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THE FRAGILE X MENTAL RETARDATION PROTEIN IN THE REGULATION OF NEURONAL mRNA TRANSLATION AND STABILITY

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SUMMARY

The Fragile X Syndrome is the most common cause of inherited mental retardation and is due to the absence of a single protein, the Fragile X Mental Retardation protein (FMRP). FMRP is an RNA-binding protein implicated in the regulation of mRNA transport, translation and stability in neurons. In the absence of FMRP, the expression of a large group of neuronal proteins is deregulated, resulting in impaired synaptic morphology and function.

Here, we investigated two critical steps of the posttranscriptional control mediated by FMRP, namely mRNA translation and stability. The modulation of protein synthesis involves the Cytoplasmic FMRP Interacting Protein 1 (CYFIP1), which represses the translation of the mRNAs associated with FMRP. Evidences reported here show that CYFIP1 may be also involved in the transport of the mRNAs and in the interplay between translation and cytoskeleton remodeling.

Besides the role in translation, FMRP regulates the decay of certain mRNA in neurons. In particular, FMRP stabilizes the mRNA encoding the PostSynaptic Density Protein 95 (PSD-95) in the hippocampus, but not in cortex. This study describes the molecular complexes associated with *PSD-95* mRNA that could account for the region-specific functions of FMRP.

In conclusions, this work provides further insights into the FMRP-dependent regulation of gene expression, therefore contributing to the understanding of Fragile X pathogenesis.

INTRODUCTION

Preface

Neurons are one of the most fascinating examples of polarized cells. The highly specialized morphology of every neuron is essential to create and maintain the neural circuits which underlie the proper functioning of the brain.

Early during development, neurons acquire the polarity. From the cell body arise two types of neurites with specific functions: a single axon, which routes the neuron's output, and one or multiple dendrites, which integrate inputs from other neurons (Arimura and Kaibuchi, 2007; da Silva and Dotti, 2002). In order to achieve their functional differentiation, the two processes acquire an array of morphological and biochemical specificities, including changes in the cytoskeleton, the membrane and the secretory pathway components (Conde and Caceres, 2009; Ye et al., 2007). Therefore, besides the cell body, which ensures the basic metabolic functions, the neurons possess subcellular compartments working as specialized domains.

The most emphasized specialization is reached at the synapses. In brain cortex, most of the excitatory synapses are formed by a bouton and a dendritic spine (Holtmaat and Svoboda, 2009). The bouton is a small axonal varicosity representing the presynaptic terminal, while the spine arises from the dendritic shaft and is the postsynaptic compartment (Holtmaat and Svoboda, 2009). Presynaptic compartments are characterized by the presence of an active zone with the synaptic vesicles containing the neurotransmitters (Ziv and Garner, 2004). The postsynaptic sites are defined by a local thickening beneath the membrane, the so-called Post Synaptic Density (PSD), which links the neurotransmitters receptors to signaling proteins and cytoskeleton (Kennedy et al., 2005).

The synapses are highly dynamic structures and undergo morphological changes in response to activity, underlying forms of experience-driven plasticity (Holtmaat and Svoboda, 2009). Such remodeling involves both the

architecture and the biochemistry and is thought to be crucial for sustaining and consolidating the processes at the base of complex phenomena, such as learning and memory.

Mental retardation

Mental retardation (MR) is a typical feature of a group of neurodevelopmental disorders defined by an early onset mental disability; the diagnostic criterion for MR is the IQ<70. In Western countries, MR affects 1.5-2% of the population and 0.3-0.5% are severely impaired (IQ<50) (Leonard and Wen, 2002; Ropers, 2008). While mild forms are likely due to complex interactions between genetic and environmental factors, severe MR is associated with circumstantial environmental events or specific genetic causes.

In fact, MR can occur as a consequence of premature birth, prenatal infections or perinatal asphyxia (Inlow and Restifo, 2004). Moreover, considering that MR is more frequent in developing countries, malnutrition, cultural deprivation and poor health care may also contribute (Leonard and Wen, 2002; Ropers, 2008).

More often, MR is due to genetic abnormalities, such as large chromosomal rearrangements (deletions, duplications or aneuploidies) or point mutations in individual genes. The chromosomal aberrations account for 15% of all cases (Leonard and Wen, 2002); for example, the Down's syndrome (trisomy of chromosome 21) remains the most common cause of MR.

MR can occur as an isolated symptom, without other consistent features (non-syndromic MR) or can be syndromic. In this case patients present other physical characteristics in addition to the intellectual disability. Moreover, MR can be inherited as autosomal dominant, autosomal recessive and X-

chromosome-linked (XLMR). Up to 15% of the intellectual disabilities are attributable to XLMR and approximately 80 genes have been already discovered (Chiurazzi et al., 2008; Gécz et al., 2009). In order to identify novel candidate genes, the coding exons on the X chromosome from 208 families with XLMR have been recently subjected to a large-scale systematic resequencing (Tarpey et al., 2009).

Although MR is a heterogenous group of pathologies, one of the key hallmarks of MR is a neuroanatomical phenotype characterized by abnormal dendritic spines. As previously mentioned, the dendritic spines undergo a series of structural changes during development and in response to neuronal activity (Holtmaat and Svoboda, 2009). Pioneering studies showed that in mentally retarded patients, the dendritic spines exhibit an immature shape, suggesting synaptic impairment (Purpura, 1974).

The Fragile X Syndrome

The Fragile X Syndrome (FXS) is the most common cause of inherited mental retardation (1 in 4000 males and 1 in 6000 females) and the most frequent form of XLMR (Chiurazzi et al., 2008).

The patients show some physical features, such as large ears, elongated face and high arched palate that have been reported in 60% of prepubertal FXS boys. Other symptoms include connective tissue anomalies, which can lead to mitral valve prolapsed, scoliosis, flat feet and joint laxity. Recurrent otitis media and strabismus are also quite common. Macro-orchidism due to a hypothalamic dysfunction affects about 90% of FXS boys by age 14 years (Jacquemont et al., 2007).

The more severe feature is the complex neurological phenotype, with a broad spectrum of cognitive and behavioral deficits. The developmental delay is the most critical feature, with a mean IQ of 42 in boys and severe

mental retardation in about 25% of cases. Since the disorder is X-linked and the penetrance is variable, females are usually in a low-normal range, with an IQ ranging from 70 to 90 (Jacquemont et al., 2007).

Behavioral symptoms include anxiety (>70%), attention deficit hyperactivity disorder (ADHD, 80%) and autism (20-30%). Although in females those anomalies are less pronounced, shyness and social anxiety are frequent (Jacquemont et al., 2007).

Autism is the most severe form of an heterogenous group of neurodevelopmental pathologies referred as autism-spectrum disorders (ASDs) (Abrahams and Geschwind, 2008). ASDs are diagnostically defined by impairments in three domains, namely social interaction, language and range of interests. First, social interactions are featured by an impaired use of nonverbal communication (facial and body language) and poor spontaneous attempts in social contacts. Second, language is delayed or absent, frequently limited to echolalia (rote repetition of words spoken by others or of memorized scripts). Third, interests are restricted and/or repetitive, such as inflexibility to routines or rituals, motor stereotypes and compulsive actions (Abrahams and Geschwind, 2008).

FXS is the most frequent monogenic cause of ASDs, including autism, Asperger's syndrome and pervasive developmental disorders not otherwise specified (Jacquemont et al., 2007). About 25% of FXS boys and 6% of girls meet criteria for ASDs, while 1-2% of patients affected by ASDs have FXS (Abrahams and Geschwind, 2008; Hatton et al., 2006). Shared neurobehavioral symptoms between ASDs and FXS are social anxiety, strong gaze avoidance, sensory hypersensitivity, tactile defensiveness, stereotypic movements, poor motor coordination, delayed speech development and echolalia (Belmonte and Bourgeron, 2006).

Epilepsy has been described in 13-18% of boys and 4% in girls and normally the seizures tend to resolve during childhood (Berry-Kravis, 2002).

Finally, the most prominent neuroanatomical feature is the dysgenesis of the dendritic spines, which appear longer and thinner than normal (Irwin et al., 2001). As mentioned, this is a key hallmark of mental retardation (Purpura, 1974).

The molecular basis of Fragile X and Tremor Ataxia Syndromes

In 1977, Sutherland described for the first time a fragile site on the q27.3 region of the X chromosome derived from cells of mentally retarded patients. Such mutation appeared to be inherited transmitted (Sutherland, 1977). Twenty years later, the molecular bases of the syndrome were discovered through the identification of the FMR1 gene (Fragile X Mental Retardation 1) (Verkerk et al., 1991). This gene is evolutionary conserved. Two autosomal paralogs, FXR1 and FXR2, and several orthologs in *Xenopus*, *Zebrafish*, *Drosophila*, chicken, mouse and human have been identified so far (Tucker et al., 2004; Verkerk et al., 1991; Zalfa and Bagni, 2004). The gene spans a region of over 40 kilobases (kb) and a 3.9 kb transcript composed by 17 exons. The mRNA presents a 5'untranslated region (5'UTR) of 0.2 kb, a coding region of 1.9 kb and a 3'UTR of 1.8 kb (Bardoni et al., 2001). Alternative splicing events on the primary transcript give rise to several protein isoforms (Denman and Sung, 2002).

In over 90% of FXS patients, the pathology is due to aberrations in the trinucleotide repeat (CGG) expansion in the 5'UTR of the FMR1 gene. This region is highly polymorphic in the normal population. Normally, the CGG expansion is within a range of 5-44 repeats, which are stably transmitted to the offspring (Fig. 1). However, for reasons still not understood yet the triplets can expand over 44 copies, giving rise to "grey-zone" alleles (45-54 repeats) or "premutation" alleles (55-200 copies) (Fig. 1). Both alleles are unstable and can evolve into a "full mutation" (>200 repeats) during the

transmission to the offspring (Jacquemont et al., 2007). It is clear that the expansion occurs during maternal transmission, since the spermatogenesis is unable to maintain the full mutations (Malter et al., 1997; O'Donnell and Warren, 2002).

While the grey-zone alleles require at least two generations before expanding to a full mutation (Fernandez-Carvajal et al., 2009), the premutation is highly unstable. In fact, the risk of transmitting an allele in the full mutation is a function of the repeat length (Hagerman and Hagerman, 2002) (Fig. 1).

Full mutation alleles are defined by the massive expansion of the triplet over 200 copies. In this condition, the CGG and the CpG islands upstream undergo hypermethylation, leading to transcriptional silencing of the gene. Therefore, no transcript and no FMRP protein are produced (O'Donnell and Warren, 2002).

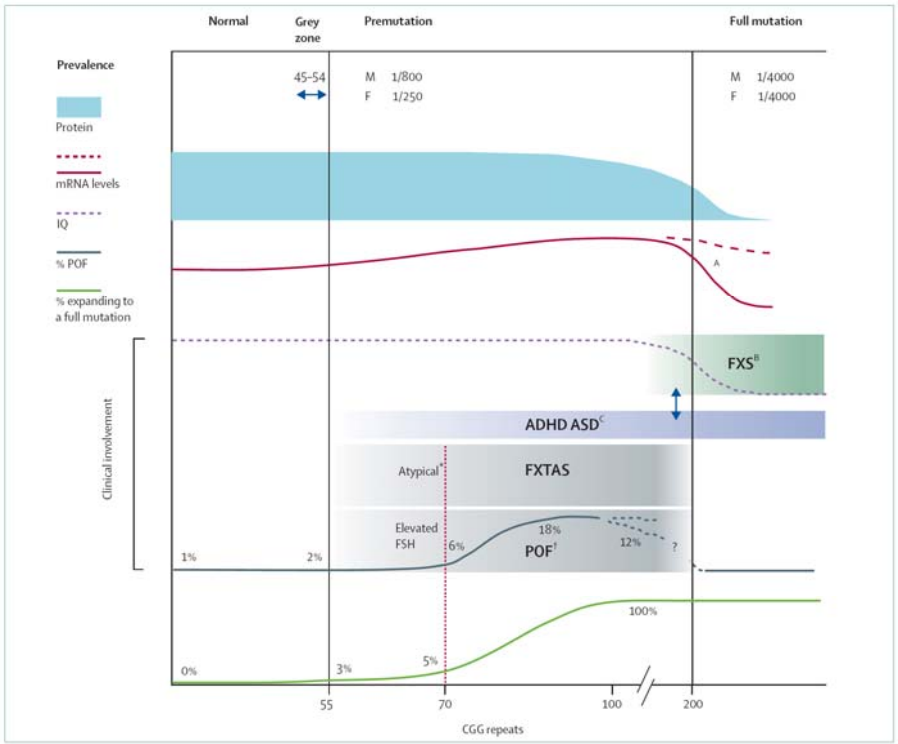
The premutation alleles do not lead to FXS phenotype, but they can cause two distinct pathologies, namely the Premature Ovarian Failure (POF) and the Fragile X Tremor/Ataxia Syndrome (FXTAS) (Fig. 1).

POF is defined as menopause or hypoestrogenic amenorrhea occurring prior to age 40. Usually, POF affects about 1% of the general population; about 6% of women with POF are positive for premutation alleles (Hagerman and Hagerman, 2002).

FXTAS is a neurodegenerative disorder mainly featured by progressive cerebellar ataxia and intention tremor. The patients also show neuropsychiatric alterations (anxiety, hostility, depression) and cognitive dysfunctions, ranging from mild frontal executive and memory deficits to global dementia (Hagerman and Hagerman, 2002; Jacquemont et al., 2007). Although FXTAS mainly affects men, clinical cases of women with FXTAS have been reported (Hagerman et al., 2004).

The etiology of both pathologies is not clear. It is known that the premutation alleles produce an aberrant CGG-expanded mRNA, leading to

slightly reduced amount of FMRP. Since the mRNA has a reduced translational efficiency (Primerano et al., 2002), its levels are significantly increased (Tassone et al., 2000) (Fig. 1). Moreover, neuroanatomical studies revealed neuronal and astrocytic intranuclear inclusions in *post mortem* brains from FXTAS patients (Tassone et al., 2004). Interestingly, these inclusions contain the expanded CGG repeat-containing *FMR1* mRNA, as



well as RNA-binding proteins. (Iwahashi et al., 2006; Tassone et al., 2004). These evidences raised the hypothesis that the premutation condition is a gain-of-function phenotype, due to RNA toxicity (Jacquemont et al., 2007; Swanson and Orr, 2007).

Fig. 1. Genotype-phenotype correlations for FMR1 alleles. Normal (5-44 CGG), grey-zone (45-54), premutation (55-200) and full mutation (>200) alleles are represented. From the top to the bottom are represented: FMRP levels (in blue), *FMR1* mRNA levels (in red), IQ (in

violet), the clinical features and the occurrence of expanding to a full mutation (in green). (Jacquemont et al., 2007)

The mouse model for the Fragile X Syndrome

Several model organisms of the FXS have been created. Since FMR1 gene is conserved along the evolution, the models are available for three organisms, namely mouse (Bakker, 1994; Mientjes et al., 2006), *Drosophila* (Zhang et al., 2001) and *Zebrafish* (Tucker et al., 2006).

The first model available was the FMR1 KO mouse created in 1994 by interrupting the exon 5 with a neomycin cassette (Bakker, 1994). Although this insertional mutation does not mimic the etiology of FXS in humans, it leads to the functional ablation of FMR1 gene. In fact, *FMR1* mRNA is not intact and does not undergo translation (Bakker, 1994). This mouse model presents an array of anatomic, behavioral and neurological similarities to those observed in FXS patients.

First, no gross anatomical abnormalities are present in FMR1 KO brains, as observed in *post mortem* studies of human Fragile X brains (Bakker, 1994; Reyniers et al., 1999). However, FMR1 KO mice show abnormal dendritic spines with a typical immature phenotype, similar to those reported in FXS (Comery et al., 1997; Irwin et al., 2001). In addition, the mutant mice show macro-orchidism from day 15 after birth and at 6 months the size of the testis exceeds 30% compared with normal mice (Kooy, 2003).

FMR1 KO mice also present impairments in synaptic plasticity, namely changes in the strength and/or number of synaptic connections in response to the activity. Experimental protocols to measure those changes are the long-term potentiation (LTP), which corresponds to an increase in the synaptic strength, and long-term depression (LTP), which results in reduced synaptic strength. In FMR1 KO mice, the LTD dependent on the metabotropic glutamate receptors (mGluRs) is enhanced in both hippocampus and cerebellum (Hou et al., 2006; Huber et al., 2002; Pfeiffer and Huber, 2009). This abnormality is also accompanied by a widespread deficit in LTP. Several studies have shown the complete absence or the reduction of LTP in neocortex and hippocampus, respectively (Pfeiffer and Huber, 2009).

However, in both regions, LTP deficits can be rescued by increasing factors involved in LTP induction, such as acute application of the brain-derived neurotrophic factor (BDNF) (Pfeiffer and Huber, 2009). Another study suggests that, in FMR1 KO animals, there is an higher threshold for LTP induction (Meredith et al., 2007).

Finally, FMR1 KO mice are more prone to epileptic seizures, a symptom associated with FXS, and this susceptibility is age-dependent (Kooy, 2003).

The Fragile X Mental Retardation Protein

The Fragile X Mental Retardation Protein (FMRP) is an RNA binding protein (RBP). This class of molecules shuttle between the nucleus and cytoplasm and is involved in the regulation of posttranscriptional steps (splicing, nuclear export, stability, localization and translation) that can occur in a coordinated manner (see the "RNA-operon" theory by (Keene, 2007).

The severe neurological phenotype exhibited in FXS highlights the key role of FMRP in brain, where is highly expressed (Devys et al., 1993). At the subcellular level, FMRP is mainly localized in the cytoplasm, but is also present at low levels in the nucleus (Feng et al., 1997). In neurons, FMRP is present in the cell body, along the dendrites and at the base of the synaptic spines (Antar et al., 2004; Feng et al., 1997; Ferrari et al., 2007), as well as in axonal growth cones and mature axons (Antar et al., 2006; Centonze et al., 2008; Price et al., 2006).

From the soma to the synapse, FMRP is part of large messenger ribonucleoprotein particle (mRNP) containing a number of protein partners and specific mRNAs and noncoding RNAs (Fig. 2). These mRNPs are probably translationally silent as they travel along the dendrites. Like other mRNPs, FMRP-containing particles have a dynamic composition that undergo a series of rearrangements with its interacting proteins (Bagni and

Greenough, 2005). Once the FMRP-silent granule reaches the synapse, the translational repression would be released upon neuronal stimulation thereby contributing to local neuronal synaptic plasticity (Bramham and Wells, 2007; Costa-Mattioli et al., 2009) (Fig. 2).

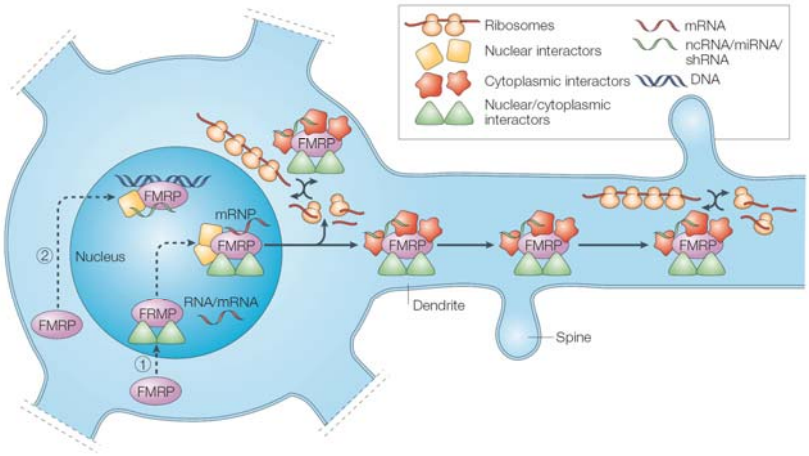


Fig. 2. Speculative model for FMRP shuttling between the nucleus and the cytoplasm. (1) FMRP enters the nucleus and form an mRNP possibly involved in the export from the nucleus to the cytoplasm. Once in the cytoplasm, the FMRP mRNP moves along the dendrites and regulates transport and translation. FMRP can also modulate mRNA stability (not shown). (2) FMRP could also be involved in the RNA-interference pathway that is associated with non coding RNAs. (Bagni and Greenough, 2005)

Biochemically, FMRP can be detected in large particles co-sedimenting with actively translating polyribosomes, in small particles co-sedimenting with silent ribosomal subunits, and with stalled (i.e., polysome associated but not translated) mRNP complexes. Cytologically, FMRP is a component of processing bodies (P bodies) and stress granules (Zalfa et al., 2006). It has been estimated that ~4% of the mRNAs in the mammalian brain are associated with FMRP (Ashley et al., 1993; Brown et al., 2001). Many FMRP target mRNAs encode important neuronal proteins; among the best

characterized are *α-CaMKII* mRNA (Dictenberg et al., 2008; Hou et al., 2006; Muddashetty et al., 2007; Napoli et al., 2008; Zalfa et al., 2003), *Arc* mRNA (Park et al., 2008; Zalfa et al., 2003), *Map1b* mRNA (Brown et al., 2001; Darnell et al., 2001; Dictenberg et al., 2008; Hou et al., 2006; Lu et al., 2004; Zalfa et al., 2003; Zhang et al., 2001), *Sapap4* mRNA (Brown et al., 2001; Dictenberg et al., 2008) and *Rac1* mRNA (Castets et al., 2005; Lee et al., 2003).

FMRP has been implicated in mRNA transport and translation, as well as in stability (Bassell and Warren, 2008; De Rubeis and Bagni, 2009). Both FMRP and associated mRNAs travel along dendrites, a dynamic process that is promoted by synaptic stimulation (Antar et al., 2004; Bassell and Warren, 2008; Ferrari et al., 2007). The transport of FMRP and associated RNAs can occur along microtubule tracks through the interactions with the motor protein kinesin (Antar et al., 2005; Davidovic et al., 2007; Dictenberg et al., 2008; Kanai et al., 2004). While some studies did not detect gross alterations in mRNA targeting in the absence of FMRP (Muddashetty et al., 2007; Steward et al., 1998; Zalfa et al., 2007), others showed that the dendritic localization of *RGS5* mRNA was impaired (Miyashiro et al., 2003). More recently, some investigations demonstrated that FMRP is involved in activity-dependent dendritic transport of several target mRNAs, such as those encoding *Map1b*, *α-CaMKII*, *Sapap4* (Dictenberg et al., 2008). From these data we can conclude that FMRP regulates mainly activity-dependent transport with the exception - so far - of *RGS5* mRNA. Moreover, FMRP regulates local protein synthesis and mRNA stability, as extensively discussed in next chapters.

The control of protein synthesis

Protein synthesis

Protein synthesis is the final step of the gene expression and is finely tuned with other processes, such as splicing, mRNA export and stability (Keene, 2007). It is a sophisticated mechanism, which requires ribosomes, general translation factors and a plethora of modulators. All together, these components orchestrate the initiation, the elongation and finally the termination of the protein synthesis (Groppo and Richter, 2009).

In eukaryotes, the initiation starts with the formation of a ternary complex consisting of GTP, the methionyl tRNA specialized for the initiation (Met-$tRNA_i$) and the initiation factor eIF2. Together with additional factors (eIF3, eIF5, eIF1 and eEF1A), the ternary complex associates with the small 40S ribosomal subunit, forming the 43S ribosomal pre-initiation complex (PIC) (Sonenberg and Hinnebusch, 2009). This complex is then recruited to the mRNA by either cap-independent or cap-dependent mechanisms.

The cap-independent translation is less used and driven by RNA sequences called internal ribosome entry sites (IRESs) that are found in both viral RNAs and cellular mRNAs (Merrick, 2004). In the cap-dependent translation, the ribosome recognizes the 5' cap structure (m7GpppX, where m is a methyl group and X is any nucleotide) through the trimeric complex eIF4F (Fig. 3). eIF4F is composed by the cap-binding protein (eIF4E), an ATP-dependent helicase which relaxes the secondary structure of the 5'UTR (eIF4A) and a scaffolding protein (eIF4G). eIF4G links the mRNA to the 43S PIC by interacting with eIF3 (Sonenberg and Hinnebusch, 2009). Moreover, the poly(A)-binding protein (PABP) associates with eIF4G, allowing the circularization of the mRNA (Tarun and Sachs, 1996). The PABP-eIF4G interaction would also promote the recognition of the 43 pre-

initiation complex by stabilizing the eIF4F binding to the cap (Sonenberg and Hinnebusch, 2007).

