these processes have been studied in considerable detail in different systems, and their mechanisms show some similarities. Although FMRP seems to have several roles in neurons, its precise place in the mechanisms of mRNA export, localization and translation remains to be defined. In particular, the specific RNA and protein partners of FMRP still need to be analysed in a frame that regards the cell as a dynamic entity in time and space. We leave the reader with several open questions, such as: does FMRP have a role, other than mRNA export, in the nucleus? Can FMRP interfere with or promote translational initiation, elongation and termination? Does FMRP have different partners at different stages of neuronal development? What proteins are involved in the modification and regulation of FMRP? Can the fragile X phenotype be improved by environmental alterations, and how does this compare with therapeutic behavioural intervention in patients? Future studies aimed at answering these questions will shed light on the mechanisms of mRNA export, localization and translation in the brain as well as in fragile X syndrome.

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Acknowledgements
We are grateful to T. Achsel, I. J. Weiler and F. Zalfa for critical reading of the manuscript and to G. Bernardi for his support. Work in C.B.'s group was supported by Telethon-Italy, Ministero dell'Istruzione, dell'Università e della Ricerca and Ministero della Salute. Work in W.G.'s laboratory was supported by the National Institute of Mental Health, the National Institute of Child Health and Human Developent and the Fragile X Research Foundation.

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The authors declare no competing financial interests.

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