Once bound, the 43 PIC scans the mRNA in 5'→3' direction, until the initiation codon. Since the association of the 40S to the large ribosomal subunit 60S is prevented by the initiation factors, they are released. This event requires the hydrolysis of the eIF2-bound GTP, promoted by the GTPase eIF5B and the GTPase-activating protein eIF5 (Sonenberg and Hinnebusch, 2009) (Fig. 3).

After the two ribosomal subunits are joined together in the 80S, the elongation starts. During this step, the ribosome moves along the mRNA and the aminoacyl-tRNAs are recruited and delivered to the ribosomes. According to the reading frame, the aminoacids are added and linked via peptide bonds, giving rise to a nascent peptide. The elongation requires only two additional factors, eEF1A and eEF2. While eEF1A contributes to delivering the aminoacyl-tRNA to the ribosomes, eEF2 facilitates the translocation of the ribosome along the mRNA (Marshall et al., 2009; Richter and Klann, 2009).

Finally, the ribosome recognizes the stop codon and is released from the mRNA. The termination is mediated by the release factor eRF1, which binds the ribosome in place of a tRNA (Richter and Klann, 2009).

The regulation of the protein synthesis is essential to keep under control the basic expression of housekeeping proteins (Sonenberg and Hinnebusch, 2007). Alterations in the basic mechanisms of translation lead to pathological conditions. Rare pathologies are caused by mutations in the components of the translation machinery, such as ribosomal proteins, translational factors, tRNAs and aminoacyl-tRNA synthetases. Although general translation should be severely affected, these pathologies do not show overlapping phenotypes involving a broad range of tissues. This raises the idea that the components of the translation machinery could have

additional functions besides their canonical, housekeeping role (Scheper et al., 2007). In addition, several diseases are due to uncontrolled protein synthesis. This is the case for cancer and in heart pathologies, frequently associated with upregulated activity and/or expression of initiation factors (Sonenberg and Hinnebusch, 2007).

Moreover, the control of protein synthesis regulates a subset of mRNAs in certain tissues and/or developmental windows. For example, during early embryogenesis, the specification of the embryonic axis, the body pattern and the cellular differentiation rely on the translational control, since transcription is quiescent (Sonenberg and Hinnebusch, 2007). This has been largely demonstrated in *Drosophila* embryo and *Xenopus* oocytes, where maternal mRNAs are spatially restricted and stored in a translational silent state (Martin and Ephrussi, 2009).

In neurons, specific mRNAs are transported along the dendrites and locally translated at synapses (Bramham and Wells, 2007). This process underlies sophisticated phenomena, such as synaptic plasticity at the basis of learning and memory (Costa-Mattioli et al., 2009).

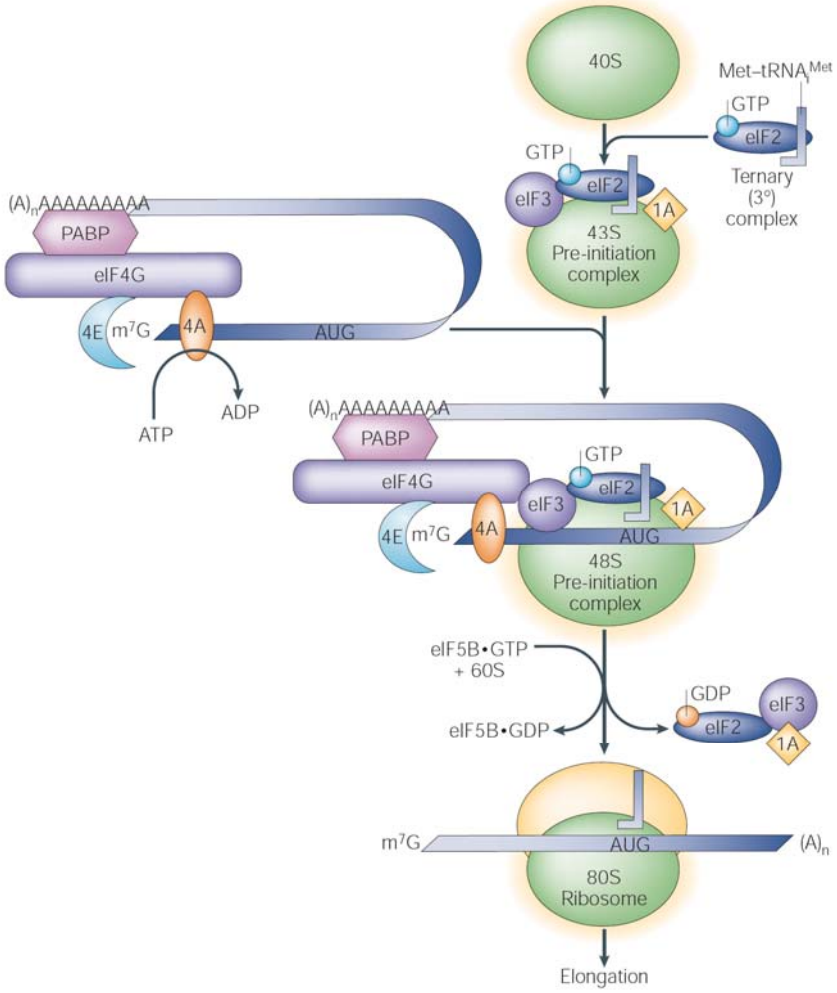


Fig. 3. Pathway of translation initiation in eukaryotes. The ternary complex (eIF2•GTP•Met-tRNAi) associates with the small ribosomal subunit (40S). This binding is promoted by additional factors, such as eIF3, eIF1 (not shown) and eIF1A (1A) and generates the 43S pre-initiation complex. The cap-binding complex, consisting of eIF4E (4E), eIF4G and eIF4A (4A), binds the cap structure at the 5' of the mRNA. eIF4G also interacts with the PABP, circularizing the mRNA. After the binding of eIF4G to eIF3 and the activation of the ATP-dependent helicase (eIF4A), the 43S pre-initiation complex binds the 5'UTR and scans the mRNA until the start codon (AUG). Once the eIF2-bound GTP is hydrolyzed by eIF5 (not shown) and eIF5B, the translational factors are released from the 40S and the joining of the two ribosomal subunits occurs (Klann and Dever, 2004)

Translational control in neurons

In the nervous system, the translational control is essential not only for basic gene expression, but also for the consolidation and storage of long-term memories. In fact, short-lasting forms of synaptic plasticity, such as the early phase of the long-term potentiation (E-LTP), rely on posttranslational modifications of pre-existing proteins. On the contrary, the long-lasting forms of plasticity, such as the late phase of the LTP (L-LTP), require *de novo* protein synthesis (Klann and Dever, 2004).

As mentioned, protein synthesis does not occur only in neuronal soma, but also along the dendrites and at synapses. In fact, in these specialized compartments there are polyribosomes, translational factors and specific mRNAs (Bramham and Wells, 2007; Steward and Schuman, 2003). Cultured neurons or purified synapses (synaptoneurosomes) stimulated with BDNF activate local protein synthesis (Aakalu et al., 2001; Schratt et al., 2004; Takei et al., 2004). Moreover, BDNF-induced LTP in hippocampal brain slices can be blocked by translational inhibitors, even when the pre- and postsynapses are severed from the soma (Kang and Schuman, 1996). Another form of synaptic plasticity, the metabotropic glutamate receptors (mGluR)-dependent long-term depression (mGluR-LTD), also depends on local protein synthesis (Huber et al., 2000).

In neurons, as well as in other cells, several mechanisms of translational control occur at the initiation steps and mainly involve eIF2 and eIF4E. Once the eIF2-bound GTP is hydrolyzed, the GDP-eIF2 is recycled to GTP-eIF2 by eIF2B, a guanine nucleotide exchange factor (GEF). This process can be inhibited by four specific kinases during certain conditions (viral infections, low heme levels, endoplasmic stress reticulum and aminoacid limitation). Upon activation, the kinases phosphorylate one of the three subunits composing eIF2, namely the α subunit (eIF2α). The phosphorylated eIF2α

acts as a competitive inhibitor of eIF2B, impairing eIF2 recycling (Costa-Mattioli et al., 2009).

In the brain, the phosphorylation of eIF2 plays an important role in long-lasting synaptic plasticity. In fact, cultured neurons treated with BDNF have decreased levels of phosphorylated eIF2α (Takei et al., 2001). Moreover, L-LTP is associated with reduced eIF2α phosphorylation and can be blocked by inhibiting eIF2α dephosphorylation (Costa-Mattioli et al., 2007). Consistently, mice expressing a mutant eIF2α with impaired phosphorylation display decreased threshold for the induction of the L-LTP and enhanced memory (Costa-Mattioli et al., 2007). The four kinases responsible for eIF2α phosphorylation, especially that one responsive to aminoacid deprivation (GCN2), are expressed in the brain (Klann and Dever, 2004). According with the previous data, GCN2 mutant mice show a phenotype similar to the eIF2α mutant mice (Costa-Mattioli et al., 2005).

The direct phosphorylation of eIF4E also contributes to the regulation of protein synthesis initiation. In fact, eIF4E can be phosphorylated on a single site and this modification decreases the cap-binding affinity (Scheper et al., 2002). This event would release eIF4E from the cap, therefore promoting the ribosome scanning (Klann and Dever, 2004). In fact, both LTP and mGluR-LTD are associated with increased eIF4E phosphorylation (Banko et al., 2006; Kelleher et al., 2004). However, the mechanisms behind this effect are still controversial (Costa-Mattioli et al., 2009).

A finely tuned mechanism to modulate protein synthesis involves the eIF4E-binding proteins (4E-BPs). The 4E-BPs and eIF4G share a canonical eIF4E-binding site (YXXXXLΦ, where X is any aminoacid and Φ is a hydrophobic aminoacid) (Costa-Mattioli et al., 2009; Marcotrigiano et al., 1999). Therefore, they compete for binding to the surface of eIF4E and the 4E-BPs prevent eIF4E-eIF4G association (Costa-Mattioli et al., 2009; Marcotrigiano et al., 1999). This compromises the formation of eIF4F and

blocks translation. An array of physiological stimuli activates the extracellular signal-regulated kinase (ERK), phosphoinositide 3-kinase (PI3K) and mammalian target of rapamycin (mTOR) pathways, leading to the phosphorylation of the 4E-BPs (see below). This event disrupts the interaction with the eIF4E, relieving the translational inhibition (Richter and Klann, 2009) (Fig. 4).

Three canonical 4E-BPs have been identified in mammals. 4E-BP1 is mostly present in adipose tissues and in pancreas, 4E-BP3 in the liver and 4E-BP2 in the brain, which expresses little or no 4E-BP1 and 3 (Banko et al., 2005; Klann and Dever, 2004). Interestingly, 4E-BP2 KO mice show impaired hippocampal LTP (the E-LTP is converted in L-LTP) and memory deficits in several behavioral tests (Banko et al., 2007; Banko et al., 2005). Such phenotype highlights the importance of the 4E-BPs mechanism in regulating translation during synaptic plasticity.

The main kinase responsible for the phosphorylation of the 4E-BPs is the mammalian Target Of Rapamycin (mTOR), which takes part in two distinct complexes, mTORC1 and mTORC2.

While mTORC1 directly modulates initiation factors, mTORC2 is indirectly implicated the regulation of those factors (see below) (Ma and Blenis, 2009). mTORC1 comprises mTOR, Raptor (regulatory associated protein of mTOR) and LST8. Raptor recruits specific targets for the subsequent phosphorylation by mTOR. In fact, Raptor can bind the 4E-BPs, as well as the ribosomal protein kinase S6K, which in turn phosphorylates the ribosomal protein S6 and eIF4B. Unlike mTORC2, mTORC1 is sensitive to the antifungal rapamycin. This drug associates with the immunophilin FKBP12 and disrupts the mTOR-Raptor interaction (Ma and Blenis, 2009).

The dominant pathway modulating mTORC1 is the PI3K signaling (Fig. 4). In response to a plethora of stimuli, PI3K is activated and converts the membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PIP_2) in PIP_3. PIP_3 recruits the kinase Akt to the membranes, where is phosphorylated

and activated by the phosphoinositide-dependent kinase 1 (PDK1) and by mTORC2. Once activated, Akt inhibits the tuberous sclerosis complex (TSC), composed by TSC1 and TSC2. TSC2 is a GTPase activating protein for the small GTPase Rheb (Ras homologue enriched in brain) and reduces its activity. When TSC2 is inactive, Rheb is in the active, GTP-bound form, which is required for mTOR activation. Thus, the 4E-BPs are hyperphosphorylated, eIF4E is no longer sequestered and translation starts (Ma and Blenis, 2009; Richter and Klann, 2009) (Fig. 4).

In addition to the PI3K pathway, the extracellular signal-regulated kinase (ERK) can also activate mTOR. In fact, ERK phosphorylates and activates p90S6K (RSK), which can in turn activate PDK. Moreover, both ERK and RSK can phosphorylate and block TSC2 (Costa-Mattioli et al., 2009).

The modulation of mTOR pathway and downstream effectors is a critical event for translation-dependent synaptic plasticity. LTP triggers activation of mTOR, resulting in increased 4E-BP2 phosphorylation and eIF4F formation (Banko et al., 2005; Kelleher et al., 2004). Moreover, LTP induces S6K and subsequent S6 phosphorylation and these events require both mTOR and ERK signaling (Hoeffer et al., 2008; Tsokas et al., 2005; Tsokas et al., 2007). These changes occur locally along the dendrites (Tsokas et al., 2005). Similar events are induced also during mGluR-LTD (Antion et al., 2008; Banko et al., 2006; Hou et al., 2006; Ronesi and Huber, 2008). Finally, genetic alterations in the components of the mTOR pathway result in deficits in synaptic plasticity and behavioral anomalies. Besides the 4E-BP2 KO mice described above, interesting phenotypes have been observed in FKBP12 conditional KO mice and TSC2 heterozygous KO mice. As mentioned, FKBP12 mediates the rapamycin effects of mTORC1; consistently, the postnatal ablation of this gene results in increased mTORC1 (Hoeffer et al., 2008). The mice display enhanced contextual fear memory and perseverative/repetitive behaviors (Hoeffer et al., 2008). Moreover, TSC2 heterozygous KO mice show an array of memory deficits, but a brief

treatment with rapamycin in adult mice rescues both synaptic plasticity and behavior alterations (Ehninger et al., 2008a).

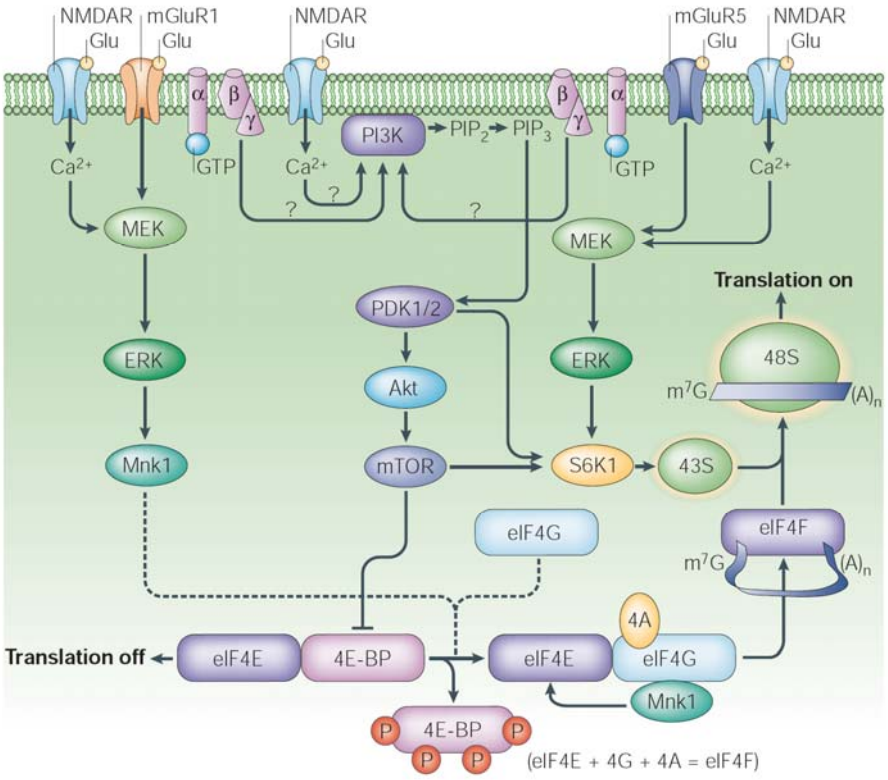


Fig. 4. Signaling pathways involved in translational regulation during L-LTP and mGluR-dependent LTD. Stimulation of mGluRs and NMDA receptors triggers the ERK and the PI3K pathways. The sequential activation of PI3K, PDK1/2, Akt and mTOR results in the phosphorylation of S6K and 4E-BPs. The phosphorylation of 4E-BPs leads to the dissociation of the eIF4E. Therefore, eIF4F (eIF4E-eIF4A-eIF4G) is formed and translation starts. Of note, these signal transduction cascades are also activated by BDNF (not shown). (Klann and Dever, 2004)

The role of FMRP in translation

As mentioned above, FMRP is a modulator of mRNA translation. First, microarray analysis revealed that 251 mRNAs associated with FMRP have an abnormal polysomal distribution in cells derived from FXS patients, indicating a role of FMRP in the regulation of protein synthesis (Brown et al., 2001). Moreover, FMRP represses protein synthesis both *in vitro* (Laggerbauer et al., 2001; Li et al., 2001) and *in vivo* (Lu et al., 2004; Muddashetty et al., 2007; Napoli et al., 2008; Zalfa et al., 2003). Therefore, in mouse brain from FMR1 KO mice, a subset of target mRNAs, namely *α-CaMKII, Arc* and *Map1b*, are distributed preferentially on the actively translating fractions (polysomes) along sucrose gradients (see below) (Zalfa et al., 2003). Consequently, the levels of the proteins encoded by those mRNAs are significantly increased in the absence of FMRP (Zalfa et al., 2003). Noteworthy, these changes are also present in purified synaptoneurosomes, indicating that FMRP negatively regulates protein synthesis at synapses (Muddashetty et al., 2007; Zalfa et al., 2003).

A key co-player in mediating FMRP-dependent repression is the *Brain Cytoplasmic 1* (*BC1*) RNA, a small non coding RNA also expressed along the dendrites (Rao and Steward, 1993). *BC1* links FMRP to some target mRNAs, such as *α-CaMKII, Arc* and *Map1b* mRNAs (Zalfa et al., 2003). In fact, *BC1* anneals with the mRNAs by base-pairing and in turn binds to FMRP (Zalfa et al., 2005; Zalfa et al., 2003).

In addition to the modulation of the basic levels of protein synthesis, FMRP is also responsible for the control of activity-dependent translation (Bassell and Warren, 2008). As extensively discussed, during BDNF-induced LTP or mGluRs-dependent LTD, synaptic protein synthesis is activated (Costa-Mattioli et al., 2009). Interestingly, the activation of group I mGluRs (mGluR1 and 5) with the agonist 3,5-dihydroxyphenylglycine (DHPG) increases FMRP at synapses by either recruiting the dendritic FMRP or activating local synthesis of *FMR1* mRNA (Antar et al., 2004;

Weiler et al., 1997). DHPG treatment activates translation in WT, but not FMR1 KO synaptosomes (Muddashetty et al., 2007; Todd et al., 2003; Westmark and Malter, 2007). Furthermore, the target mRNAs are not longer recruited on polysomes in FMR1 KO synaptoneurosomes upon stimulation, indicating that the lack of FMRP impairs mGluR-induced translation (Muddashetty et al., 2007). In conclusion, FMRP controls both basal and activity-dependent synaptic protein synthesis (Muddashetty et al., 2007; Zalfa et al., 2003).

These observations, together with the deficits in mGluR-dependent LTD observed in FMR1 KO animals (Pfeiffer and Huber, 2009), contribute to create the so-called "mGluR theory" (Bear et al., 2004). This theory suggests that FMRP normally represses translation downstream of mGluRs and, in the absence of FMRP, uncontrolled protein synthesis results in excessive AMPA internalization and increased LTD (Bear et al., 2004). In support of this theory, some morphological, physiological and behavioral features of FXS can be rescued in the model organisms either by administration of a mGluR antagonist (MPEP) (McBride et al., 2005; Tucker et al., 2006; Yan et al., 2005) or genetic reduction of mGluR5 (Dolen et al., 2007).

The mechanisms behind FMRP-mediated translational control, namely whether FMRP intervenes during initiation or elongation, are still debated. Sedimentation along sucrose gradients, which measures complex size after ultracentrifugation, indicates the translational state of an mRNP (Zalfa et al., 2006). Usually, translational initiation complexes are smaller than a ribosome (80S) and are referred as light mRNPs; actively translating particles (polysomes) are bigger than the 80S. However, stalled initiation complexes, such as stress granules and P bodies, can aggregate in particles as heavy as polysomes (Zalfa et al., 2006). Some studies detected FMRP co-fractionating with polysomes (Ceman et al., 2003; Khandjian et al., 2004; Stefani et al., 2004), while some others showed co-sedimentation with

mRNPs (Ishizuka et al., 2002; Siomi et al., 2002; Siomi et al., 1996; Zalfa et al., 2003).

New insights into the posttranslational modifications of FMRP could reconcile these observations. FMRP can be either phosphorylated (Ceman et al., 2003) and methylated (Dolzhanskaya et al., 2006; Stetler et al., 2006). Ceman et al. (2003) reported that exogenous FMRP associates with apparently stalled polysomes, while mutants that cannot be phosphorylated are run-off from those fractions (Ceman et al., 2003). It is tempting to hypothesize that FMRP could shuttle between the two fractions depending on whether it is posttranslationally modified or not, although the mechanism still needs to be elucidated. Interestingly, the change in phosphorylation does not affect FMRP association to RNA. Moreover, two recent reports indicate that the endogenous phosphorylation of FMRP through the PP2A/S6K1 pathway would modulate the translational repression of FMRP mRNA targets in response to mGluRs signaling (Narayanan et al., 2007; Narayanan et al., 2008).

Furthermore, the distribution of FMRP on both mRNPs and polysomes could account for different functions of FMRP in either repressing or activating translation (Zalfa et al., 2006). In fact, Brown and colleagues found that out of 251 mRNAs displaying different polysomal distribution in cells from FXS patients, 136 were increased on polysomes and 115 decreased (Brown et al., 2001). Additionally, a recent report suggested that FMRP could promote the translation of a novel target mRNA, namely the mRNA encoding the superoxide dismutase 1 (SOD1) (Bechara et al., 2009).

The idea that FMRP is implicated in the repression of the initiation is also supported by the recent discovery that the Cytoplasmic FMRP Interacting Protein 1 (CYFIP1) is a novel neuronal 4E-BP (Napoli et al., 2008). CYFIP1 was early identified as a partner of FMRP in neurons (Schenck et al., 2003; Schenck et al., 2001) and as a crucial component of the actin cytoskeleton (Takenawa and Suetsugu, 2007). In fact, CYFIP1 is part of the complex with

the WASP-family verprolin-homologous (WAVE) family of proteins and could be the linker between upstream signaling and the activation of the complex (Eden et al., 2002; Kobayashi et al., 1998; Schenck et al., 2003).

Our laboratory found that CYFIP1, as well as FMRP, co-fractionates with light mRNPs and that it is part of the cap-binding complex, together with eIF4E and PABP (Napoli et al., 2008). Interestingly, the association of CYFIP1 with eIF4E is competed by exogenous 4E-BP2. In addition, CYFIP1 binds directly eIF4E, although it does not have the canonical eIF4E-binding sequence (YXXXXLΦ, where X is any aminoacid and Φ is a hydrophobic aminoacid) (Costa-Mattioli et al., 2009; Marcotrigiano et al., 1999). Surprisingly, the "non canonical" sequence is predicted to form a peculiar "reverse L shaped" with two α helices turns; this structure overlaps with the region of the 4E-BPs fitting into the eIF4E pocket (Marcotrigiano et al., 1999; Napoli et al., 2008). The FMRP-CYFIP1-eIF4E complex is present also in synaptoneurosomes and contains *BC1* RNA, as well as the FMRP targets *α-CaMKII*, *Map1b*, *App* and *Arc*. Noteworthy, stimulation with either BDNF or DHPG reduces the binding of CYFIP1 to eIF4E and the presence of *Map1b* mRNA and *BC1* RNA in the complex (Napoli et al., 2008). According to all these observations, CYFIP1 downregulation in cultured neurons, as well as genetic depletion of CYFIP1 (CYFIP1 +/- mice), causes a significant increase in the protein levels of α-CaMKII, MAP1B and APP (Napoli et al., 2008). All these data support the model that at synapses FMRP tethers specific mRNAs on CYFIP1, which in turn sequesters eIF4E and represses translation initiation. Upon synaptic stimulation, FMRP-CYFIP1 dissociates from eIF4E and translation takes place (Fig. 5).

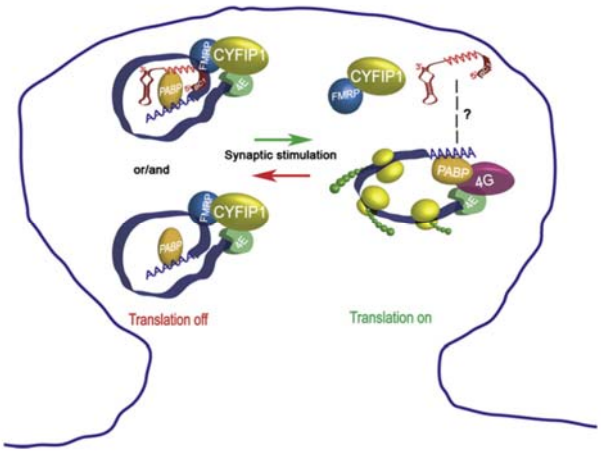


Fig. 5. Proposed model for mRNA translational repression and activation by CYFIP1-FMRP complex. CYFIP1-FMRP and CYFIP1-FMRP-*BC1* mRNPs are transported to the synapses in a translationally dormant state. After synaptic stimulation, CYFIP1-FMRP complex is released from eIF4E and local translation takes place. (Napoli et al., 2008)

CYFIP is not the only "specific" 4E-BP. In fact, the model proposed by Napoli and colleagues closely resembles the mechanism described for two regulatory complexes: vertebrate Maskin/Neuroguidin-CPEB and *Drosophila* Cup-Bruno. In such cases, a protein (CYFIP1, Maskin or Neuroguidin, Cup) sequesters the initiation factor eIF4E and simultaneously binds an RNA-binding protein (i.e., FMRP, CPEB, and Bruno, respectively); this configuration tethers the repression complex to a specific subset of mRNAs (Richter and Klann, 2009) (Fig. 6).

Besides the well-documented role in translational control, FMRP has been recently implicated in the regulation of mRNA stability (De Rubeis and Bagni, 2009).

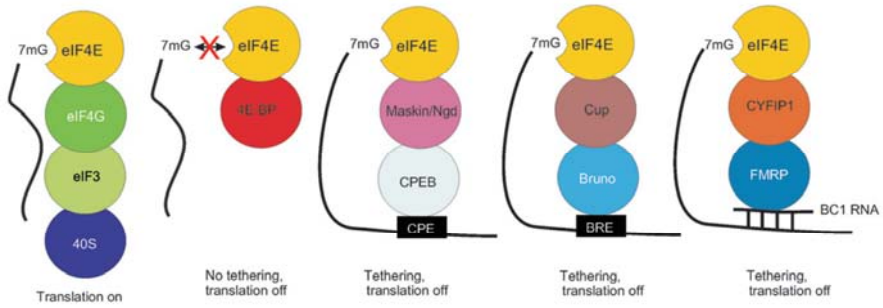


Fig. 6. Translational control by 4E-BPs. Cap-dependent translation depends on interactions between eIF4E, eIF4G, eIF3 and the small ribosomal subunit 40S. The eIF4E-eIF4G interaction can be disrupted by the "canonical" 4E-BPs (4E-BP1/2/3) or by "specific" 4E-BPs, such as Maskin, Neuroguidin (Ngd), Cup or CYFIP1. Because of specific RNA-binding proteins, such as CPEB, Bruno or FMRP, the inhibitory complex is driven on a subset of mRNAs. (Richter and Klann, 2009)

The control of mRNA stability

The regulation of protein stability and degradation

mRNA stability is a highly regulated posttranscriptional step tightly coordinated with mRNA translation. A specific RNA surveillance mechanism, the Nonsense-Mediated Decay (NMD), removes mRNAs harboring premature stop codons to prevent the accumulation of aberrant, possibly dominant-negative, proteins (Behm-Ansmant and Izaurralde, 2006). Once mRNAs overcome NMD control, they can be translated or sequestered such that they can undergo translation or degradation at a later time. In the latter case, several factors can modulate the decay rate of mRNAs and mediate the communication between translation and degradation.

Moreover, the interplay between translation and degradation may take place in cytoplasmic foci referred as P bodies, where mRNAs can be degraded or stored to later re-enter the translating pool of mRNAs (Parker and Sheth, 2007). These bodies are functionally related to other cytoplasmic aggregates, namely stress granules, which are composed by stalled translational pre-initiation complex (Kedersha et al., 2005). The nature of these bodies remains to be elucidated.

Normally, housekeeping genes produce invariantly stable transcripts while the turnover of some mRNAs undergoes a tight regulation to rearrange gene expression to certain cellular and/or developmental stimuli. Many mRNAs have a fast decay rate, resulting in a low steady-state level of protein under basal conditions. After stimulation, the mRNAs are rapidly induced by transcriptional activation and a modest increase in the amount leads to a significant variation in their expression (Khabar, 2007). In neurons, among the mRNAs regulated at the stability level are those encoding proteins related to neuronal growth (GAP-43, NGF, Tau), enzymes or enzyme inhibitors (acetylcholinesterase, neuroserpin), receptors (D2 dopamine receptor, m4 muscarinic receptor, β_1-adrenergic receptor) and transcription factors (c-Fos, N-Myc/c-Myc) (Bolognani and Perrone-Bizzozero, 2008).

The decay rate of mRNAs depends on *cis*-acting elements frequently located in their 3'UTRs as well as their associated *trans*-acting factors. A well characterized sequence involved in mRNA stability is a 50-150 nucleotide sequence rich in adenosine and uridine, the so-called AU-rich element (ARE). These sequences are located in the 3' UTRs of the mRNAs that are regulated by the AU-rich RNA binding proteins (AUBPs). In some cases, these sequences are rich in different residues such as GU or C (Kim and Gorospe, 2008; Vlasova et al., 2008). The importance of AREs in regulating gene expression is highlighted by the fact that 5-8% of human genes encode ARE-containing transcripts (Bakheet et al., 2001). Although

AREs were originally defined as an AUUUA core associated with instability (Shaw and Kamen, 1986), it became clear over the years that ARE motifs can vary somewhat and regulate mRNA stability in both directions (Barreau et al., 2005). In fact, the interaction between ARE sequences and ARE-binding proteins can block or enhance the recruitment of the mRNA decay machinery and lead to a rapid modification of gene expression in response to environmental and developmental conditions.

Several RNA binding proteins that associate with these RNA elements are AU-binding factor 1 (AUF1) (Zhang et al., 1993), Tristetraprolin (TTP) (Carballo et al., 1998), Hu/ELAV (Dalmau et al., 1990), CUG triplet RNA-binding protein 1 (CUG-BP1) (Vlasova et al., 2008), and butyrate response factor-1 (BRF1) (Stoecklin et al., 2002). While the first two proteins are detected in all tissues, some of the Hu protein are neuro-specific (Hambardzumyan et al., 2009). In general AUF1, TTP, CUG-BP are destabilizing factors that decrease the half-life of mRNA while the Hu proteins are stabilizing factors that promote stability and translation. AUF-1 might promote stability and degradation depending on the mRNA and the cell type (Sela-Brown et al., 2000; Xu et al., 2001). In addition, microRNAs also affect mRNA stability. In a genome-wide microarray analysis it has been shown that some microRNAs downregulate many target mRNAs (Lim et al., 2005). Further studies have also identified the molecular mechanism and the protein complex(es) involved (Bagga et al., 2005; Behm-Ansmant et al., 2006; Wu et al., 2006).

In mammals, two major pathways for mRNA degradation have been described. The first step is the removal of the poly(A) tail, which opens both 5’ and 3’ ends for exonucleolytic attack (Fig. 7). In fact, the interaction of the poly(A) and the 5’ end of the mRNA, via the PABP-eIF4G complex, forms a closed-loop state of the mRNA that is not accessible to the exonucleases (Mazumder et al., 2003).

In the first pathway, after deadenylation, the decapping enzymes Dcp1 and Dcp2 eliminate the 5' cap and the mRNA body is degraded by the 5'→ 3' exonuclease Xrn (Wilusz and Wilusz, 2004). Alternatively, the decay occurs in 3'→ 5' direction catalyzed by the exosome, a large exonucleolytic complex. The residual cap is degraded by the scavenger enzyme DcpS (Wilusz and Wilusz, 2004) (Fig. 7).

One of the most well described neuronal mRNAs regulated at the stability level is that encoding Growth-Associated Protein 43 (GAP-43), a developmentally-regulated protein involved in axon elongation in both developing and regenerating neurons (Korshunova and Mosevitsky, 2008). The expression of GAP-43 is posttranscriptionally regulated by HuD, a neuronal ARE-binding protein belonging to the family of ELAV/Hu proteins. HuD recognizes an U-rich element in the 3'UTR of *GAP-43* mRNA and stabilizes it by interfering with the removal of the poly(A) tail (Beckel-Mitchener et al., 2002; Chung et al., 1997). Indeed, increased levels of HuD correspond to higher GAP-43 expression (Anderson et al., 2001; Bolognani et al., 2006; Pascale et al., 2004), leading to changes in neurite outgrowth (Anderson et al., 2001) and in synaptic plasticity (Bolognani et al., 2007b; Pascale et al., 2004).

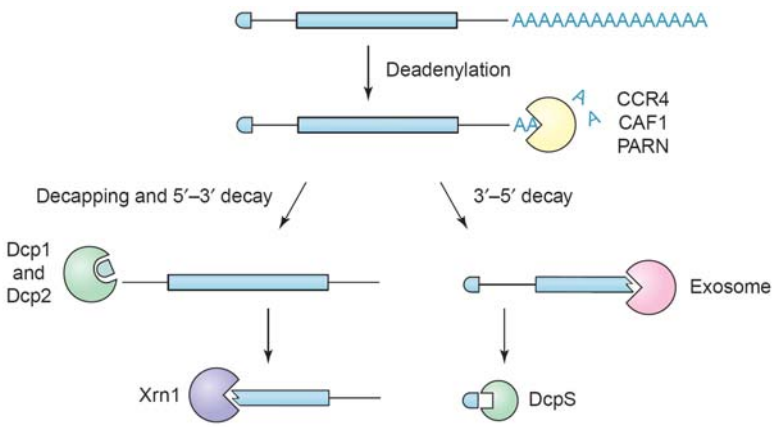


Fig. 7. The pathways of the mRNA decay. The first step is the deadenylation, followed by 5'→3' or 3'→5' degradation. The 5'→3' decay starts with the removal of the cap by the Dcp1 and Dcp2 enzymes, followed by the degradation via the exonuclease Xrn1. The 3'→5' decay is catalyzed by a large complex, the exosome, and the remaining cap is degraded by the scavenger protein DcpS. (Wilusz and Wilusz, 2004)

Neuronal regulators of mRNA stability

Hu/ELAV proteins. One of the few specific proteins that have been described as key regulators of mRNA turnover in brain is the Hu family of proteins. The Hu proteins were first described as autoantigens in patients affected by paraneoplastic encephalomyelitis (Dalmau et al., 1990). The human proteins were then recognized to be orthologous of the *Drosophila* Embryonic Lethal Abnormal Vision (ELAV) protein, a splicing regulator important for neural development (Simionato et al., 2007). In mammals, four ELAV/Hu proteins have been described: HuR (alias HuA), HuB, HuC and HuD (Brennan and Steitz, 2001). While HuR is ubiquitous and HuB is present in the brain and in germ cells, the expression of HuC and HuD is restricted to neurons. The neuronal ELAV/Hu proteins (nELAV) are essential for development of the nervous system. In fact, Hu proteins are reported as early markers of neuronal commitment and show a specific timing of expression in the developing brain (Okano and Darnell, 1997; Pascale et al., 2008). Studies *in vivo* or in primary neurons in which the nELAV expression has been perturbed by either overexpression or downregulation directly implicate these proteins in neuronal differentiation as well as in learning and memory (Akamatsu et al., 2005; Anderson et al., 2001; Bolognani et al., 2004; Bolognani et al., 2007a; Pascale et al., 2004; Quattrone et al., 2001). In neurons, the ELAV proteins are expressed in the cell body and along the dendrites (Bolognani et al., 2004; Tiruchinapalli et al., 2008a; Tiruchinapalli et al., 2008b); HuD has been also found in the neurites of in PC12 cells (Aranda-Abreu et al., 1999; Smith et al., 2004). Like many other RNA-binding proteins, the Hu proteins are detected both in

the nucleus and in the cytoplasm, suggesting different roles in different compartments. Although a role in splicing and nuclear polyadenylation is possible, the best characterized functions are the control of mRNA decay and the regulation of protein synthesis (Hinman and Lou, 2008). Focusing on the mRNA stability process, the Hu proteins can positively modulate the half-life of a subset of mRNAs critical for neuronal differentiation and maintenance. This group includes mRNAs encoding key transcription factors (c-Fos, c-Myc), molecules involved in neurite outgrowth and synapse functionality (the above-mentioned GAP-43, Tau, acetylcholinesterase, neuroserpin) and determinant of neural differentiation (Musashi-1) (Pascale et al., 2008).

AUF1 and KSRP. While the Hu proteins are mostly implicated in the stabilization of mRNAs, other neuronal decay-promoting factors have also been described. One of the first identified is the AU-binding factor 1 (AUF1), also known as hnRNP D (Zhang et al., 1993). AUF1, which shuttles between the nucleus and cytoplasm, consists of four isoforms generated by alternative splicing of a single transcript (Sarkar et al., 2003a; Wagner et al., 1998). The different isoforms are expressed in the brain and show specific activities in binding and modulating the decay of ARE-containing transcripts (Dobi et al., 2006; Raineri et al., 2004; Sarkar et al., 2003b). Although the mRNAs associated with AUF1 are well characterized in non-neuronal cells (Bhattacharya et al., 1999; Mazan-Mamczarz et al., 2009), few target mRNAs have been identified in the brain. One example is the mRNA encoding the α_2 subunit of the nitric-oxide sensitive guanylyl cyclase in the cerebellar granule cells; the messenger is bound by AUF1 but upon the activation of the N-methyl-D-aspartate (NMDA) glutamate receptors, AUF1 is downregulated and the α_2 mRNA is stabilized (Jurado et al., 2006). In this case, there is an activity-dependent regulation mediated by AUF1. It has also been suggested that AUF1 is involved in integrating genetic and epigenetic signals during cortical development. It is specifically expressed in subsets of

proliferating neural precursors and differentiating postmitotic neurons of the developing cortex (Lee et al., 2008). Recently AUF-1 has been shown to promote the degradation of some target mRNAs but increase the stability and translation of other mRNAs ; this duality may be due to relative abundance of AUF1 (Mazan-Mamczarz et al., 2009).

Another destabilizing factor present in neurons is the human K homology splicing regulatory protein (KSRP), a protein originally described as a splicing regulator (Min et al., 1997). KSRP is homologous to the murine Zipcode Binding Protein 2 (ZBP2), involved in *β-actin* mRNA localization in neurons (Gu et al., 2002). The protein is expressed in neurons and in glia and it is distributed is both the nucleus and cytoplasm where it can interact with mRNAs and enhance their turnover (Chou et al., 2006; Gherzi et al., 2004; Snee et al., 2002). Despite the fact that KSRP is neuronal, no specific target mRNAs in the brain have been characterized.

The role of FMRP in stability control

In addition to its role in mRNA transport and translation, recent evidence shows that FMRP assumes an activity related to mRNA decay. Three studies have shown that mRNA abundance is affected when FMRP expression is abolished. Although such reports do not directly demonstrate a role of FMRP in mRNA stability, they provided a first cue that mRNA levels are altered in the absence of FMRP. First, in a high-throughput screen to identify FMRP target mRNAs, Brown et al. (2001) found that the levels of 144 genes were changed in lymphoblastoid cells from Fragile X patients (Brown et al., 2001).

In another analysis, Miyashiro et al. (2003) identified some of the same mRNAs as Brown et al. (2001) and described their expression pattern; 3 out of 11 mRNAs were reduced in the *FMR1* KO versus wild type mouse

hippocampus (Miyashiro et al., 2003). For two of these mRNAs, those encoding the ribosomal component p40/LRP and the G-protein-coupled receptor kinase 4 (GRK4), the subcellular distribution was unaffected while for the dystroglycan-associated glycoprotein 1 (*DAG1* mRNA), an altered dendritic localization as well as reduced abundance was observed (Miyashiro et al., 2003).

Moreover, FMRP loss may also lead to an impaired expression of $GABA_A$ receptors. The *δ subunit* mRNA, previously identified by Miyashiro et al. (2003), has been found to be downregulated in *FMR1* KO neurons in a genome-wide expression profiling study (Gantois et al., 2006) as well as in other studies addressing its localization (Dictenberg et al., 2008). Consistent with these results, El Idrissi et al. (2005) reported a decreased expression of the GABA β subunit in several brain areas from *FMR1* KO mice (El Idrissi et al., 2005). Interestingly, a further indication of FMRP as neuronal mRNA stabilizing factor came from D'Hulst et al. (2006) who reported that the mRNAs encoding 8 out of the 18 known GABA subunits (α_1, α_3, α_4, β_1, β_2, γ_1, γ_2 as well as the above mentioned δ) were significantly reduced in the cortex, but not in the cerebellum, from FMRP-lacking mice. In addition, the expression of all the three subunits conserved in Drosophila appears to be compromised as well (D'Hulst et al., 2006).

Finally, two recent reports implicate FMRP as a direct modulator of mRNA turnover (Fig. 7). First, FMRP has been shown to be involved in the regulation of *PSD-95* mRNA stability in hippocampus (Zalfa et al., 2007). PSD-95 encodes a key scaffolding protein of the post-synaptic density (PSD), the signal transduction machinery at glutamatergic synapses (Kim and Sheng, 2004). Because PSD-95 loss compromises both the structure and the function of dendritic spines (Migaud et al., 1998; Vickers et al., 2006), alterations in PSD-95 expression could contribute to the cognitive impairment caused by the absence of FMRP. *PSD-95* mRNA is part of the FMRP mRNP *in vivo*; the C-terminal domain of FMRP binds a G-rich

structure in its 3'UTR (shown not be structured as a G-quartet) (Zalfa et al., 2007). By inhibiting transcription in primary neurons with actinomycin D, it has been shown that FMRP protects *PSD-95* mRNA from decay specifically in the hippocampus (Figure 7A). The stabilizing effect of FMRP can also be enhanced upon neuronal activity, such as the stimulation of metabotropic glutamate receptors (mGluRs) (Zalfa et al., 2007).

Furthermore, the region-specific effect of FMRP on *PSD-95* mRNA stability is consistent with other reports indicating that PSD-95 synaptic translation is affected in the cortex of FMRP-lacking mice (Muddashetty et al., 2007; Todd et al., 2003) (Fig. 7A). Thus, it is possible that FMRP has multiple independent functions in the regulation of posttranscriptional control of gene expression (i.e. stability, mRNA translation) depending on the cellular context and developmental timing.

The FMRP complex regulating *PSD-95* mRNA turnover has not yet been identified. The FMRP-binding site in the 3'UTR is close to three U-rich tracts; two of them contain AREs. These motifs could be crucial for *PSD-95* mRNA half-life and the function of FMRP could prevent the action/binding of other destabilizing factors (Figure 7A). Moreover, FMRP is not a general regulator of mRNA decay. The stability assay revealed that only two out of 12 known FMRP targets (*PSD-95*, *Map1b*, *α-CaMKII*, *Fxr1*, *G3bp*, *App*, *RhoA*, *Ef-1α*, *Vdac1*, *HnRNPA2*, *Sapap4* and *Mbp*) have a compromised half-life in the absence of FMRP (Zalfa et al., 2007). The observation that *Myelin basic protein* (*Mbp*) mRNA has also a decreased stability extends this function to oligodendrocytes (Zalfa et al., 2007). Considering that previous work by Kooy and collaborators (D'Hulst et al., 2006; Gantois et al., 2006) has shown that in absence of FMRP a subset of mRNAs encoding the GABA receptors is downregulated, it is tempting to hypothesize that this reduced expression could occur at the level of mRNA stability.

FMRP has also been reported to contribute to *Nxf1* mRNA stability (Zhang et al., 2007). *Nxf1* mRNA encodes the large subunit of the mRNP export receptor involved in the transport of mature transcripts from the nucleus to the cytoplasm. NXF1 is the predominant component of the nuclear export factor family, which also includes NXF2, previously identified as a direct partner of FMRP in both testis and brain. The authors initially proposed that FMRP could act as an adaptor protein recruiting a specific subclass of mRNPs to NXF2 and to facilitate the nuclear export (Lai et al., 2006). More recently, using a mouse neuroblastoma cell line (N2a cells), they showed that upon NXF2 overexpression, the turnover of the messenger increased and consequently the mRNA levels were reduced. Because this effect was abolished by silencing FMRP in the neuroblastoma cell line, the authors proposed that FMRP could contribute to the degradation induced by NXF2 (Zhang et al., 2007) (Figure 7B).

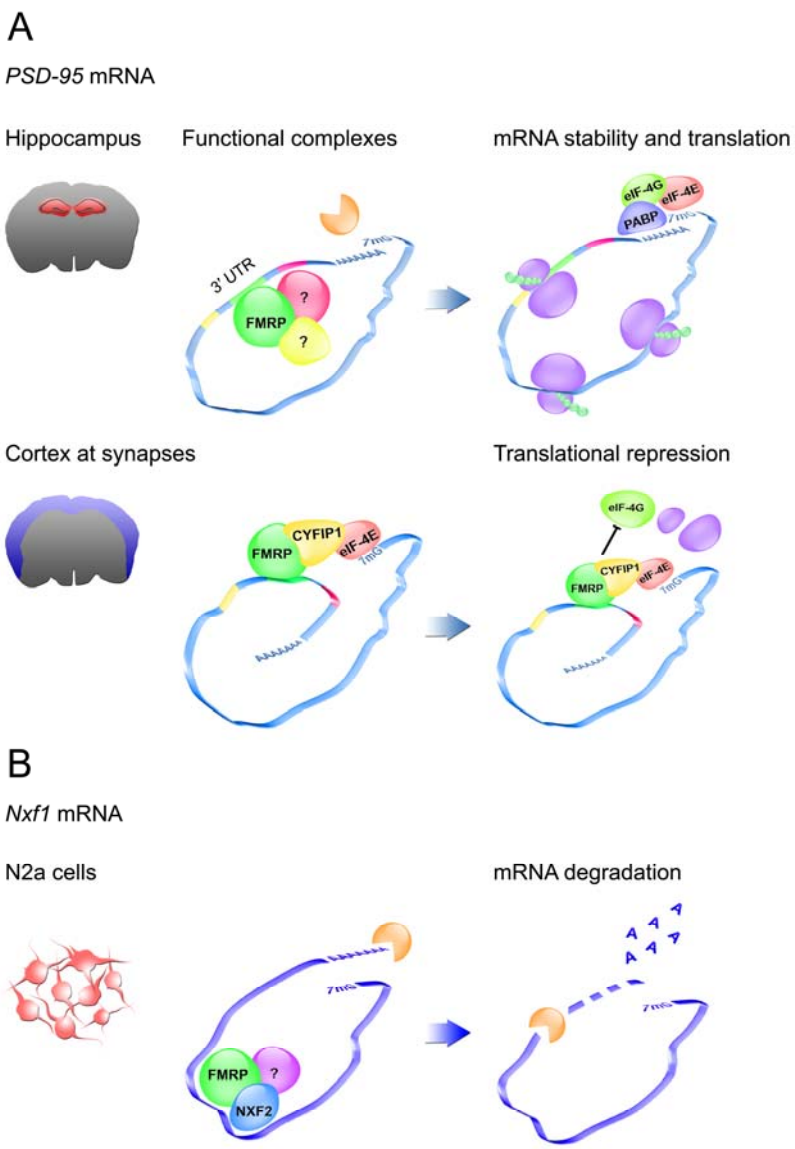


Fig. 7. FMRP in the control of mRNA stability. Panel A shows the regulatory effect of FMRP on *PSD-95* mRNA in the hippocampus (upper panel, Zalfa et al., 2007) and in the cortex (lower panel, Muddashetty et al., 2007, Napoli et al., 2008). FMRP (in green) directly interacts with the 3'UTR of *PSD-95* mRNA. The FMRP-binding site (G-rich region, in green)

is close to a U-rich stretch (in yellow) and two AU-rich tracts (in light blue and purple). In the hippocampus (upper panel), the FMRP complex, which could include other factors involved in the control of mRNA turnover (in yellow and purple), would block the entry of the exonucleases (in orange) and consequently mRNA degradation. Consequently, the stable mRNA can undergo translation. In the cortex (lower panel), the FMRP complex would tether *PSD-95* mRNA to a repression complex. One possibility is via CYFIP1. In this case, CYFIP1 would prevent eIF4E (in red) interacting with eIF4G (in light green), therefore inhibiting the initiation of translation. PSD-95 could also be possibly repressed via other mechanisms i.e. microRNAs (not shown here). Panel B depicts the role of FMRP in the regulation of *Nxf1* mRNA half-life in a neuronal cell line (Zhang et al., 2007). FMRP (in green) directly binds NXF2 (in blue) and the complex associate with the messenger. It is still unknown which component of the complex mediate the recognition of the mRNA. The FMRP-NXF2 complex and possibly other proteins (purple) would facilitate the degradation of *Nxf1* mRNA (De Rubeis and Bagni, 2009).

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PUBLICATIONS

In the following pages, I enclosed the publications related to my work on the Fragile X Syndrome. During my PhD I have also collaborated with Prof. Piacentini (University of Rome Tor Vergata) on a non-related topic and I am co-author of a paper published in Oncogene (Fazi et al, Oncogene, 2009).

- Zalfa F., Eleuteri B., Dickson K.S., Mercaldo V., **De Rubeis S.**, Di Penta A., Tabolacci E., Chiurazzi P., Neri G., Grant S.G.N. and Bagni C. (2007). A new function for the fragile X mental retardation protein in regulation of PSD-95 mRNA stability. *Nat. Neurosci.*, 10 (5), 578-587.

- Napoli I., Mercaldo V., Pilo Boyl P., Eleuteri B., Zalfa F., **De Rubeis S.**, Di Marino D., Mohr E., Massimi M., Falconi M., Witke W., Costa-Mattioli M., Sonenberg N., Achsel T. and Bagni C. (2008). The Fragile X Mental Retardation Protein represses activity-dependent mRNA translation through CYFIP1, a new 4E-BP. *Cell*, 134(6), 1042-1054.

- **De Rubeis S.** and Bagni C. "Synaptosome". Essay for the Encyclopedia of Neuroscience, 2008. Eds Binder M.D., Hirokawa N., Windhorst U., Hirsch, M.C., Springer.

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ARTICLES

nature neuroscience

A new function for the fragile X mental retardation protein in regulation of *PSD-95* mRNA stability

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Fragile X syndrome (FXS) results from the loss of the fragile X mental retardation protein (FMRP), an RNA-binding protein that regulates a variety of cytoplasmic mRNAs. FMRP regulates mRNA translation and may be important in mRNA localization to dendrites. We report a third cytoplasmic regulatory function for FMRP: control of mRNA stability. In mice, we found that FMRP binds, *in vivo*, the mRNA encoding PSD-95, a key molecule that regulates neuronal synaptic signaling and learning. This interaction occurs through the 3′ untranslated region of the *PSD-95* (also known as *Dlg4*) mRNA, increasing message stability. Moreover, stabilization is further increased by mGluR activation. Although we also found that the *PSD-95* mRNA is synaptically localized *in vivo*, localization occurs independently of FMRP. Through our functional analysis of this FMRP target we provide evidence that dysregulation of mRNA stability may contribute to the cognitive impairments in individuals with FXS.

FXS is caused by a trinucleotide expansion in the X-linked fragile X mental retardation gene (*FMR1*) that leads to the subsequent loss of FMRP, and it is the most common cause of X-linked mental retardation. FMRP has multiple RNA-binding motifs and is thought to be involved in mRNA localization and translational regulation in neurons, two processes required for synaptic plasticity (reviewed in ref. 1). Because the only obvious abnormality in the brains of individuals with FXS is the presence of longer, immature-appearing spines[1], current models have focused on the possible dysregulation of synaptic mRNAs as an underlying cause of FXS mental deficits.

A wide variety of mRNAs have been identified as potential targets of mammalian FMRP, both *in vitro* and *in vivo*[2,3]. FMRP binds various mRNA elements[1], including a G-rich RNA structure (G-quartet)[4–6] and U-rich stretches[7]. FMRP is also indirectly recruited to some target mRNAs via binding to the noncoding RNAs *BC1* and *BC200* (refs. 8–11). Finally, both mammalian and *Drosophila* FMRP are present in microRNA (miRNA) complexes[12] and may be recruited to mRNAs that are bound to miRNAs.

In the FMRP protein, the RGG box recognizes G-quartet sequences present in some FMRP targets[4], whereas the N-terminus recognizes a bulge in *BC* RNAs[10]. Notably, although FMRP contains two KH domains, a known RNA-binding motif, no endogenous neuronal targets that are recognized by this domain have been identified[13]. Functionally, FMRP acts as a translational repressor of a subset of neuronal mRNAs[3], and it may be involved in synaptic mRNA localization, as FMRP is present in mRNP localization complexes[14]. A limited number of studies also suggest that FMRP may regulate transcription[15–17].

Despite much research, it remains unclear precisely how the loss of FMRP leads to alterations in the neuronal mechanisms responsible for cognition. One proposal suggests that alterations in metabotropic glutamate receptor (mGluR)-mediated signaling might underlie a number of the cognitive deficits associated with FXS[18]. Disruption of NMDA receptors[19] or associated signaling components[20–22] can also lead to impairments in synaptic plasticity. Notably, mGluRs and NMDA receptors coexist in a large-scale signaling complex[23]. PSD-95 (DLG4), a component of the MAGUK family of adaptor proteins that includes SAP102 (DLG3) and PSD-93, binds directly to the NMDA receptor and links other adaptors to mGluRs[24]. Mice lacking PSD-95 have impairments in learning[20] and cortical plasticity[21]. Similarly, SAP102 mutant mice show learning impairments[25], and mutations in human *SAP102* are implicated in mental retardation[26]. Notably, PSD-95 mutant mice also show dendritic spine abnormalities in the striatum and hippocampus[27], one of the key alterations seen in humans with FXS and in FMRP mutant mice[1]. A quantitative neuroimaging study also found larger right and left hippocampal volumes in individuals with FXS compared with controls, suggesting that this region may be involved in the behavioral and cognitive abnormalities associated with FXS[28].

One report has indicated that FMRP regulates PSD-95 protein levels in response to mGluR signaling[29]. However, putative FMRP-binding sites were identified by sequence analysis, and direct interactions

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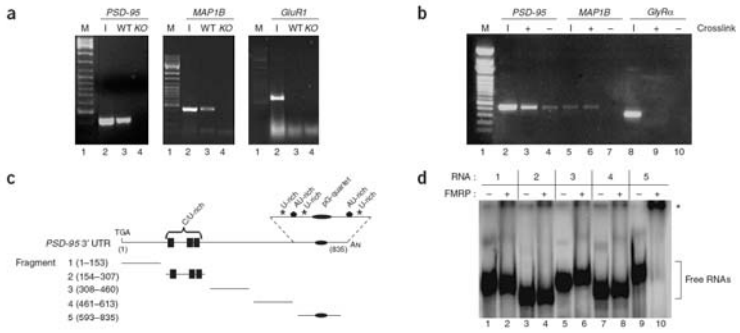

Figure 1 FMRP interacts directly with the 3′ UTR of *PSD-95* mRNA. (**a**) Brain lysates from wild-type (WT) and *FMR1* knockout mice (KO) were immunoprecipitated with FMRP antibodies. RT-PCR was performed using oligonucleotides specific for the *PSD-95*, *MAP1B* and *GluR1* mRNAs. Input (1/5) is reported in lane 2. Lanes that were not relevant to this experiment were removed between the marker and lanes 1 and 2. (**b**) CLIP assay. Hippocampal cell extracts were immunoprecipitated with FMRP antibodies. RT-PCR was performed using oligonucleotides for the *PSD-95*, *MAP1B* and *GlyRα* mRNAs. Input (1/5) is reported in lanes 2, 5 and 8. (**c**) *PSD-95* 3′ UTR fragments used in EMSA experiments. Potential functional motifs are indicated. (**d**) ^{32}P-radiolabeled fragments (1–5) of the *PSD-95* 3′ UTR were incubated in the presence of FMRP (+, lanes 2, 4, 6, 8 and 10). Control reactions were performed in buffer alone (−, lanes 1, 3, 5, 7 and 9). RNA-protein complexes were resolved on native polyacrylamide gel. Unbound RNA fragments (|) and RNA-protein complexes (*) are indicated.

between the *PSD-95* mRNA and FMRP were not tested. Although the authors concluded that these effects were due to translational regulation of *PSD-95* mRNA, the above mentioned results could not formally distinguish between effects on mRNA export, stability or translation.

In this study we provide a detailed assessment of the role that FMRP has in controlling PSD-95 expression. We have found that FMRP interacts directly with the 3′ UTR of the *PSD-95* mRNA, providing evidence that FMRP is important in increasing the stability of the *PSD-95* message. This stabilization is further increased by mGluR activation. These findings suggest that, in addition to dysregulation of translation targets, some of the FXS impairments may arise as a result of alterations in the stability of FMRP target mRNAs.

RESULTS

PSD-95 mRNA interacts directly with FMRP

To address whether FMRP directly regulates *PSD-95* mRNA, we examined whether *PSD-95* mRNA was present in the FMRP complex in mice. We found *PSD-95* mRNA in FMRP immunoprecipitates from wild-type mice but not from *FMR1* knockout mice (**Fig. 1a**). A known FMRP-interacting mRNA, *MAP1B* (refs. 8,30,31), was also coprecipitated (**Fig. 1a**), whereas a negative control mRNA (*GluR1*, also known as *Gria1*) was not (**Fig. 1a**). Using reversible cross-linking–immunoprecipitation (CLIP)[32] from primary hippocampal neurons (**Fig. 1b**), we showed that FMRP bound directly to the *PSD-95* mRNA, as cross-linking occurred only if FMRP and *PSD-95* were in close proximity *in vivo*. *MAP1B* mRNA, but not *GlyRα* (also called *Glra1*) mRNA, was also cross-linked to FMRP (**Fig. 1b**).These data indicate that *PSD-95* mRNA is part of the FMRP mRNP complex *in vivo*.

To map the FMRP–*PSD-95* mRNA interaction, we carried out direct protein-RNA binding assays. We focused on the 3′ untranslated region (UTR) of *PSD-95* mRNA because *in silico* analysis of this region had shown the presence of a putative G-quartet[29] and three U-rich stretches[33] (**Fig. 1c** and **Supplementary Fig. 1** online), sequence elements previously shown to recruit FMRP to RNAs[4,7]. Of the five short RNAs that spanned the entire 3′ UTR of the mouse *PSD-95* mRNA (**Fig. 1c**), only fragment 5 had FMRP-binding ability in electrophoretic mobility shift assays (EMSAs) with purified baculovirus-expressed human FMRP protein (**Fig. 1d**). This RNA fragment was also bound by mouse brain extracts (data not shown). The lack of FMRP binding to fragments 1–4 (**Fig. 1d**) and the antisense strand (data not shown), and the ability of excess unlabeled fragment 5 RNA to compete, indicated that the FMRP-RNA interaction was specific and did not simply reflect general RNA affinity. The protein-binding ability of fragment 5 RNA was also specific, as it did not bind other RNA binding proteins (the microbial transcription and translation modulator NusG or the spliceosomal 15.5-kDa (hSnu13p) protein; data not shown).

We also investigated which protein domain of FMRP (N-terminus, KH1, KH2 or C-terminus)[34] was involved in binding to the *PSD-95* mRNA (**Fig. 2a**). We found that only the C-terminus contained *PSD-95* mRNA–binding ability (**Fig. 2b**). This domain bound with high affinity, as binding remained present under high-stringency conditions (50 mM LiCl) (**Fig. 2c**). The binding was specific, as the C-terminus did not interact with fragment 1, and binding to fragment 5 was competed with by excess unlabeled fragment 5, but not fragment 1 (**Fig. 2c**).

We further mapped the mRNA region in fragment 5 that was responsible for FMRP binding by scrambling the G-rich region to eliminate all similarity to the G-quartet consensus and converting the U-rich regions into mixed sequences (**Fig. 3a**). High lithium (50 mM), a condition that destabilizes G-quartet structures[4,35], did not interfere with FMRP binding to either the wild-type (**Fig. 3b**) or the mutagenized fragment 5 (**Fig. 3b**). Notably, mutagenesis of all three U-rich regions did not prevent FMRP binding (**Fig. 3b**).

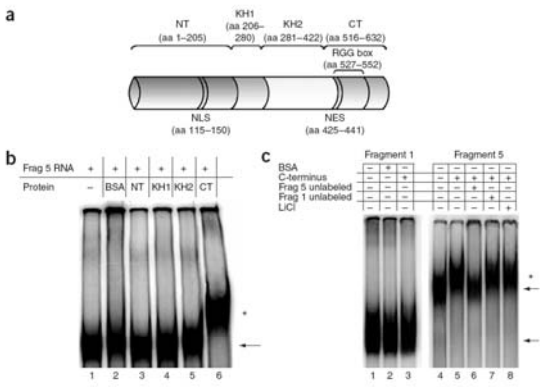

Figure 2 The C-terminal domain of FMRP is able to specifically interact with the *PSD-95* mRNA 3′ UTR. (**a**) Schematic representation of FMRP and its principal domains: the N terminus (NT), KH1, KH2 and the C terminus (CT) containing the RGG box. The nuclear localization sequence (NLS) and the nuclear export sequence (NES) are also indicated. (**b**) ^{32}P-radiolabeled fragment 5 of the *PSD-95* 3′ UTR was incubated alone (lane 1), in the presence of BSA (lane 2) or in the presence of FMRP domains: N terminus (lane 3), KH1 (lane 4), KH2 (lane 5) and C terminus (lane 6). (**c**) ^{32}P-radiolabeled fragments 1 and 5 of the *PSD-95* 3′ UTR were incubated alone (lane 1 and 4) or in the presence of FMRP C terminus (lane 3 and 5). Fragment 1 was also incubated in the presence of BSA (lane 2). To assess the specificity of interaction between the fragment 5 and the C-terminus, we carried out the RNA binding assay in the presence of competitor RNAs (unlabeled fragment 5, lane 6; fragment 1, lane 7) or in the presence of the chaotropic salt LiCl (lane 8). RNA-protein complexes were resolved on a native polyacrylamide gel. Unbound RNA fragments (←) and RNA-protein complexes (*) are indicated.

Because previous studies suggested that FMRP has a high affinity for poly(rG) *in vitro*[34], we further examined the G-rich region. Although the entire G-rich region showed binding to FMRP (**Figs. 3c,d**; I + II G-rich), even in the presence of high lithium salt (**Fig. 3d**), no binding was detected when we used two short RNA fragments (**Figs. 3c,d**) of that region (**Fig. 3c**). Our findings argue that FMRP recognizes a structured G-rich sequence in the 3′ UTR of the *PSD-95* mRNA or a region spanning the two fragments, and that this structure does not form a G-quartet.

Similar *PSD-95* polysomal profile in wild type and knockout

FMRP can act as a translational repressor[3,31] and local translation of synaptic mRNAs has been increasingly implicated in neuronal plasticity, learning and memory formation (reviewed in ref. 36). Notably, a number of localized mRNAs encode synaptic proteins (for example, Arc, MAP1B, αCaMKII and SAPAP4) that are translationally repressed by FMRP[8,16]. Thus far, our experiments indicate that FMRP can directly interact with the *PSD-95* mRNA, but do not address the functional role of this interaction.

We assessed whether *PSD-95* mRNA translation was regulated by FMRP, as was previously proposed[29], by carrying out sucrose gradient fractionation of cytoplasmic (**Fig. 4a**) and hippocampal (**Fig. 4b**) brain extracts from wild-type and *FMR1* knockout mice. Unexpectedly, the percentage of *PSD-95* mRNA associated with polysomes did not change in the *FMR1* knockout animals compared with wild-type animals in either whole brain or hippocampal extracts. Although the profile of the negative control, *β-actin* (*ACTB*) mRNA, also remained unchanged, *Arc* mRNA, which is known to be translationally regulated by FMRP[8], showed the expected shift toward a more translationally active polysome pool in *FMR1* knockout extracts. We cannot formally rule out the possibility that FMRP changes the translation efficiency of the *PSD-95* mRNA without changing the percentage messenger on polysomes (PMP) ratio (for example, by altering miRNA-regulated translation; reviewed in ref. 37). However, because other FMRP-regulated mRNAs (such as *Arc*) do change their PMP ratio, the above findings indicate that FMRP does not regulate PSD-95 protein synthesis in a manner similar to those of other well-studied FMRP targets.

PSD-95 mRNA is dendritically localized with FMRP *in vivo*

It has been estimated that hundreds of mRNAs are present in dendrites, but whether the entire population or only a subset are localized near synapses is currently unknown[38]. Because this list includes mRNAs that are known targets of FMRP (for example, *Arc*, *αCaMKII*), and because PSD-95 is an integral component of the postsynaptic density, we assessed whether the *PSD-95* message was localized in dendrites and, if so, whether this localization was dependent on FMRP.

By analyzing the presence of *PSD-95* mRNA in synaptoneurosomes from total brain, we found that *PSD-95* mRNA showed a marked dendrite/soma enrichment ratio (**Supplementary Fig. 2** online), suggesting that the mRNA was localized at synapses. This was further confirmed by *in situ* hybridization in neuronal cultures (**Fig. 5**). We found that *PSD-95* mRNA localized in both cell bodies and along dendrites of hippocampal (**Fig. 5a**) and cortical (data not shown) neurons with a typical punctate pattern. Similarly, a recent large-scale screen also suggested putative targeting of the *PSD-95* mRNA to both proximal and distal dendrites[39]. Unexpectedly, although *PSD-95* mRNA largely colocalized with FMRP throughout the cell and into neurites (**Fig. 5a**), the *PSD-95* mRNA was still localized in dendrites from *FMR1* knockout hippocampal (**Fig. 5a**) and cortical (data not shown) cultures. Control experiments indicated that we could specifically detect dendritic (*αCaMKII*) and cell body (*α-tubulin*) mRNAs[38] (**Fig. 5b**), and that the sense probes did not show any specific mRNA staining (**Supplementary Fig. 3** online). These data further confirm that the *PSD-95* message is part of an FMRP mRNP complex, but suggest that FMRP function is not necessary to localize the *PSD-95* message.

We confirmed that *PSD-95* mRNA was dendritically localized using both digoxigenin (DIG) RNA labeling (data not shown) and radioactive *in situ* hybridization (**Fig. 6**) on brain slices. *PSD-95* mRNA was present in the hippocampus, cortex (**Fig. 6a**) and cerebellum (**Fig. 6b**). The unlocalized control mRNA (*α-tubulin*) stained only cell bodies in the hippocampus and dentate gyrus (**Fig. 6c** and **Supplementary Fig. 4** online). Although *PSD-95* mRNA localization was distinct from another localized mRNA (*αCaMKII*; **Fig. 6d** and **Supplementary Fig. 4**), *PSD-95* mRNA was clearly present in hippocampal dendrites of both wild-type and *FMR1* knockout mice in a region corresponding

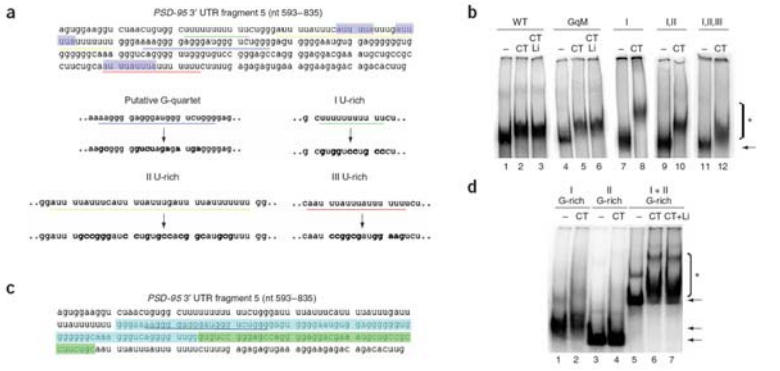

Figure 3 A G-rich region in the *PSD-95* 3′ UTR is responsible for FMRP C-terminus binding. (**a**) Sequence of fragment 5 (nt 593–835) and mutagenesis of the putative G-quartet and U-rich regions. The first U-rich region is underlined in green, the second U-rich region in yellow, the third U-rich in red and the putative G-quartet in blue. The blue highlighted regions represent two AREs. Bold characters represent introduced substitutions. (**b**) Wild-type fragment 5 (WT) and the putative G-quartet mutated fragment 5 (GqM) were incubated alone (lanes 1 and 4), with FMRP C terminus (lanes 2 and 5) or in the presence of LiCl 50 mM (lanes 3 and 6). The first U-rich mutant (I), the first and second U-rich double mutant (I and II) and the triple U-rich mutant (I, II and III) were incubated alone (lanes 7, 9 and 11) or with the C-terminus (lanes 8, 10, 12). Unbound RNA fragments (←) and RNA-protein complexes (*) are indicated. (**c**) The first G-rich region is highlighted in blue and the second in green. (**d**) The first G-rich region (nt 666–741) of fragment 5, the second G-rich region (nt 742–786) or the entire G-rich region (nt 666–786) were incubated alone (lanes 1, 3 and 5) or with the C-terminus of FMRP (lanes 2, 4 and 6). The C-terminus and the entire G-rich were incubated in the presence of 50 mM of LiCl (lane 7).

to the stratum lacunosum-moleculare (**Fig. 6a** and **Supplementary Fig. 4**). A control *PSD-95* mRNA sense probe did not show any specific mRNA staining (**Supplementary Fig. 3**). Notably, quantification of *PSD-95* mRNA levels showed a clear, though nonsignificant, reduction in hippocampal mRNA in the *FMR1* knockout animals relative to cortical mRNA levels (**Fig. 6a**, $P > 0.05$). This tendency was not observed when comparing *PSD-95* cerebellar with cortical mRNA (**Fig. 6b**) or *α-tubulin* hippocampal with cortical mRNA (**Fig. 6c**) ratios between wild-type and *FMR1* knockout mice.

Together, these data provide evidence that the *PSD-95* mRNA is localized in dendrites *in vitro* and *in vivo*. As there is less *PSD-95* mRNA in the stratum lacunosum-moleculare in *FMR1* knockout mice (**Fig. 6a**), we cannot exclude the possibility that FMRP might be involved in a subtle modulation of *PSD-95* mRNA localization.

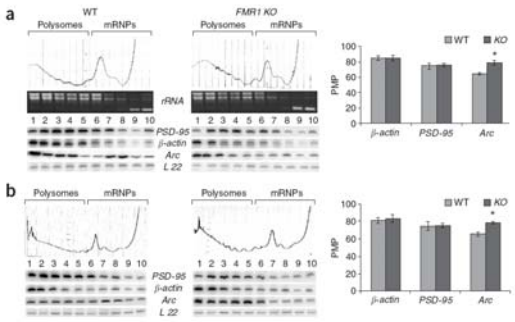

Figure 4 The *PSD-95* mRNA polysomal profile is similar in wild-type and *FMR1* knockout mice. (**a**) Cytoplasmic brain extract was fractioned by centrifugation on a 5–70% sucrose gradient. Ten fractions were collected while 254-nm absorbance was recorded. RNA was extracted from each fraction and visualized on a denaturing agarose gel. *rRNA* 28S, 18S and 5S/tRNA are shown in each fraction. Radioactive RT-PCR analyses of total RNA in each fraction was carried out with primers specific for *PSD-95*, *β-actin*, *Arc* and *L22* RNAs. The efficiency of translation is reported as a graphic profile of PMP, which was calculated, after normalization to *L22*, by comparing the radioactivity of the first five fractions containing active polysomes with the radioactivity from all ten fractions. The *PSD-95*, *β-Actin* and *Arc* PMP in each fraction of wild-type or *FMR1* knockout gradients was normalized for *L22* RNA. (**b**) Same as in **a**, using cytoplasmic extracts from the hippocampus. PMP value of three independent experiments with standard error is reported. *, $P < 0.05$ for knockout versus wild type by Student's *t*-test.

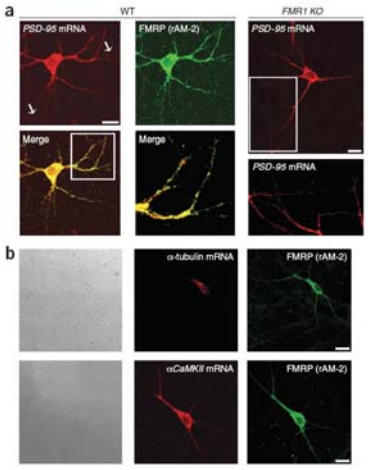

Figure 5 *PSD-95* mRNA is dendritically localized in neuronal cell cultures. (**a**) Left, *in situ* hybridization carried out using an antisense riboprobe specific for *PSD-95* mRNA (red), combined with an immunofluorescence for FMRP (green) on wild-type (WT) hippocampal cultures (10 DIV). A merged image and a merged magnification are also shown (yellow). Right, *in situ* hybridization for *PSD-95* in *FMR1* knockout hippocampal cultures (top) and magnification (bottom). (**b**) Upper, bright-field image (left) and *in situ* hybridization carried out using an antisense riboprobe specific for the cell body–specific α-*tubulin* mRNA (red, middle), combined with an immunofluorescence for FMRP (green, right) on hippocampal cultures (10 DIV). Lower, bright-field image (left) and *in situ* hybridization carried out using an antisense riboprobe specific for the dendritically localized α*CaMKII* mRNA (red, middle), combined with an immunofluorescence for FMRP (green, right) on hippocampal cultures (10 DIV).

However, as *PSD-95* mRNA is clearly present in dendrites in the absence of FMRP (**Figs. 5a** and **6a**), our data suggest that the FMRP does not have a primary role in *PSD-95* mRNA localization.

Impaired *PSD-95* mRNA and protein levels in *FMR1* knockout

Our results suggest that FMRP does not directly regulate translation (**Fig. 4**) or transport (**Figs. 5** and **6**) of *PSD-95* mRNA. Earlier reports, however, have suggested that FMRP might also control mRNA abundance via transcriptional regulation[15–17]. Notably, our radioactive *in situ* hybridization data indicated a possible decrease in *PSD-95* mRNA intensity in hippocampal neurons from *FMR1* knockout mice (**Fig. 6a**), suggesting that mRNA abundance may be regulated by FMRP.

To determine whether FMRP controls mRNA abundance, we first examined the total *PSD-95* mRNA level in wild-type and *FMR1* knockout mice. In total brain, *PSD-95* mRNA levels were significantly decreased in *FMR1* knockouts compared with wild-type mice (**Fig. 7a**). Notably, quantitative RT-PCR analyses carried out on the three principal brain areas (hippocampus, cerebellum and cortex) showed that the decrease in *PSD-95* mRNA was very pronounced in the hippocampus, less so in the cerebellum and not observed in the cortex (**Fig. 7b**). Quantitative RT-PCR analyses of the *PSD-95* mRNA from hippocampal neurons of wild-type and *FMR1* knockout mice confirmed this hippocampal-specific decrease in *PSD-95* mRNA (**Fig. 7c**). Although there was a subtle trend toward lower abundance of *PSD-95* mRNA in the hippocampus as detected with radioactive *in situ* hybridization (**Fig. 6**), this was not statistically significant, and we believe that these differences may be due to different sensitivities of the techniques. Differential *PSD-95* expression was also reflected at

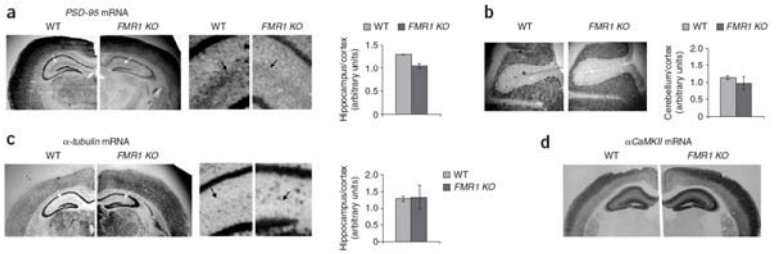

Figure 6 *PSD-95* mRNA is dendritically localized *in vivo*. Radioactive *in situ* hybridization on transversal brain sections from wild-type (WT; left image in each case) and *FMR1* knockout mice (KO; right image in each case) for (**a**,**b**) *PSD-95*, (**c**) α-*tubulin* and (**d**) α*CaMKII* mRNAs for *PSD-95* mRNA in both wild-type and knockout mice. Arrows point to the stratum lacunosum-moleculare. Right, an enlargement of the CA2 areas marked by the white arrows in the smaller image is shown. Black arrows point to the hippocampal region enriched in *PSD-95* mRNA. Quantification of *PSD-95* mRNA level in hippocampus relative to cortex is shown (average value from three sections are reported, with s.e.). (**a**,**b**) Cerebellar sections are shown. Quantification of *PSD-95* mRNA level in cerebellum relative to cortex is shown (average values from three sections are reported, with s.e.). (**c**) *In situ* hybridization on brain sections from wild-type and *FMR1* knockout mice for α-*tubulin* mRNA. A blown-up of the areas marked by the white arrows is shown. Black arrows indicate the lack of any detectable signal in this area. Quantification of α-*tubulin* mRNA level in hippocampus relative to cortex is shown (average value from three sections are reported, with s.e.). (**d**) *In situ* hybridization on brain sections from wild-type and *FMR1* knockout mice for α*CaMKII* mRNA.

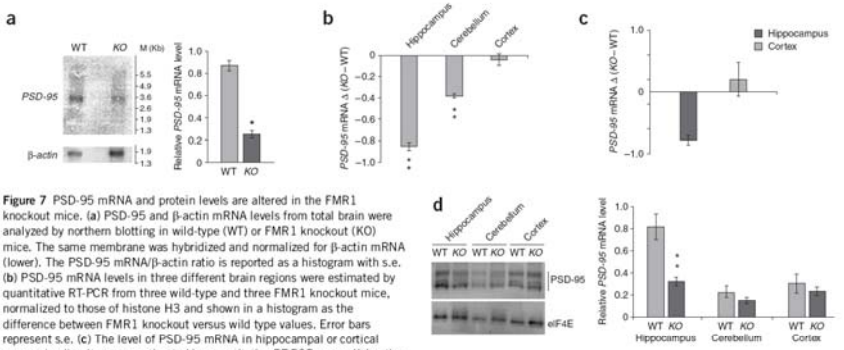

Figure 7 PSD-95 mRNA and protein levels are altered in the FMR1 knockout mice. (**a**) PSD-95 and β-actin mRNA levels from total brain were analyzed by northern blotting in wild-type (WT) or FMR1 knockout (KO) mice. The same membrane was hybridized and normalized for β-actin mRNA (lower). The PSD-95 mRNA/β-actin ratio is reported as a histogram with s.e. (**b**) PSD-95 mRNA levels in three different brain regions were estimated by quantitative RT-PCR from three wild-type and three FMR1 knockout mice, normalized to those of histone H3 and shown in a histogram as the difference between FMR1 knockout versus wild type values. Error bars represent s.e. (**c**) The level of PSD-95 mRNA in hippocampal or cortical neuronal cell culture was estimated by quantitative RT-PCR, normalizing the values to histone mRNA. The histogram represents the difference between the values for FMR1 knockout and wild type, and the bars represent the s.e. of three independent measurements. (**d**) Protein extracts from cortex, hippocampus or cerebellum of four wild-type and four FMR1 knockout mice were analyzed for PSD-95 and eIF4E proteins. Left, western blot from one of the four independent mice analyzed for PSD-95 and eIF4E. Right, histogram showing results with s.e. *, $P < 0.05$ and **, $P < 0.01$ for knockout versus wild type by Student's *t*-test in all panels.

the protein level, with a statistically significant decrease occurring in the hippocampus and a nonsignificant decrease in the cerebellum (**Fig. 7d**, $P > 0.05$).

These data suggest that either transcription or stability of the *PSD-95* mRNA is regulated by FMRP in the hippocampus. Critically, the hippocampus is important for the learning processes that are altered in people with FXS[40], and the loss of PSD-95 results in hippocampal-dependent learning defects[20].

Activity-dependent FMRP control of *PSD-95* mRNA stability

To directly assess whether this change in mRNA level was a result of altered transcription or mRNA stability, we examined the half-life of the *PSD-95* message in cortical and hippocampal primary cultured neurons. Notably, after transcriptional blockade with actinomycin D, *PSD-95* mRNA abundance was significantly and selectively reduced in hippocampal cultures in the absence of FMRP (**Fig. 8a** and **Supplementary Fig. 5** online). The stability of *PSD-95* mRNA was unaffected in *FMR1* knockout cortical cultures (**Supplementary Fig. 6** online), in agreement with prior results (**Fig. 6a** and **7b,c**). These results were not due to nonspecific cell death effects, as the morphology of hippocampal cells from wild-type and *FMR1* knockout mice were the same (**Supplementary Fig. 7** online) and cell survival was the same in both genotypes (**Fig. 8b**), although we did note that after 12 h both the wild-type and *FMR1* knockout neurons showed some increase in the

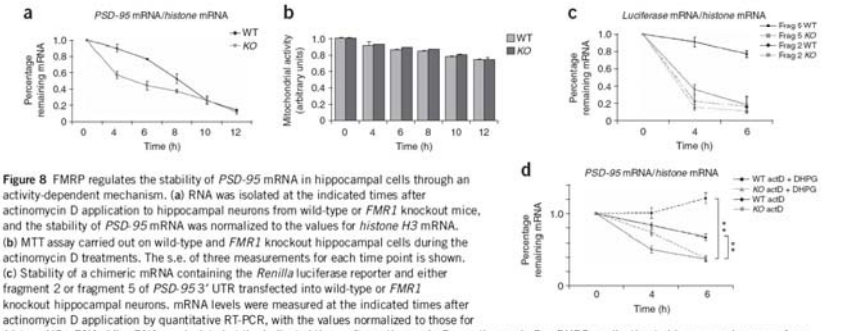

Figure 8 FMRP regulates the stability of *PSD-95* mRNA in hippocampal cells through an activity-dependent mechanism. (**a**) RNA was isolated at the indicated times after actinomycin D application to hippocampal neurons from wild-type or *FMR1* knockout mice, and the stability of *PSD-95* mRNA was normalized to the values for *histone H3* mRNA. (**b**) MTT assay carried out on wild-type and *FMR1* knockout hippocampal cells during the actinomycin D treatments. The s.e. of three measurements for each time point is shown. (**c**) Stability of a chimeric mRNA containing the *Renilla* luciferase reporter and either fragment 2 or fragment 5 of *PSD-95* 3′ UTR transfected into wild-type or *FMR1* knockout hippocampal neurons. mRNA levels were measured at the indicated times after actinomycin D application by quantitative RT-PCR, with the values normalized to those for *histone H3* mRNA. (**d**) mRNA was isolated at the indicated times after actinomycin D or actinomycin D + DHPG application to hippocampal neurons from wild-type or *FMR1* knockout mice. The stability of *PSD-95* mRNA in wild-type or *FMR1* knockout hippocampal cells was measured by quantitative RT-PCR. **, $P < 0.01$ for knockout versus wild type by Student's *t*-test.

amount of cell death (≈25%). Together, these results suggest that FMRP functions to stabilize the *PSD-95* mRNA specifically in the hippocampus. Furthermore, the stability of a reporter (*Renilla reniformis* luciferase) RNA carrying the FMRP-interacting portion of the *PSD-95* 3′ UTR (fragment 5) was more stable when transfected into wild-type versus *FMR1* knockout hippocampal neurons (**Fig. 8c**), whereas a reporter RNA containing another *PSD-95* 3′ UTR that does not bind FMRP (fragment 2) was equally unstable in both cultures (**Fig. 8c**). These data strongly suggest that a direct interaction between FMRP and the *PSD-95* 3′ UTR is necessary to confer mRNA stabilization.

Because FMRP has not been previously shown to regulate mRNA stability, we also assessed the stability of 11 other FMRP targets and 2 synaptic scaffolding proteins whose mRNAs are localized in dendrites (*Homer1a* and *Shank1*). Of these mRNAs (**Supplementary Table 1** online), only myelin basic protein mRNA (*MBP*) changed its stability. *MBP* mRNA is a target of FMRP regulation[41], and is present only in glia cells, which also express FMRP[8,41]. We detected the *MBP* mRNA because our primary neurons were cocultured with glial cells. Notably, although this list is clearly not exhaustive, our analyses suggest that FMRP-mediated mRNA stabilization is a highly selective mechanism with respect to both cell type and target mRNA, and that it works in both neurons and glia.

Because FMRP is regulated by mGluR activation (for example, see refs. 29,42,43), we also investigated whether mGluR stimulation would alter FMRP-dependent *PSD-95* mRNA stabilization. Using two independent protocols (see Methods for details), we found that the presence of (S)-3,5-dihydroxyphenylglycine (DHPG) further stabilized *PSD-95* mRNA in wild-type cells at both time points measured (**Fig. 8d**). In *FMR1* knockout cells, the addition of DHPG provided only transient stabilization that did not persist at the later time point, suggesting that DHPG might also have a transient, FMRP-independent effect on mRNA half-life. Quantification of three independent experiments indicated that there was a significant DHPG-dependent stabilization effect only in the wild-type neurons and that this effect was mostly lost in *FMR1* knockout hippocampal cells (**Fig. 8d**). Together, the data suggest that there is a long-lasting FMRP-dependent stabilization effect via mGluR-specific neuronal activity.

DISCUSSION

In this paper we have shown that FMRP interacts directly with the 3′ UTR of *PSD-95* mRNA. However, we found that *PSD-95* mRNA polysomal association remained the same in wild-type and *FMR1* knockout mice and that the *PSD-95* mRNA was still dendritically localized in *FMR1* knockout neurons. Although translation of the *PSD-95* mRNA may decrease as a result of postinitiation mechanisms (that is, as in the case of some miRNAs; reviewed in ref. 37) that we cannot detect with the current assay, this translation mechanism would be different from that previously documented for other FMRP targets (such as *Arc*).

Notably, we found that the FMRP–*PSD-95* mRNA interaction resulted in a stabilization of the *PSD-95* message that can be further increased via mGluR stimulation. In *FMR1* knockout mice the *PSD-95* message was less stable, resulting in a reduction of this critical synaptic protein. These observations are consistent with previous circumstantial evidence suggesting that FMRP could potentially control mRNA abundance. A microarray study identified 113 FMRP-associated mRNAs whose abundances are reduced in fragile X cell lines, yet whose polysome profile remained unchanged[16]. Another study found decreases in the levels of some FMRP-target mRNAs in the absence of FMRP[17]. Although neither group examined these mRNAs further, it is possible that reduced levels of these mRNAs actually reflect a loss of mRNA stability in the absence of FMRP. Our finding that at least one other mRNA (*MBP*) was destabilized in the absence of FMRP lends support to this idea.

Unexpectedly, we found that the stabilization of the *PSD-95* message was dependent on the area of the brain examined. The effect was most prominent in the hippocampus, present to a minor extent in cerebellum and nonexistent in the cortex. This lack of a cortical effect is consistent with previous findings that PSD-95 protein levels are the same in wild-type and *FMR1* knockout cortical cells[29]. That study also observed an FMRP-dependent increase in PSD-95 protein in cortical cells shortly after DHPG treatment, but found that protein levels fell back to baseline by 4 h[29], suggesting that there was a transient surge in PSD-95 expression. In hippocampal neurons, we observed that the relative level of the *PSD-95* mRNA rose slightly after 4–6 h of DHPG exposure, suggesting that there is an additional activity-dependent increase in RNA stability. Together, these data suggest that FMRP can regulate, according to the physiological state (DHPG-treated or not) and cell type (cortical or hippocampal), both a rapid rise in PSD-95 translation (cortex) and a more prolonged rise in *PSD-95* mRNA levels as a result of an increase in stability (hippocampus), and suggest that FMRP could have multiple independent roles.

We have mapped the binding site of FMRP to a G-rich element that is flanked by two AU-rich elements (AREs), well-known *cis*-acting mRNA elements that regulate mRNA half-life. Several *trans*-acting factors that aid in both stabilization and destabilization of target mRNAs are known to bind to AREs[44]. Notably, regulation of HuD, a member of the Hu class of ARE-binding proteins[44], during neuronal development results in temporal regulation of *GAP-43* (ref. 45). Similarly, regulation of mRNA stability is often the result of competition between stabilizing and destabilizing factors[44]. It is therefore plausible that the region-specific regulation of the *PSD-95* message is a result of interference between the stabilizing role of FMRP and the stabilizing and destabilizing functions of other binding factors. In support of this notion, we found that the hippocampus and cortex contained different forms of Hu-family proteins (**Supplementary Fig. 8** online). Combinatorial models are an emerging theme explaining RNA-protein binding specificity (reviewed in ref. 46,47), and in our case may explain why FMRP does not stabilize all of its known binding targets (**Supplementary Table 1**).

We also found that the *PSD-95* mRNA was localized in dendrites *in vivo*, but that its localization was not dependent on FMRP, further highlighting the complexity surrounding FMRP's many roles in the cytoplasm. Several factors are known to bind to FMRP and are presumed to aid it in these cytoplasmic regulatory functions. To date, however, only one of these interactions has been shown to aid FMRP function. Cooperative binding between FMRP and the *BC1* RNA leads to the translational repression of a subset of mRNAs, and *BC1* functions as a repressor of translational initiation in rabbit reticulocyte assays[3]. We expect further binding partners to collaborate with FMRP to aid translational repression, mRNP localization and this newly identified mRNA stabilization function.

Although a large number of putative FMRP target mRNAs have been isolated in the past 5 years, relatively few are known to be involved in regulating synapse structure and function. Our results strengthen the idea that FMRP function is extremely important for the correct formation of the postsynaptic compartment. These results also support the notion that the underlying cause of FXS, and potentially other forms of mental retardation, may involve direct interference with synaptic signaling that leads to spine dysmorphogenesis and ultimately to memory defects[1]. Notably, the mRNA encoding a PSD-95 associated

protein, SAPAP4, has also been shown to be in a complex with FMRP[16]. In addition, PSD-95, SAPAP4, Arc and αCaMKII are all components of the large-scale NMDA receptor signaling complex that links NMDA receptors to the mGluR signaling pathway[23], and disruption of *PSD-95* (ref. 20), *Arc*[48] and *αCaMKII* (ref. 22) all result in learning impairments. This is of interest in light of the evidence suggesting that alterations in glutamate receptor signaling via mGluRs might underlie a number of the cognitive deficits associated with FXS[23]. Furthermore, various other cases of neurological deficits also result in a decrease in PSD-95 expression (for example, refs. 49,50), suggesting that strict regulation of PSD-95 expression is required for proper brain function. PSD-95 is important in both behavioral memory and dendritic spine morphology[27], both features of FXS. Together, these results suggest that FMRP may regulate NMDA and mGluR receptor signaling through several proteins, including PSD-95, and that the cognitive and anatomical defects in FXS may arise from the disruption of this complex.

METHODS

Animal treatment. Animal care conformed to institutional guidelines in compliance with national and international laws and policies (European Community Council Directive 86/609, Oja L 358,1, December 12, 1987; NIH Guide for the Care and Use of Laboratory Animals). All animals were 3-week-old males (C57/BL6 wild type and two *FMR1* knockout strains on C57/BL6 and FVB background).

Western blots. We used standard methodologies with an FMRP monoclonal antibody (MAB2160) from Chemicon and a polyclonal antibody (rAM2) produced in our laboratory[8]. The PSD-95 antibody was from Upstate (1:1,000) and the eIF4E antibody from Cell Signaling (1:10,000). All secondary antibodies were from Promega. The proteins were revealed using ECL Plus and a phosphoimager (both from Amersham).

cDNA constructs. We obtained a mouse *PSD-95* cDNA construct with the 3′ UTR from the IMAGE consortium (ID 10318) and also isolated *PSD-95* coding and 3′ UTR fragments via RT-PCR from mouse brain extract and T/A cloning (Promega Easy T/A cloning kit; pT/A-Fragment 1–5). Details of constructs and mutagenesis are reported in the **Supplementary Methods** online. FMRP protein domains were previously reported[34].

EMSA. We carried out binding reactions using full-length human FMRP protein in binding buffer (300 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, 0.5% glycerol, 20 mM HEPES pH 7.5 and 300 ng ml^{-1} tRNA), incubating at 25 °C or 4 °C for 30 min. We added heparin (0.3 mg) for 5 min before separation on a 6% native polyacrylamide gel. We carried out binding reactions with FMRP domains in the same buffer plus 100 or 300 mM KCl and 50–100 ng recombinant protein.

***In vitro* transcription.** We carried out these reactions using standard protocols (Ambion SP6/T7 Mega-Script) with [α-^{32}P]UTP, [α-^{35}S]UTP and UTP-Cy5 for EMSA, northern blotting and *in situ* hybridization, respectively.

Primary cultures. We prepared primary cortical and hippocampal neuronal cultures from embryonic mice (embryonic day 15, cortical; embryonic day 19, hippocampal) using standard protocols.

Neuronal transfection. We transfected hippocampal neurons at 14 days *in vitro* (DIV) using a standard Ca^{2+} phosphate precipitation protocol. We washed the precipitate using Hanks' balanced saline (HBSS) and carried out actinomycin D experiments 48 h later.

FISH, immunofluorescence and immunohistochemistry. We fixed primary hippocampal and cortical neurons at 20–25 °C for 15 min (4% paraformaldehyde, 2 mM $MgCl_2$, 5 mM EGTA in 1× PBS) and then UV irradiated and permeabilized the cells (1× PBS containing 0.1% Triton X-100). We prehybridized neurons (50% formamide, 2× SSC, 10 mM NaH_2PO_3) and then hybridized at 42 °C (30% formamide, 10 mM NaH_2PO_3, 10% dextran sulfate, 2× SSC, 0.2% BSA, 0.5 mg ml^{-1} yeast tRNA and 500 μg ml^{-1} salmon sperm DNA, and *in vitro* synthesized Cy5-labeled riboprobe). We carried out immunofluorescence and immunohistochemistry preincubation in 2% donkey serum, 0.2% Triton X-100, and then incubation in 1% BSA with antibodies specific for FMRP antibodies[8], followed by Cy3-labeled secondary antibodies specific for rabbit IgG (Jackson ImmunoResearch). We analyzed neurons by confocal scanning microscopy (Zeiss LSM 510).

Radioactive *in situ* hybridization. We cryostat sectioned, fixed (4% paraformaldehyde), permeabilized (1 mg ml^{-1} proteinase K) and acetylated (0.25% of acetic anhydride in 0.1 M triethanolamine, pH 8.0) brains before prehybridization and hybridization using standard protocols (55 °C in 50% formamide, 1× Denhardt's solution, 10% dextran sulfate, 0.3 M NaCl, 5 mM EDTA, 0.5 mg ml^{-1} yeast tRNA, 20 mM Tris HCl pH 8.0, 50 mM DTT and 10^5 cpm $μl^{-1}$ of [α-^{35}S]UTP-riboprobe). Slides were emulsified (Kodak autoradiography emulsion NTB2) and developed (ILFORD PQ developer) after 7–15 d of exposition. We analyzed sections by microscopy using a Zeiss Axioskop (1.25× or 5× objectives), acquired images with a Canon S50 digital camera and quantified the signal using ImageQuant and ImageJ.

Immunoprecipitation and RT-PCR. Whole brain was homogenized in 10 mM HEPES pH 7.4, 700 mM NaCl, 0.5% Triton X-100, 30 mM EDTA, protease inhibitors (Sigma-Aldrich) and 30 U ml^{-1} RNasin. We preblocked 20 μl protein A–Sepharose (0.1 mg ml^{-1} BSA, 0.1 mg ml^{-1} yeast tRNA and 0.1 mg ml^{-1} glycogen) for 1 h and then immunoprecipitated with specific FMRP antibodies[8]. DNase I (RNase-free, Amersham Pharmacia Biotech) was added during washes. We treated the immunoprecipitate with 50 μg proteinase K (Sigma-Aldrich) before RNA extraction and precipitation. First-strand synthesis was performed using p(dN)6 and 100 U of M-MLV RTase (Invitrogen). RT-PCR was performed as described in ref. 8. Radioactive semiquantitative RT-PCR reactions were performed in nonsaturating conditions in the presence of 0.2 $μ^{Ci}$[α-^{32}P]dCTP, 1 mM dCTP and 10 mM each dATP, dGTP and dTTP and analyzed on a 5% polyacrylamide gel.

Reversible cross-linking. We performed experiments as previously described[32]. Briefly, we washed hippocampal neurons at 10 DIV with Neurobasal medium containing 2% B27, and cross-linked them in 0.5% formaldehyde (J.T. Baker) for 30 min at 20–25 °C and quenched with 0.25 M glycine (Bio-Rad). We harvested cells by centrifugation, PBS washing and resuspension in RIPA buffer (see ref. 32). We immunoprecipitated cross-linked complexes with an FMRP antibody[8]. Before RT-PCR, we reversed cross-linking by treatment at 70 °C.

Polysomal analysis and RT-PCR. We analyzed cytoplasmic brain extract (of total brain and hippocampi) as previously described[8]. See **Supplementary Methods** for details.

mRNA stability assay. We treated primary cortical or hippocampal cultures (10 DIV) from time 0 with actinomycin D (10 μg ml^{-1}) for the indicated times. We washed cultures in PBS, extracted RNA with Trizol and analyzed RNA by quantitative RT-PCR. We used a NIKON C1 with plan-neofluar 20× to analyze both wild-type and *FMR1* knockout cultures for morphology. We assessed mRNA stability after DHPG treatment in two different ways. First, we added DHPG (100 μM) to cultures pre-exposed to actinomycin D for 3.5 h or 5.5 h. After 30 min of DHPG treatment, we collected mRNA for quantitative RT-PCR analysis. Second, we added DHPG (50 μM) and actinomycin D jointly to cells at time 0 and collected RNA 4 or 6 h later for quantitative RT-PCR analysis.

Quantitative RT-PCR. We carried out reactions with MoMLV–reverse transcriptase (Invitrogen) and the TaqMan Universal PCR Master Mix (ABI 4304437) using dual-labeled TaqMan probes (Applied Biosystems). We detected mouse *PSD-95* mRNA using the Pre-Developed TaqMan probe Mm00492193_m1 and compared with the endogenous control mRNA (mouse *H3f3b* mRNA Pre-Developed TaqMan probe Mm00787223_s1). Cycle parameters were as suggested by the manufacturer. Relative *PSD-95* mRNA levels, normalized to *H3f3b*, were calculated as follows: $2^{-[\mathrm{DeltaCt(treated)} - \mathrm{DeltaCt(untreated)}]} = 2^{-\mathrm{DeltaCt}}$, where DeltaCt equals Ct(*PSD-95*) − Ct(*H3f3b*). *β-actin* mRNA was detected with Pre-Developed TaqMan probe

Mm00607939_s1, and *Renilla* luciferase mRNA was detected using primers specifically designed by Applied Biosystems (See **Supplementary Methods**).

Primers. We provide a table listing the primers used in this study in the **Supplementary Methods**.

Neuronal cell survival (mitochondrial activity). We measured the mitochondrial activity using the colorimetric MTT assay by incubating hippocampal cultures for 30 min at 37 °C with 1 ml of Locke's buffer (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5.6 mM glucose, 5 mM HEPES, pH 7.4) containing 300 µg MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium, Sigma). We then dissolved cultures with 700 µl of DMSO and tested viable neurons by production of the purple MTT cleavage product, formazan. We took three independent measurements of sample optical density using a VICTOR 3V 1420 Multilabel Counter at 490 nm and reported the mean with s.d. The value of each culture is divided by the reference value (control culture at time 0).

Northern blot analysis. We probed 2 µg of poly(A)$^+$ RNA from the entire brain or 20 µg of total RNA using a mouse [α-^{32}P]UTP *PSD-95* mRNA antisense probe to fragment 5 (nucleotides (nt) 2,820–3,061 of BC014807), the entire 3′ UTR (nt 2,227–3,061 from BC014807) or the coding region (nt 61–2,226 of BC014807). We probed the same membrane with a *β-actin* cDNA antisense fragment (nt 258–837 of X03672) and quantified radioactive signals with a phosphoimager (Amersham).

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

F.Z. contributed to the conclusions drawn in **Figures 1**, **4**, **6**, 7 and **8**. B.E. contributed to the conclusions drawn from **Figures 5–8**. K.S.D. provided intellectual input, contributed to the conclusions drawn from **Figures 1–3** and **8**, contributed a portion of the funding and contributed to the writing of this manuscript. V.M. contributed to the conclusions drawn from **Figures 5–7**. S.D.R. contributed to the conclusions drawn from **Figures 2**, 3 and 7. A.D.P. contributed to the conclusions drawn from **Figures 1** and 7. E.T. and P.C. contributed to the conclusions drawn from **Figures 7** and **8**. G.N. contributed with intellectual inputs. S.G.N.G. contributed some initial funding for this work and intellectual inputs. C.B. provided intellectual input, funding, coordination of the project and contributed to the writing of this manuscript.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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The Fragile X Syndrome Protein Represses Activity-Dependent Translation through CYFIP1, a New 4E-BP

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SUMMARY

Strong evidence indicates that regulated mRNA translation in neuronal dendrites underlies synaptic plasticity and brain development. The fragile X mental retardation protein (FMRP) is involved in this process; here, we show that it acts by inhibiting translation initiation. A binding partner of FMRP, CYFIP1/Sra1, directly binds the translation initiation factor eIF4E through a domain that is structurally related to those present in 4E-BP translational inhibitors. Brain cytoplasmic RNA 1 (*BC1*), another FMRP binding partner, increases the affinity of FMRP for the CYFIP1-eIF4E complex in the brain. Levels of proteins encoded by known FMRP target mRNAs are increased upon reduction of CYFIP1 in neurons. Translational repression is regulated in an activity-dependent manner because BDNF or DHPG stimulation of neurons causes CYFIP1 to dissociate from eIF4E at synapses, thereby resulting in protein synthesis. Thus, the translational repression activity of FMRP in the brain is mediated, at least in part, by CYFIP1.

INTRODUCTION

The construction of neuronal circuits in the developing brain requires the correct assembly of trillions of synaptic connections. Finely regulated protein synthesis may be required to obtain the correct set of proteins at synapses and to modulate their activity in response to different developmental cues or synaptic stimulations. Indeed, accumulating evidence indicates that local (synaptodendritic) protein synthesis modulates synaptic plasticity (Martin et al., 2000; Steward and Schuman, 2003; Pfeiffer and Huber, 2006; Lin and Holt, 2008). Although the general translational machinery has been found at or near synapses, components that might control specific mRNA translation in that compartment are largely unknown.

One protein implicated in neuronal mRNA translation is the fragile X mental retardation protein (FMRP), the absence of which causes the fragile X syndrome (FXS) that is characterized at the cellular level by a deficit in synapse maturation. FMRP is an RNA-binding protein with roles in mRNA localization, translation (Bagni and Greenough, 2005), and stability (Zalfa et al., 2007; Zhang et al., 2007). It recognizes mRNAs by directly interacting with them through G quartets and/or U-rich sequences or through small, noncoding RNA adaptors such as the brain cytoplasmic RNA *BC1* and possibly microRNAs (Bagni and Greenough, 2005). Strong evidence indicates that FMRP represses translation, although how it does so is enigmatic.

Two well-characterized pathways that affect local protein synthesis in neurons involve activation of the TrkB receptors (Steward and Schuman, 2003; Schratt et al., 2004) by the neurotrophin BDNF and stimulation of the group 1 metabotropic glutamate receptors (mGluRs) (Weiler and Greenough, 1993). BDNF treatment activates the translation of two dendritic FMRP target mRNAs that encode Arc/Arg3.1 and αCaMKII (Aakalu et al., 2001; Yin et al., 2002; Zalfa et al., 2003; Schratt et al., 2004). On the other hand, FMRP is also regulated in response to mGluR stimulation (Weiler et al., 1997; Antar et al., 2004; Ferrari et al., 2007); moreover, long-term depression (LTD), triggered by activation the of mGluRs, is enhanced in the hippocampus of mutant mice lacking FMRP (Huber et al., 2002).

One possible mechanism for regulating translation is by modulation of the interactions between factors required for translational initiation. Cap-dependent translation, which requires the association of the eIF4A-eIF4G-eIF4E (eIF4F) complex with the 5′ terminal m^7G cap, is known to be particularly important in neurons (Richter and Sonenberg, 2005). eIF4F assembly is often regulated by the 4E binding proteins (4E-BPs), which interfere with the eIF4E-eIFG interaction (Richter and Sonenberg, 2005; Richter and Klann, 2007; Banko et al., 2007). eIF4E-binding proteins, including 4E-T and eIF4G, share a motif that is responsible

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for their association with eIF4E. Although 4E-BP1, BP2, BP3, and 4E-T, which all block the eIF4G-eIF4E interaction, probably act as general regulators, other proteins such as *Xenopus* Maskin and *Drosophila* Cup act as mRNA-specific 4E-BPs (Richter and Sonenberg, 2005). Thus far, only Neuroguidin has been identified as a 4E-BP in the nervous system (Jung et al., 2006).

Here, we demonstrate that FMRP-mediated repression of translation requires an interaction with *C*ytoplasmic *FMRP I*nteracting *P*rotein CYFIP1 (Schenck et al., 2001; Schenck et al., 2003) also known as Sra-1 (Kobayashi et al., 1998), which also binds the cap-binding factor eIF4E. The eIF4E-interacting domain of CYFIP1 forms the characteristic "reverse L shaped" structure that is also assumed by the canonical eIF4E-binding motif (Marcotrigiano et al., 1999). Modulation of CYFIP1 levels affects general mRNA translation in mammalian cells. In the brain, however, CYFIP1 forms a complex with specific FMRP-target mRNAs; reduced levels of CYFIP1 cause an increase in the synthesis of MAP1B, αCaMKII, and APP, whose mRNAs are known to be regulated by FMRP (Bagni and Greenough, 2005; Hou et al., 2006; Westmark and Malter, 2007). Our data indicate that an eIF4E-CYFIP1-FMRP complex is present at synapses and that synaptic activity releases CYFIP1 from eIF4E, as well as from bound RNAs, resulting in the alleviation of translation repression.

RESULTS

FMRP Cosediments with Light mRNPs in a Complex Possibly Containing Both eIF4E and CYFIP1

To investigate the mechanism by which FMRP represses translation in neurons, we examined the distribution of FMRP in sucrose gradients. As shown in Figure 1A, FMRP cofractionated with mRNPs with sedimentation values ranging from 40S to 80S. This profile is similar to the one observed for translational regulators such as mammalian p27/eIF6 and eIF4E (Figure 1A and Figure S1 available online). Interestingly, CYFIP1, which interacts with FMRP in the cytoplasm (Schenck et al., 2001), partially cofractionated with FMRP and eIF4E (Figure 1A, lanes 6–9, and Figure S1). Poly(A)-binding protein (PABP), which associates with translationally active and inactive mRNAs, cosedimented with mRNPs (Figure 1A), as well as with heavy polysomes (Figure S1). These sedimentation experiments suggest that FMRP, CYFIP, and eIF4E might reside in a common complex, perhaps with mRNA.

The FMRP-CYFIP1 Complex Binds eIF4E and PABP in Brain

We next investigated whether the FMRP-CYFIP1 complex could be retained with eIF4E on m^7GTP-Sepharose. When the beads were incubated with total brain cytoplasmic extracts, both FMRP and CYFIP1 were recovered from them but only after specific elution with m^7GTP (Figure 1B, lane 3). Whereas the recovery of FMRP varied according to salt concentration (data not shown), CYFIP1 recovery was not particularly salt sensitive. PABP was also present in the m^7GTP eluate, whereas two other proteins not involved in mRNA translation, β-tubulin and reticulon 1C, were not specifically eluted. Interestingly, WAVE, a cytoplasmic protein interacting with CYFIP1 (Bogdan et al., 2004), was also mostly not retained on the beads (Figure 1B, lane 3), suggesting a specific function of the FMRP-CYFIP1-eIF4E complex. Furthermore, neither CYFIP1 nor FMRP (Figure 1B, compare lanes 5 and 6) was present in the last wash; GTP greatly reduced the yield of the eluted complex (Figure 1B, lane 7), demonstrating the efficiency and specificity of the m^7GTP elution.

The FMRP-CYFIP1-eIF4E complex was also coprecipitated from brain extracts with specific FMRP antibody (Ferrari et al., 2007). A specific interaction among FMRP, eIF4E, and CYFIP1 was detected in wild-type (Figure 1C, lane 4) but not in *FMR1* knockout (KO) mice (Figure 1C, lane 3) (Bakker et al., 1994). RNase A treatment did not destroy the CYFIP1-FMRP-eIF4E interaction as assessed by CYFIP1 antibody coprecipitation (Figure 1D, compare lanes 2 and 6, and Figure S2), although a decrease in the FMRP-CYFIP1 association did occur (see below). These data indicate that the FMRP-CYFIP1-eIF4E complex is maintained primarily by protein-protein interactions.

To investigate whether the binding of the CYFIP1-FMRP complex to m^7GTP was mediated by FMRP, we performed m^7GTP chromatography with *FMR1* KO brain extracts (Figure 1E). The eIF4E-CYFIP1 association did not require FMRP (Figure 1E, lane 3). Furthermore, addition of exogenous human 4E-BP1 (Haghighat et al., 1995) to brain extracts decreased the amount of FMRP-CYFIP1 bound to eIF4E (Figure 1F, lanes 2–4), suggesting that they competed for the same site on eIF4E. This was confirmed with a 4E-BP mutated in the eIF4E-binding site (Figure S3). We conclude from these data that the FMRP-CYFIP1 complex binds eIF4E.

Translational repression occurs when 4E-BPs bind eIF4E to the exclusion of eIF4G (Marcotrigiano et al., 1999; Richter and Sonenberg, 2005). Consequently, we investigated whether the absence of a functional eIF4GI impaired the binding of CYFIP1-FMRP complex to eIF4E. After inactivation of eIF4GI by cleavage (Gradi et al., 1998) (Figure 1G, lane 1, asterisk), CYFIP1 and FMRP still bound eIF4E (Figure 1G, lane 2), indicating that binding of CYFIP1 to eIF4E does not require functional eIF4G.

PABP at the 3′ terminus of mRNA interacts with the 5′ cap-binding complex and circularizes mRNAs (Mazumder et al., 2003). Furthermore, the noncoding RNA *BC1*, which resides in the FMRP complex (Zalfa et al., 2003, 2005; Gabus et al., 2004; Johnson et al., 2006; Centonze et al., 2007b) also binds PABP (West et al., 2002). To test whether the FMRP-CYFIP1 complex simultaneously interacts with PABP, we used poly(A)-Sepharose beads to isolate PABP and associated factors from brain extracts. The FMRP-CYFIP1 complex was recovered together with PABP (Figure S4A). The binding of CYFIP1 to PABP was independent of FMRP and *BC1* RNA, as shown by the use of *FMR1* and *BC1* KO (Skryabin et al., 2003) brain extracts (Figure S4A). Moreover, the recovery of the FMRP-CYFIP1 complex was not due to nonspecific binding of CYFIP1 or FMRP to the polyribonucleotides affixed to the beads (Figure S4B). PABP and FMRP were also coimmunoprecipitated (Figure 1C, lane 4), indicating that FMRP and CYFIP1 are present in a complex containing both eIF4E and PABP.

CYFIP1 Binds Directly and Specifically to eIF4E

Because CYFIP1 bound m^7GTP (Figure 1B) independently of FMRP (Figure 1E), we hypothesized that CYFIP1 might be a novel

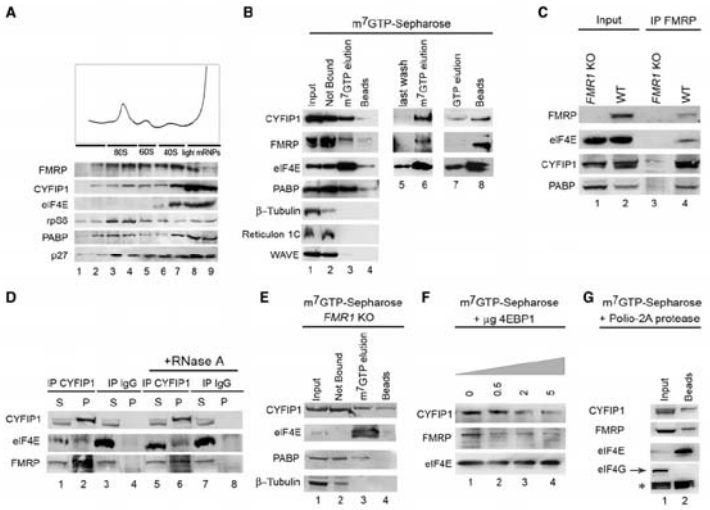

Figure 1. FMRP-CYFIP1 mRNP Interacts with the Translational Initiation Complex

(A) Cosedimentation of proteins on a 5%–25% sucrose gradient. The 80S monosome, the 60S and 40S subunits, and the very light mRNPs were detected by absorbance of 254 nm light. FMRP, CYFIP1, eIF4E, ribosomal protein S6 (rpS6), PABP, and eIF6/p27 were detected by immunoblotting.

(B) The initiation complex contains CYFIP1. Mouse brain proteins eluted from m^{7}GTP-Sepharose by free m^{7}GTP were analyzed by immunoblotting for CYFIP1, FMRP, eIF4E, PABP, β-Tubulin, Reticulon 1C, and WAVE. Lane 1, input (1/20); lane 2, unbound proteins (1/20); lane 3, specific elution with free m^{7}GTP; and lane 4, proteins retained on the beads after m^{7}GTP elution (beads). CYFIP1 and FMRP are absent from the last wash (lane 5) and in the GTP elution (lane 7). Proteins recovered after specific (lane 6) and nonspecific (lane 7) elution. Lane 8, proteins bound to the beads after nonspecific elution.

(C) CYFIP1 and eIF4E are both present in the FMRP complex in vivo. Western blot of proteins from *FMR1* KO and wild-type (WT) mouse brain extracts (Input [1/10], lanes 1 and 2, respectively) and proteins recovered after immunoprecipitation of the FMRP complex. Lane 4 shows detection of FMRP, eIF4E, CYFIP1, and PABP. Lane 3 is as above in the *FMR1* KO extracts.

(D) CYFIP1-eIF4E and CYFIP1-FMRP interactions resist RNase treatment. Lanes 2 and 6 show western blot of proteins recovered after immunoprecipitation of the CYFIP1 complex from WT mouse brain extracts. Lanes 1 and 3 show supernatants (1/20) after immunoprecipitation with CYFIP1 antibody and rabbit IgG. Lanes 5 and 7 show supernatants (1/20) after CYFIP1 and IgG immunoprecipitation in the presence of RNase. Lanes 4 and 8 show immunoprecipitations with rabbit IgG.

(E) FMRP absence does not interfere with CYFIP1-eIF4E complex formation. The same experiment as in (B) was performed with *FMR1* KO mouse brain extract. Lane 1, input (1/20); lane 2, unbound proteins; lane 3, m^{7}GTP-eluted proteins; and lane 4, proteins recovered from the beads after m^{7}GTP elution (Beads).

(F) Human 4E-BP1 competes with CYFIP1 for eIF4E binding. Lane 1, specifically eluted proteins (same as in [B]). Lanes 2–4, increasing amounts of wild-type human 4E-BP1 were added to brain extracts before m^{7}GTP chromatography (lanes 2–4). Western blotting was used to detect the levels of bound CYFIP1, FMRP and eIF4E.

(G) CYFIP1 binds the eIF4E complex independent of eIF4G. Lane 1, input (1/20) of the HeLa cytoplasmic extracts incubated with Polio-2A protease; lane 2, proteins retained on the column.

eIF4E-binding protein. eIF4G and several characterized 4E-BPs share a short consensus motif that is responsible for binding to eIF4E (Richter and Sonenberg, 2005). Therefore, we used multisequence alignments to search for a similar peptide that is conserved in the CYFIP protein family. A candidate peptide (residues 733 to 751, human CYFIP1, Swissprot annotation Q14467) was identified in the central region of CYFIP1. Here, several amino acids are conserved among CYFIP and human 4E-BPs, 4G-I, and 4G-III (Figure 2A). To validate the functional significance of this similarity, we compared a structural arrangement of the CYFIP1 peptide spanning residues 733 to 751 with the known structure of eIF4E when complexed with the 4E-BP1 peptide (Tomoo et al., 2005). Indeed, the CYFIP1 peptide (blue ribbon in Figure 2B) could potentially adopt the peculiar "reverse L shaped" structure with two α-helical turns located at its center that are stabilized by two internal salt bridges between residues Asp742-Arg744 and Glu748-Lys750. Moreover, the CYFIP1 peptide docks onto the molecular binding surface of eIF4E (red

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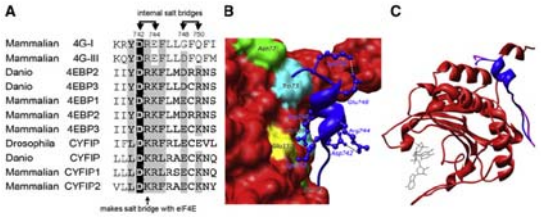

Figure 2. Multiple Alignment of Mammalian, Zebrafish, and *Drosophila* CYFIP1 and Canonical 4E-BPs

(A) The gray and black-boxed amino acids in the multiple sequence alignment are conserved and identical residues, respectively, that fold and interact with eIF4E. Black arrows denote internal salt bridges and one salt bridge with eIF4E.

(B) Hypothetical interactions between eIF4E, represented as a red molecular surface, and CYFIP1 peptide, shown as a blue spiral ribbon.

(C) Structural prediction of a complex among 4E-BP (red), the m^7GTP cap (gray molecule), and the CYFIP1 peptide (blue). The 4E-BP peptide (purple) found in the crystal structure almost perfectly overlaps with the CYFIP1 peptide.

(B) and (C) were produced with the UCSF Chimera program (Pettersen et al., 2004).

ribbon; Figures 2B and 2C), where 12 out of the 16 interactions formed between eIF4E and 4E-BP1 (Marcotrigiano et al., 1999; Tomoo et al., 2005) are conserved. In particular, the internal salt bridge Asp742-Arg744 (Figure 2B and Figure S6A) restricts the available conformations of the basic residue Lys743 and is thus locked in a favorable position for the formation of a salt bridge with Glu132 of eIF4E (Figure 2B). The 4E-BP1 peptide (purple ribbon, Figure 2C) and the CYFIP1 binding peptide (blue ribbon, Figure 2C) overlap in their "reverse L shaped" predicted structure and fit into the eIF4E pocket. The m^7GTP-Sepharose chromatography (Figure 1) and the in silico analysis (Figure 2) predict that residues 733 to 751 of CYFIP1 may fold into an eIF4E-binding domain even though the amino acid sequence does not conform to the previously established consensus YxxxxLL (Mader et al., 1995; Altmann et al., 1997). The charged residues implicated in the interaction are important for establishing the recognition between CYFIP1 and eIF4E. Interestingly, this pattern is common among three different protein families (CYFIP, 4G, 4E-BPs) that interact with eIF4E (Figure 2A).

To determine whether CYFIP1 is a 4E-BP, we performed GST-Sepharose pull-down assays using in vitro-synthesized ^{35}S-methionin-labeled proteins. Like 4E-T (Ferraiuolo et al., 2005) (Figure 3A, lane 7), CYFIP1 was precipitated specifically by GST-eIF4E (Figure 3A, compare lane 4 with lanes 2 and 3), indicating that eIF4E directly interacts with CYFIP1. The binding of CYFIP1 to the GST-eIF4E W73A mutant was reduced by 70%, similar to the effect on 4E-T (Figure 3B). eIF4E simultaneously interacts with CYFIP1 and m^7GTP, because both proteins were retained on m^7GTP-Sepharose beads. The retention of CYFIP1 was insensitive to RNase and DNase (Figure S5). To verify the importance of the putative eIF4E-binding region for the eIF4E-CYFIP1 interaction, we introduced several mutations in the CYFIP1 sequence (Figure 3C), followed by chromatography on m^7GTP-Sepharose (Figure 3D). First, the Asp742 and Arg744 residues were both mutated to alanine, thus removing the salt bridge at the beginning of the α helix and presumably allowing more flexibility of the critical Lys743. This mutant (A-A) bound 20% less efficiently than did the wild-type. We introduced other mutations that affect both internal salt bridges (Asp742Lys; Arg744Glu; Glu748Lys) and invert the electric charge of the critical lysine that is predicted to interact with eIF4E (Lys743Glu). Binding of this quadruple mutant (KEE-K) was reduced even further (by 60%), indicating that this region of CYFIP1 is involved in eIF4E binding. Finally, we studied the importance of the lysine that presumably interacts with eIF4E, mutating it to glutamate (-E-). This single substitution had an effect similar to that of the quadruple mutation; a reduction of 70% (compare the last two bars in Figure 3D, right panel). Because the substitution of Lys743 with a residue of opposite charge did not induce destabilization of the "reverse L shaped" structure of the CYFIP1 peptide, the strong inhibition of eIF4E binding indicates that Lys743 is involved in the interaction of CYFIP1 with eIF4E, as predicted by sequence and structural analysis (Figure S6). On the contrary, the two salt bridges, Asp742-Arg744 and Glu748-Lys750 (Figure 2A), stabilize the CYFIP1 peptide in a conformation that is able to interact with the eIF4E protein surface through Lys743. Mutagenesis of CYFIP1 where a 4E-BP motif similar to the YxxxxLL consensus was found (WFREFFL) (Figure S7) did not significantly change the binding to eIF4E.

To assess whether the isolated CYFIP1 peptide, which includes the eIF4E-binding domain, binds purified eIF4E, we synthesized wild-type (WT) and two mutant peptides (K-EE-K and -E-, Figure 3C) affixed to biotin. The western blot in Figure 3E shows that under stringent conditions (300 mM NaCl), no eIF4E bound to either the control (lane 2) or the CYFIP1 mutant peptides (lanes 4 and 5), whereas the streptavidin beads containing the WT peptide efficiently bound purified human eIF4E (lane 3). In less stringent conditions (150 mM NaCl, lower panel) the mutant peptides also bound some eIF4E but with lower efficiency than did the WT peptide. Therefore, the region of CYFIP1 with structural homology to the 4E-BPs does indeed bind eIF4E. CYFIP1 specifically and directly interacts with eIF4E through the noncanonical motif DKRLRSECK, where the lysine at the second position is critical for the interaction.

The FMRP-CYFIP1 Complex Is Consolidated by Binding to RNAs

To address the role of CYFIP1 in vivo, we transfected mammalian cells with DNA encoding this protein; as expected, overexpression of CYFIP1 repressed general translation. Silencing of CYFIP1 increased general translation (Figure S8B). Targeting

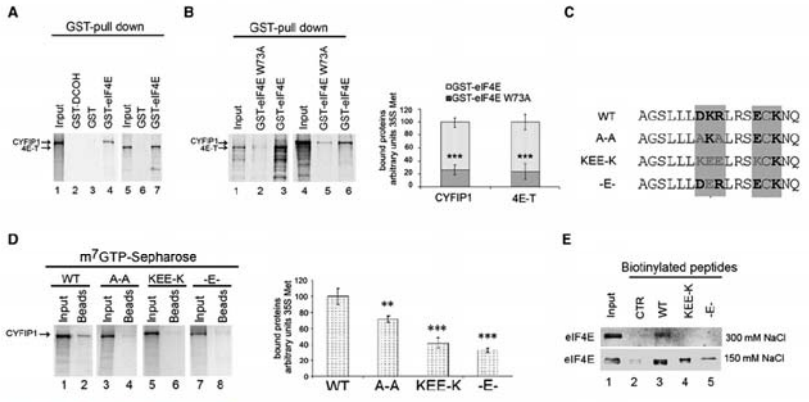

Figure 3. eIF4E and CYFIP1 Interact Directly

(A) GST pull-down assays. *E. coli*-expressed GST, GST-DCOH, and GST-eIF4E were immobilized on glutathione-Sepharose and incubated with ^{35}S-methionine-labeled CYFIP1 and 4E-T. Lanes 1 and 5, inputs. GST-eIF4E bound CYFIP1 (compare lanes 2–4) and 4E-T (positive control, lane 7).

(B) GST fusion protein binding assays. GST-eIF4EW73A mutant protein (lanes 2 and 5) or wild-type eIF4E (lanes 3 and 6) was incubated with ^{35}S-methionine-labeled 4E-T or CYFIP1 proteins. The relative intensities of CYFIP1 or 4E-T (bound proteins:input) were quantified. Proteins bound to GST-eIF4E W73A (dark gray bars) were compared to proteins bound to GST-eIF4E (100% binding, light-gray bars) (n = 5) ***p < 0.001, Student's t test.

(C) CYFIP1 amino acid sequence of the WT and mutant eIF4E-binding peptide. A-A, KEE-K, and -E- mutants are shown.

(D) m^{7}GTP binding assays. ^{35}S-methionine-labeled wild-type or mutant CYFIP1 was applied to m^{7}GTP-sepharose beads. Lanes 1, 3, 5, and 7 represent the input of WT and mutant CYFIP1 proteins, respectively (1/10). Lanes 2, 4, 6, and 8 represent the CYFIP1 proteins bound to m^{7}GTP beads. Relative intensities (bound: input) of WT and CYFIP1 mutants were quantified as described before (n = 8). ***p < 0.001, **p < 0.01, ANOVA and Dunnett's multiple comparison tests.

(E) Peptide binding assay. Human GST-eIF4E was applied to streptavidin beads containing wild-type or mutant CYFIP1 biotinylated peptides (sequences in [C]) or unrelated peptide with a biotin added to the N terminus. The bound eIF-4E was detected by immunoblotting. Lane 1, input (1/20); lane 2, unrelated peptide; lane 3, WT peptide; and lanes 4 and 5, K-EE-K and -E- mutant peptides, respectively.

of CYFIP1 to a specific mRNA further reduces its expression (Figures S8A–S8C).

According to our model, FMRP recruits CYFIP1 to mRNAs. To test this model, we used ^{35}S-labeled FMRP and CYFIP1 proteins and the FMRP target mRNA encoding *Arc/Arg3.1*. GST-eIF4E pull-down experiments showed that eIF4E did not bind significantly to FMRP (Figure 4A, compare lane 3 with lane 7), whereas it binds to CYFIP1, as shown before (compare lane 4 to lane 8). When capped-*Arc* mRNA was added, CYFIP1 and especially FMRP interacted more efficiently with eIF4E (Figure 4A, lane 6). The specificity was confirmed by the reduced recovery of the FMRP-CYFIP1 complex in presence of a nonneuronal mRNA (capped-*luciferase* mRNA), as shown in Figure 4A (lane 10). Some residual FMRP and CYFIP1 bound also to *luciferase* mRNA, in agreement with the fact that CYFIP1 also inhibits *luciferase* mRNA (by 25%; Figure S8). Thus, the interaction between FMRP/CYFIP1 and eIF4E is increased and possibly stabilized by the presence of target mRNAs, consistent with the decreased coprecipitation of FMRP with CYFIP1 after RNase treatment (Figure 1D).

Blocking of BC1 RNA in vitro reduces the affinity of FMRP to at least some of its target mRNAs (Zalfa et al., 2003). We therefore tested whether the absence of *BC1* RNA interferes with the binding of FMRP-CYFIP1 to eIF4E. When m^{7}GTP-Sepharose and brain extracts from *BC1* KO mice were used, there was a significant decrease (by 60%) in the amount of recovered FMRP (Figure 4B, lane 7). This effect is consistent with the decreased coprecipitation of FMRP with CYFIP1 after RNase treatment (Figure 1D). Furthermore, CYFIP1 formed an RNP containing *BC1* RNA (Figure 4B and Figure S9), and in the absence of FMRP, the association of *BC1* RNA with CYFIP1 decreased (Figure 4C), indicating that FMRP and *BC1* require each other for optimal interaction with CYFIP1 and eIF4E.

We then assessed whether CYFIP1 is associated with mRNAs in the brain. Figure 4D (lanes 2 and 3) shows that *Map1b* mRNA (Bagni and Greenough, 2005) was detected in the CYFIP1 immunoprecipitate. One negative control was neuronal *D$_2$DR* mRNA, whose metabolism is not affected by FMRP (Centonze et al., 2007a). Other FMRP target mRNAs were also investigated. The association of *Map1b*, *αCaMKII*, and *Arc* (Zalfa et al., 2003), but possibly not of *App* (Westmark and Malter, 2007), with the CYFIP1 complex was decreased in the absence of BC1 RNA (Figure 4D, Figure S9). RT-Q-PCR was also performed for *Map1b* mRNA (Figure 4E), confirming and extending our previous data

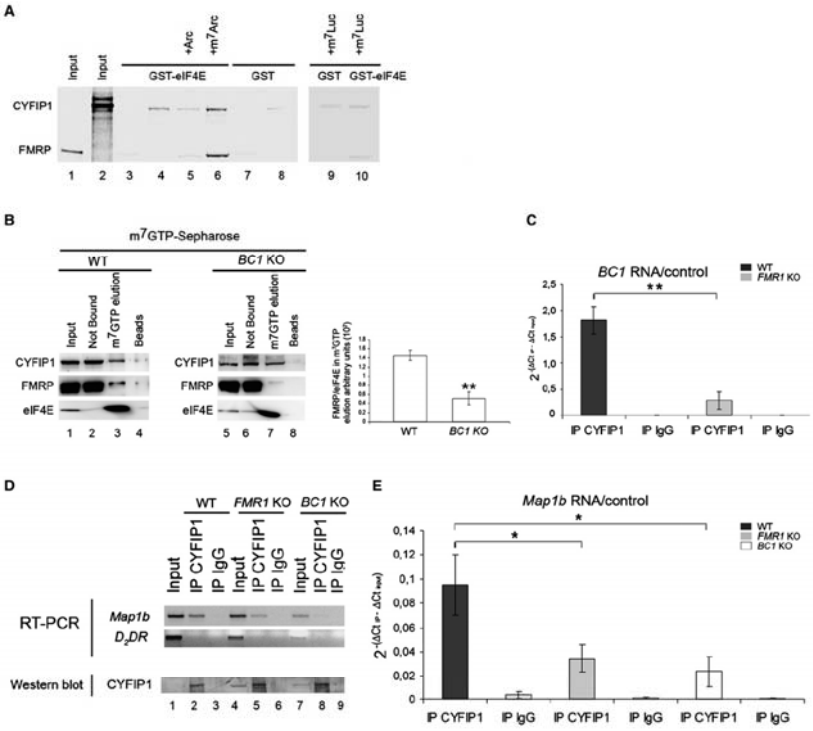

Figure 4. RNA Facilitates CYFIP1-FMRP Complex Formation

(A) *Arc* mRNA facilitates the CYFIP1-FMRP-eIF4E complex formation. Lanes 1 and 2, inputs (1/10) of ^{35}S-methionine-labeled FMRP and CYFIP1; lane 3, FMRP binding to the GST-eIF4E; lane 4, CYFIP1 binding to GST-eIF4E; lane 5, FMRP + CYFIP1 binding to GST-eIF4E in presence of uncapped *Arc* mRNA; lane 6, FMRP + CYFIP binding to the GST-eIF4E in the presence of m^{7}G-capped *Arc* mRNA; lane 7, FMRP binding to GST; lane 8, CYFIP1 binding to GST; lane 9, FMRP + CYFIP1 binding to GST in the presence of firefly m^{7}G-capped *luciferase* mRNA; and lane 10, FMRP + CYFIP1 binding to GST-eIF4E in the presence of firefly m^{7}G-capped *luciferase* mRNA.

(B) *BC1* RNA increases eIF4E-CYFIP1-FMRP complex formation. Extracts from WT or *BC1* KO brains were incubated with m^{7}GTP-Sepharose and m^{7}GTP-eluted proteins were separated by SDS-PAGE. CYFIP1, FMRP, and eIF4E were detected by immunoblotting. Lanes 1 and 5, inputs (1/20), lanes 2 and 6, unbound proteins (1/20); lanes 3 and 7, specific elution with free m^{7}GTP; and lanes 4 and 8, proteins bound to the beads after specific elution. The level of FMRP was normalized for the amount of eIF4E in the specific elution (lanes 3 and 7). Average of independent experiments is plotted on the histogram (n = 6, right panel). $^{**}p < 0.01$, Student's t test.

(C) CYFIP1 is part of a neuronal RNP. RT-Q-PCR of *BC1* RNA from wild-type (WT) and *FMR1* KO brain extracts was performed after CYFIP1 or IgG immunoprecipitation (n = 4). $^{**}p < 0.01$, Student's t test.

(D) After CYFIP1 immunoprecipitation from WT, *FMR1*, and *BC1* KO brain extracts, the RNA was extracted, and RT-PCR was used to detect *Map1b* and *D$_2$DR* mRNAs. Lanes 1, 4, and 7 represent the inputs (1/10). Lanes 2, 5, and 8 contain the immunoprecipitated RNA from WT, *FMR1*, and *BC1* KO extracts, respectively. Lanes 3, 6, and 9 reflect the mRNA associated with the control rabbit IgG. Lower panel: a western blot from the immunoprecipitated CYFIP1 performed from one-fourth of the same experiment used for the RT-PCR.

(E) RT-Q-PCR of CYFIP1-associated *Map1b* mRNA. RT-Q-PCR of *Map1b* from WT, *FMR1*, and *BC1* KO brain extracts was performed after CYFIP1 or control IgG immunoprecipitation (n = 7). $^{*}p < 0.05$, ANOVA and Dunnett's multiple comparison tests.

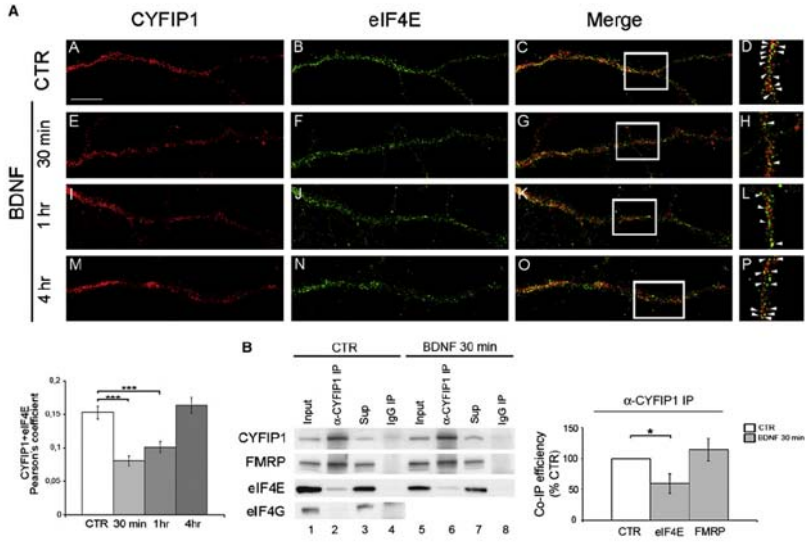

Figure 5. The CYFIP1-eIF4E Complex Is Activity-Regulated

(A) CYFIP1-eIF4E colocalization in dendrites under resting and stimulated conditions. Cortical neurons (14 DIV) were stained for CYFIP1 (red) and eIF4E (green) (colocalization is in yellow) under control conditions (CTR) (subpanels A–D) or after BDNF stimulation (subpanels E–P) at three different times. Subpanels D, H, L, and P show enlargements of the areas boxed in white with white arrowheads pointing to colocalization of CYFIP1 and eIF4E. Quantification of four independent experiments (a total of 320 neurites for each condition) was performed with the ImageJ program package and reported as a histogram. ANOVA, post hoc Scheffé test; ***$p < 0.0001$ and ***$p < 0.001$ at 30 min and 1 hr, respectively, and $p = 0.99$ at 4 hr. The scale bar represents 10 μm.

(B) CYFIP1-eIF4E-FMRP association in resting and stimulated neuronal cultures. CYFIP1 was immunoprecipitated under control conditions (lane 2, bottom panel) or after BDNF treatment (lane 6), and its presence with eIF4E and FMRP was analyzed by western blotting. Under control conditions, eIF4G was absent from the complex (lane 2). Quantification of eight independent experiments to detect eIF4E and four to detect FMRP is reported in the histogram. The amount of coimmunoprecipitated eIF4E or FMRP was normalized for the immunoprecipitated CYFIP1. *$p < 0.05$, one-sample Student's t test.

that a significant (60%) decrease in the association of this transcript with CYFIP1 occurred in *BC1* KO mice. Another putative FMRP target is *Fmr1* mRNA (Schaeffer et al., 2001; Miyashiro et al., 2003), which we did not detect in the neuronal CYFIP1 complex (Figure S9). Taken together, these data show that CYFIP1 requires *BC1* RNA and FMRP for optimal association with—and translational repression of—some key brain mRNAs.

The FMRP-CYFIP1 Complex Is Activity-Regulated in Neurons

Next, we analyzed the distribution of FMRP-CYFIP1, FMRP-eIF4E, and CYFIP1-eIF4E in primary cultures of hippocampal neurons. eIF4E was colocalized with CYFIP1 and FMRP in cell bodies and dendrites (Figure S10; see Figure S11 for specificity of antibody reaction). In some cases, synaptic activity and/or developmental transitions can alleviate translational repression (Gebauer and Hentze, 2004; Richter and Sonenberg, 2005); consequently, we investigated whether BDNF stimulation caused release of eIF4E from the FMRP-CYFIP1 complex. Resting neuronal cultures are shown in Figure 5A, subpanels A–D. BDNF was added to hippocampal neurons for 30 min, 1 hr, or 4 hr, followed by eIF4E and CYFIP1 immunodetection (Figure 5A, subpanels E–P). The overlap of CYFIP1 and eIF4E signals changed significantly with BDNF treatment; it decreased by 30% after 1 hr of treatment. Over 4 hr, however, the difference from baseline was not significant (Figure 5A, subpanels M–P). To verify that the diminished colocalization corresponded to a decrease in the CYFIP1-eIF4E complex, we performed CYFIP1 immunoprecipitations with similarly treated neurons (Figure 5B). As expected, the yield of coprecipitating eIF4E, normalized for the amount of precipitated CYFIP1, was reduced (by 40%) after 30 min of BDNF treatment, although FMRP levels did not change (Figure 5B). These data suggest that diminished colocalization reflects a decrease in complex formation. Moreover, eIF4G did

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not coprecipitate with CYFIP1 (Figure 5B, bottom panel), further indicating that CYFIP1, like other 4E-BPs, competes with eIF4G for binding to eIF4E. These observations indicate that the inhibitory FMRP-CYFIP1-eIF4E complex is dynamically regulated in an activity-dependent manner to repress and then possibly release dendritic mRNAs for translation.

The FMRP-CYFIP1-eIF4E Complex Is Present and Active at Synapses

To address the functional significance of the FMRP-CYFIP1-eIF4E complex at synapses, we prepared synaptoneurosomes (Pilo Boyl et al., 2007), the enrichment of which was monitored by PSD-95 levels in the synaptic fraction compared to total extracts (Figure S12). m^7GTP-Sepharose beads were then incubated with extracts from cortical synaptoneurosomes. Figure 6A shows that CYFIP1 bound to the beads was specifically eluted with m^7GTP, and FMRP was also detected in this complex (Figure 6A, lane 3).

We then stimulated cortical synaptoneurosomes with BDNF, immunoprecipitated CYFIP1, and examined coprecipitated eIF4E or associated mRNAs. In control experiments, eIF4E was associated with CYFIP1 (Figure 6B, lane 2). After 30 min of BDNF stimulation, a fraction of the eIF4E was released from the CYFIP1 complex (Figure 6B, lane 5 and right panel). The disassembly of the CYFIP1-eIF4E complex suggests that target mRNA translation was activated; indeed, around 80% of *Map1b* and *BC1* RNAs, as measured by RT-Q-PCR, was released from the CYFIP1 complex after stimulation (Figure 6C).

Because FMRP responds to mGluR stimulation (Weiler et al., 1997; Huber et al., 2002; Dolen et al., 2007), we treated hippocampal and cortical synaptoneurosomes with the group I mGluR agonist DHPG. After 5 min of stimulation, a significant decrease of eIF4E in the CYFIP1 complex was observed (Figure 6D). Longer stimulations (DHPG for 15 min) caused a significant increase in CYFIP1-eIF4E complex formation (Figure S13), suggesting that mGluR stimulation induced an initial release of CYFIP1-eIF4E-regulated translation followed by rapid overcompensation. We also electroporated primary cortical neurons with siRNAs directed against *CYFIP1*; reduction of CYFIP1 protein lead to an increase of MAP1B (Figure S14). Finally, extracts from *CYFIP1* heterozygote mice (*CYFIP1*$^{+/-}$) were analyzed; in this case, MAP1B protein increased by ~25% compared to the WT. Furthermore, the translation of αCaMKII and APP proteins was also upregulated by ~70% and ~90%, respectively (Figure 6E, compare lanes 1 and 2). Taken together, these data show that CYFIP1 regulates the expression of *Map1b* and other FMRP target mRNAs.

DISCUSSION

Although CYFIP1 was identified as an FMRP-interacting factor as well as a component of the WAVE complex involved in actin polymerization, its molecular function was unknown (Schenck et al., 2001; Kunda et al., 2003). We show that CYFIP1 binds eIF4E in brain extracts, synaptoneurosomes (Figures 1 and 6), and in vitro (Figure 3A). CYFIP1 contains a peptide that is predicted to exhibit structural similarity to the canonical eIF4E-binding domain (Richter and Sonenberg, 2005) (Figure 2A). Indeed, the integrity of this sequence motif is required for efficient eIF4E binding (Figures 3C–3E). CYFIP1 is not the first eIF4E-binding protein that does not contain a well-conserved eIF4E-binding peptide; the *Xenopus* oocyte maturation factor Maskin (Richter and Sonenberg, 2005) is another example. It seems therefore likely that by convergent evolution, several protein families developed a surface domain that can efficiently block access of eIF4G to eIF4E.

FMRP Recruitment of CYFIP1 Represses Translation

The 4E-BPs bind eIF4E independently of other factors and thus downregulate the translation of many mRNAs that have no obvious sequence similarity (Richter and Sonenberg, 2005). In other cases, eIF4E-binding proteins are recruited by specific proteins present only on a subset of mRNAs. For example, Maskin requires the RNA binding protein CPEB (Richter and Sonenberg, 2005), and Cup requires Bruno (Nakamura et al., 2004) or other proteins (Nelson et al., 2004), depending on the developmental stage. Here, we show that CYFIP1 inhibits the translation of associated mRNAs through FMRP (Figure 4). We propose that in the brain, FMRP helps recruit and/or stabilize CYFIP1 on the 5′ end of specific mRNAs to repress translation. Several observations support this point: the two proteins form a heterodimer (Schenck et al., 2001); FMRP increases the affinity of CYFIP1 for capped mRNAs (Figure 4A); *BC1*, an RNA involved in FMRP-mRNA complex formation (Zalfa et al., 2003, 2005; Gabus et al., 2004), also increases the FMRP-CYFIP1-eIF4E interaction (Figure 4B); CYFIP1 is associated with *BC1* RNA, *Map1b*, *αCaMKII*, *Arc*, and *App* mRNAs in the brain; in *FMR1* or *BC1* KO mice, the interaction of these mRNAs with CYFIP1 is decreased (Figures 4C–4E, Figure S9, and data not shown). Finally, reduction of CYFIP1 in the brain leads to an increase of MAP1B, APP, and αCaMKII (Figure 6E). These increases are consistent with those observed in the absence of FMRP in the mammalian brain (Zhang et al., 2001; Zalfa et al., 2003; Lu et al., 2004; Hou et al., 2006; Westmark and Malter, 2007). *Fmr1* mRNA, an FMRP target (Schaeffer et al., 2001; Miyashiro et al., 2003), is not part of the CYFIP1 mRNP, and consequently FMRP expression does not change upon CYFIP1 reduction (Figure 6E and Figures S9 and S14). Perhaps *Fmr1* mRNA is regulated by a different FMRP complex or is not controlled at the translational level.

The FMRP-CYFIP1-eIF4E Complex Responds to Synaptic Stimulation

One major issue concerning translation in neurons is regulation by synaptic stimulation. Protein synthesis is activated by several synaptic stimuli such as BDNF and DHPG. BDNF stimulates translation via mTOR and ERK-MAPK at synapses and likely involves modulation of initiation. Moreover, BDNF activates the translation of *Arc* and *αCaMKII* mRNAs in dendrites and at synapses (Aakalu et al., 2001; Yin et al., 2002; Schratt et al., 2004). After application of BDNF to cultured primary neurons and synaptoneurosomes, we observed a dissociation of eIF4E and CYFIP1, which coincided with the release of associated (m)RNAs (Figures 5, 6B, and 6C). In our model (Figure 7), this disassembly would free eIF4E to initiate translation.

DHPG stimulation of mGluR activity also induces translation initiation via ERK and subsequent eIF4E phosphorylation (Richter and Klann, 2007). DHPG stimulation of synaptoneurosomes

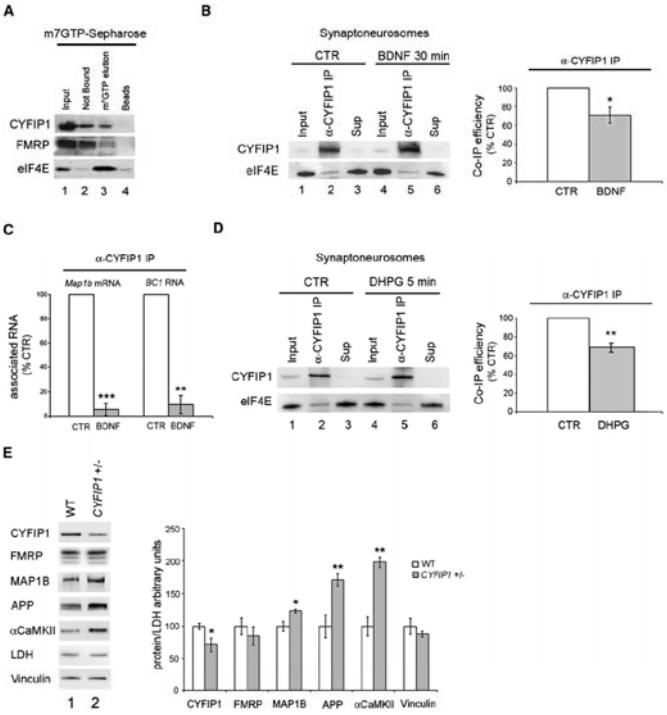

Figure 6. The FMRP-CYFIP1-eIF4E Complex Is Activity Regulated at Synapses

(A) CYFIP1-eIF4E complex is detected in synaptoneurosomes. m^7GTP-Sepharose beads were incubated with synaptic extracts; the recovered proteins were immunoblotted for CYFIP1, FMRP, and eIF4E. Lane 1, input (1/20); lane 2, unbound proteins; lane 3, specific elution with free m^7GTP; and lane 4, proteins bound to the beads after specific elution (beads).

(B) The CYFIP1-eIF4E complex is activity regulated at synapses. Proteins coimmunoprecipitating with CYFIP1 (lanes 2 and 5), respective supernatants (lanes 3 and 6), and inputs (1/40, lanes 1 and 4) were analyzed by immunoblotting. Quantification of four independent experiments is represented in the histogram. $^*p < 0.05$, one-sample Student's t test.

(C) CYFIP1 is part of a synaptic BDNF-sensitive mRNP complex. Shown is the amount of *Map1b* mRNA and *BC1* RNA, as determined by RT-Q-PCR, that coprecipitates with CYFIP1 after mock or BDNF stimulation of synaptoneurosomes (n = 4). Values were normalized for the mock control of each experiment. $^{***}p < 0.001$, $^{**}p < 0.01$, one-sample Student's t test.

(D) eIF4E is released from the CYFIP1 complex after DHPG stimulation. The experiments were performed as in (B) with DHPG stimulation for 5 min. n = 5, $^{**}p < 0.01$, one-sample Student's t test.

(E) CYFIP1 affects protein levels encoded by some FMRP target mRNAs. Brain proteins from WT (lane 1) or CYFIP1$^{+/-}$ mice (lane 2) were analyzed by immunoblotting to detect CYFIP1, FMRP, MAP1B, APP, αCaMKII, LDH, and vinculin levels (left panel). Quantified proteins were normalized for LDH. This ratio in WT mice was set at 100%. Histogram in the right panel shows the quantification of five independent experiments. $^{**}p < 0.01$, $^*p < 0.05$, Student's t test.

caused CYFIP1-eIF4E dissociation (Figure 6D), confirming a response of FMRP translational repression to mGluR signaling (Huber et al., 2002; Hou et al., 2006; Dolen et al., 2007). Interestingly, we detected the release of CYFIP1 from eIF4E only after a short stimulation (Figure 6D), which could be due to a short pulse of FMRP dephosphorylation observed under similar

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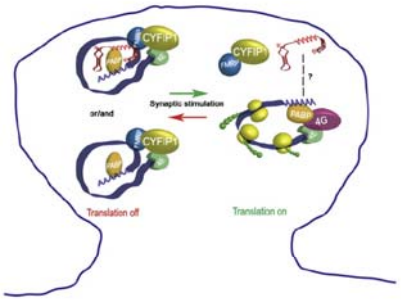

Figure 7. Proposed Model for mRNA Translational Repression and Activation by the CYFIP1-FMRP Complex

The CYFIP1-FMRP or CYFIP1-FMRP-*BC1* mRNA complex is transported in dendrites as translationally silent mRNPs. Some of these mRNPs are also present at synapses. After synaptic stimulation, and possibly after CYFIP1 and/or FMRP protein modifications, the CYFIP1-FMRP complex is released from eIF4E, and local mRNA translation ensues.

conditions (Narayanan et al., 2007). Alternatively, because Rac1 in its GTP form disassembles the FMRP-CYFIP1 complex in *Drosophila* (Schenck et al., 2003), it is tempting to speculate that a similar mechanism could also mediate eIF4E dissociation from CYFIP1.

Relevance for the Fragile X Syndrome

We propose that in the absence of FMRP, which causes FXS, there would be decreased binding of CYFIP1 to FMRP target mRNAs. This would relieve translational suppression and induce higher than normal levels of proteins whose synthesis is under the control of FMRP. On the other hand, the fact that CYFIP1 associates with the WAVE complex, which plays a role in actin polymerization (Bogdan et al., 2004), implicates the FMRP regulatory complex in synapse maturation. In support of this notion, synaptic abnormalities observed in mutants affecting Rac1 signaling pathways resemble those in the FXS (Tashiro and Yuste, 2004). Furthermore, the role of CYFIP1 as coregulator of FMRP may also help to explain the autistic features of FXS because CYFIP1 has been recently implicated in autism (Nishimura et al., 2007; Nowicki et al., 2007). Further work is needed to understand the functional consequences of impaired local protein synthesis in the developing brain and how this correlates to the autistic and FXS phenotypes.

EXPERIMENTAL PROCEDURES

Animals

All animals were treated according to institutional and international guidelines (see the Supplemental Data). The C57/BL6 *FMR1* KO mice were provided by Ben Oostra (Bakker et al., 1994), the 129Sv *BC1* KO mice by Juergen Brosius (Skryabin et al., 2003), and the CYFIP1$^{+/-}$ mice by Walter Witke (M.M. and W.W., unpublished data). All of the animals used in this study were 3 weeks old.

GST Pull-Down Assay

Plasmids encoding GST, GST-DCOH, GST-eIF4E, and GST-eIF4E Trp73Ala (see the Supplemental Data) were expressed in *E. coli* BL21; lysates were clarified and mixed with glutathione-Sepharose 4B beads (Amersham-Biosciences) for 1 hr at 4°C. The beads were collected and washed in PBS 1×. Glutathione beads containing the same amount of fusion proteins (5–10 μg) were incubated in binding buffer (20 mM Tris-HCl [pH 8.0], 100 or 200 mM NaCl, 1 mM ethylenediaminetetraacetic acid [EDTA], and 0.5% NP-40). Proteins bound to the beads were resolved by SDS-PAGE. The same amounts of protein were subsequently incubated in 250 μl of binding buffer in the presence of 5 μl of lysate containing in vitro-synthesized protein (TNT System Promega). After 90 min incubation at 4°C, the beads were washed in binding buffer and resolved by SDS-PAGE and phosphorimaged.

Protein Extract Preparation

Total mouse brain and cultured primary neurons were homogenized in lysis buffer (100 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris-HCl [pH 7.5], 1 mM dithiothreitol, 30 U/ml RNasin, 1% Triton X-100, 0.5 mM Na-orthovanadate, 10 mM β-glycerophosphate, and 10 μg/ml Sigma protease inhibitor), incubated 5 min on ice, and centrifuged at 12,000 g for 8 min at 4°C, and the supernatant was used for immunoprecipitations and m^{7}GTP-Sepharose chromatography. For total protein analysis, brains were homogenized in Laemli buffer, boiled, and vortexed. The procedures were repeated five times.

Immunoprecipitation

Brain (500–800 μg) or cell (200 μg) extracts were used for immunoprecipitation experiments. For immunoprecipitation of FMRP, brains were prepared as described above, and a reversible immunoprecipitation system (Catch and Release v2.0, Upstate) was used. For CYFIP1, the lysis buffer contained 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1.5% Triton X-100, and protease inhibitors (Sigma). The lysates were centrifuged first at 1000 rpm and then at 10,000 rpm, each for 10 min at 4°C. The extracts were incubated with 4 μg of CYFIP1 polyclonal antibody (see Table S1 for antibodies) overnight at 4°C. Twenty microliters of protein A Sepharose (Amersham) previously saturated with 1% BSA in PBS was incubated with the extract for 90 min at 4°C. The beads were washed three times in 1 ml of buffer (150 mM NaCl, 50 mM Tris-HCl [pH 8.0], and 1% Triton X-100). The same amount of rabbit IgG was used as control. Immunoprecipitation experiments with CYFIP1 antibody followed BDNF (30 ng/ml) stimulation of cortical neurons for 30 min before collecting the cells.

Cosedimentation of Proteins on Polysome-mRNPs Gradients

Total mouse brain was homogenized in lysis buffer (see Protein Extract Preparation). The supernatants were centrifuged through 5%–25% sucrose gradients as described in the Supplemental Data. Fractions were analyzed by immunoblotting.

m^{7}GTP Chromatography

m^{7}GTP-Sepharose beads (Amersham Biosciences) were equilibrated with buffer A (100 or 200 mM KCl, 50 mM Tris-HCl [pH 7.5], 5–10 mM $MgCl_2$, and 0.5% Triton X-100) plus BSA (0.1 mg/ml) at 4°C for 30 min. The resin was washed and incubated with 500–900 μg of protein extract from mouse brain for 60 min at 4°C; GTP (100 μM) was added to reduce nonspecific binding. The beads were washed with 0.4 ml of buffer A and then incubated for 30 min with 200 μM of m^{7}GTP. In some cases, 100 μg of RNase A (Sigma) and 1000 U of RNase T1 (Roche) per mg of protein were added (Nakamura et al., 2004).

Assay with Biotinylated Peptides

Biotinylated CYFIP1 wild-type ([Btn]AGSLLLDKRLRSECKNQ), mutant ([Btn]AGSLLLKEELRSKCKNQ; [Btn]AGSLLLDERLRSECKNQ), and control ([Btn]GSAPTRPPPLPP) peptides (Sigma) were dissolved in Tris-buffered saline (TBS)/Tween (0.02%); each peptide (25 μg) was incubated with streptavidin-conjugated magnetic beads (30 μl, Invitrogen) for 15 min at room temperature in buffer (150 or 300 mM NaCl, 50 mM Tris-HCl [pH 7.5], and 0.1% Triton X-100). The beads were then washed twice in the same buffer and mixed with

purified human GST-eIF4E (200 or 400 ng) for 1 hr at 4°C. The beads were then washed, and the protein was eluted, immunoblotted, and probed for eIF4E.

Immunoprecipitation Followed by RT-PCR and RT-Q-PCR Analysis

For RT-PCR and RT-Q-PCR analysis of immunoprecipitates, the beads were saturated in 1% BSA in PBS and heparin (1 mg/ml). CYFIP1 antibody was incubated with the beads (90 min at 4°C), washed three times (150 mM NaCl, 50 mM Tris-HCl [pH 8.0], and 1% Triton X-100), and incubated with 300 μg of brain extract or 200 μg of synaptic brain extract plus heparin (0.1 mg/ml) for 1 hr at 4°C. They were then washed, and RNA was eluted in 0.2 M NaAcetate, 1 mM EDTA, and 0.2% SDS for 5 min at 95°C. RNA was extracted (Trizol, Invitrogen) and used for p(dN)6-primed RT and PCR with mRNA-specific primers (Table S2). Real-time PCR was performed with an ABI 7900 Sequence Detector with dual-labeled TaqMan probes (Applied Biosystems). See the Supplemental Data for further details.

Neuronal Culture, Stimulation, and Image Analysis

Primary mouse cortical neurons (E15) were prepared as described (Ferrari et al., 2007); 14 DIV cells were treated with brain-derived neurotrophic factor (BDNF) (30 ng/ml, Alomone Laboratory) for 30, 60, or 240 min as described (Takei et al., 2001). They were then fixed with paraformaldehyde (1%–4%), permeabilized with Triton X-100 (0.2%), and analyzed for CYFIP1 and eIF4E. See Table S1 for details of the antibodies. The images were acquired with a confocal laser scanning microscope (LSM510, Zeiss and BIORAD Radiance 2010) with plan neofluar 40× or 63× oil objectives. Quantitative analysis in double-labeled material was performed blind from four different stimulated cultures by counting of 80 cells and 20 neurons (for each condition). A total of 320 dendrites over a length of 50 μm starting 20 μm from the nucleus was analyzed. Quantification was performed with ImageJ program (1.37v version) (from ImageJ for microscopy: http://www.macbiophotonics.ca/imagej/colour_analysis.htm#coloc_ica). See the Supplemental Data for further details.

Synaptoneurosomes

Synaptoneurosomes were prepared by homogenization of fresh cortex tissue in ice-cold buffer as described (Pilo Boyl et al., 2007). They were resuspended in HEPES-Krebs buffer (10 mM HEPES [pH 7.4], 150 mM NaCl, 3 mM KCl, 10 mM glucose, 2 mM $MgSO_4$, and 2 mM $CaCl_2$), equilibrated at 37°C for 5 min, and incubated at 37°C for 30 min with 200 ng/ml BDNF or for 5 min and 15 min with 100 μM DHPG. Stimulated and control (buffer treated) synaptoneurosomes were then lysed as previously described.

Homology Modeling Methods

A multialignment was carried out between a human 4E-BP1 fragment and twelve sequences of CYFIP 1 and 2, chosen among different species and isoforms (data not shown). In this multialignment, only the CYFIP sequences with a length comparable to that of human CYFIP1 were considered (KIAA0068, accession number D38549). A region showing a high conservation of residues that can be considered for the binding with eIF4E was found and modeled with the 4E-BP1 fragment present depicted by X-ray crystallography used as a template (Tomoo et al., 2005). The sequence numbering of CYFIP1 residues used throughout the analysis was made relative to the sequence of human CYFIP1 (accession number Q14467). See the Supplemental Data for further details.

Statistical Analysis

Differences between groups were determined by ANOVA and then Sheffé multiple comparison post hoc test, Dunnett's test, or one-sample Student's t test, where appropriate. Student's t test was used for comparisons where only two groups were analyzed. Correlations were determined with Pearson's correlation analysis. Significance was accepted at $p < 0.05$. Error bars represent the SEM.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, 14 figures, and two tables and can be found with this article online at http://www.cell.com/cgi/content/full/134/6/1042/DC1/.

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S

Synaptosome

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Synonyms

Synaptoneurosome; Synaptodendrosome; Neurosecretosome

Definition

The ►synaptosome is a subcellular particle deriving from the interruption of the axonal termini (see ►Axon) after the brain tissue has been homogenized in a buffer isoosmotic with the plasma. It represents mainly the presynaptic compartment or presynaptic spine but often retains part of the postsynaptic components according to the experimental condition used.

The ►synaptoneurosome is a composite particle containing one or more presynaptic compartments (synaptosome) attached to a postsynaptic element (neurosome) [1]. In the ►synaptodendrosome, the axon terminal adheres to a larger portion of the postsynaptic compartment (dendrite) (see ►Dendrite) [2]. Finally, the ►neurosecretosomes are a subtype of synaptosomes isolated from neurosecretory neurons such as neurons from the neurohypophysis [3].

Characteristics

Quantitative Description

The synaptosomal particles have a variable size according to their composition. The simplest synaptosomes are small bodies with a mean diameter of 0.6 μm or up to 1 μm for the neurosecretosomes [1,3]. As the neurosome vesicle measures around 1 μm, the complete synaptoneurosome has a mean diameter of 1.6 μm [1].

Pre and Postsynaptic Compartment Purification

The quality and composition of the synaptosomal fraction depends on the purification method used. The use of one or multiple step gradients as well as the homogenization of the brain, performed manually or mechanically, leads to a different population of synaptosomes with variable intact postsynaptic compartments.

The traditional procedure utilizes the separation of particles deriving from brain tissue homogenate, through an isoosmotic density gradient [4]. The homogenate is loaded on a sucrose gradient that is then centrifuged at high speed. During the centrifugation, each particle sediments at a specific location along the gradient according with the size and weight leading to the separation of four major subcellular fractions. The bottom fraction (P1) contains mainly nuclei and cell debris, whereas the middle fraction (P2) is a heterogeneous population including myelin fragments, synaptosomes and free mitochondria. At the top, the microsomes, ribosomes and smaller entities form two distinct fractions (P3 and P4). The further separation of the middle fraction (P2) leads to the isolation of synaptosomes.

To improve the quality of synaptosoneurosomes fractions, recently, a second density gradient has been employed that makes use of chemicals based on the iodixanol [5]. While the purity of the preparation is very high, the synaptoneurosome recovery is quite low. An alternative method, frequently used for the isolation of synaptoneurosomes, makes use of subsequent filtration steps in isoosmotic buffer. The brain homogenate is passed first through a 100 μm nylon mesh filter and then through a 5 μm filter [1].

Higher Level Structures

The synaptosomes appear as spherical or elongated particles containing the nerve terminal often joined to a partial or complete postsynaptic compartment [4,5]. The particle is coated by a membrane, which seals off the particle at the point where the axon is fractured. This continuous envelope preserves the integrity of synaptoneurosome and thus both the presynaptic and postsynaptic compartments retain their main structural features [4].

The presynaptic element (see ►Chemical synapse, ►presynaptic structure) contains a pool of synaptic vesicles (see ►Synaptic vesicle) that are organized in the active zone (see ►Active zone) close to the presynaptic membrane [1,4].

As mentioned, although in the synaptosome preparation the postsynapse is often not well preserved, the sealed presynaptic compartment is frequently attached to a residual of postsynaptic membrane. In the intact

synaptoneurosomes the postsynaptic element is well conserved as shown by a sealed membrane containing a dense structure beneath the membrane, known as postsynaptic density (PSD) (see ►Postsynaptic density) [1]. Figure 1 shows: (i) a drawing of a synaptic contact (pre and postsynaptic compartments) that is piched off during the isolation of synaptoneurosomes, (ii) an image, acquired at the electron microscopy, of a synaptoneurosome obtained with a protocol previously described [5] and (iii) a colored drawing of the same electron microscopy image.

Lower Level Components

At the ultrastructural level, the synaptosomal particles retain the cytoplasm components and organules.

In vivo, the presynaptic element presents an active zone (see ►Active zone) containing numerous synaptic vesicles 40–250 nm in diameter: a storage of releasable neurotransmitters (see ►Neurotransmitter). Generally, the synaptosomes contain small vesicles (40–60 nm in diameter), both clear-core vesicles with acetylcholine or amino acid transmitters, and dense-core vesicles (see ►Dense core vesicles) with catecholamines [6,7]. Sometimes larger dense-core vesicles (see ►Large dense core vesicles) have been observed containing neuropeptides (up to 250 nm in diameter) or biogenic amines [6,7]. In the neurosecretosomes the hormones are packaged in large neurosecretory granules (around 150 nm in diameter), with a dense core surrounded by a clear zone [3].

The presynaptic element preserves also the molecular machinery for exocytosis of synaptic vesicles (see ►Presynaptic exocytosis) [7] and the synaptosomal fractions are highly enriched in proteins, involved in neurotransmitter release, such as synaptophysin (see ►Synaptophysin) [5]. In intact synaptosomes, the exocytosis apparatus can maintain its functionality (see below). Within the presynaptic compartment one or more mitochondria are found and supply the energy for the local metabolism [4,7].

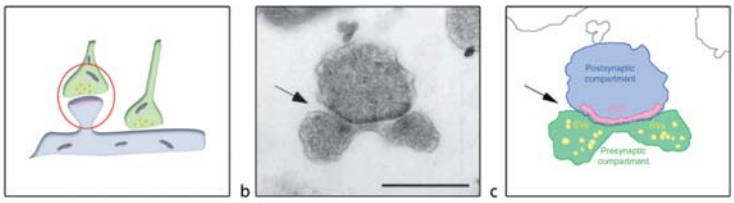
The pre and postsynaptic elements in the synaptoneurosome are joined together, as expected in a chemical synapse, (see ►Chemical synapse). As mentioned, the postsynaptic membrane shows a local thickening, the so-called Post Synaptic Density (PSD), which links the neurotransmitters receptors to signaling protein and cytoskeleton. In fact, the key component of this machinery, known as postsynaptic density protein 95 (PSD-95), (see ►PSD-95) is highly enriched in synaptosomal fractions [5].

In polarized cells, the mRNAs are delivered to specific subcellular compartments to be locally translated. In neurons, mRNAs as well as the translational machinery have been found in dendrites and in axons, especially in growth cone (see ►Dendritic protein synthesis) [8 and references therein]. In synaptoneurosomes, ►dendritic mRNAs, polyribosomes [8 and references therein] and translational factors [9] have been detected and messenger RNAs can be locally translated upon synaptic stimulation (see below) [5,8 and references therein]. Lately, small non-coding RNAs such as microRNAs and the Brain Cytoplasmic RNA 1, *BC1*, have also been detected at synapses [2,10].

Higher Level Processes

The synaptosomes maintain their viability and metabolic activity in media isotonic to plasma for some hours after isolation [6]. The high membrane potential and the low intracellular calcium concentration indicate the integrity of synaptosomal particles [7].

Importantly, the synaptosomes include mitochondria that supply the energy needed for the metabolic activities of presynaptic terminals. In fact the synaptosomal mitochondria possess a stable membrane potential which



Synaptosome. Figure 1 (a) Drawing of a synaptic contact. The *red circle* indicates the point of rupture of the axon-dendrite contact. The postsynaptic compartment is shown in *light blue* with a visible postsynaptic density (PSD) in *pink*; the presynapse is shown in *green* and contains synaptic vesicles in *yellow*. (b) Electron micrograph of a synaptoneurosome from Bagni et al. (2000) J Neurosci. Copyright 2000 Society for Neuroscience (c) Colored drawing of the previous micrograph. Postsynapse (*light blue*), PSD (*pink*), presynaptic terminal (*green*), synaptic vesicles (SVs) (*yellow*). Scale bar is 0.5 µm.

sustains the ATP production for the bioenergetic metabolism of particle [7]. As the synaptosomal particles are vulnerable to osmotic shock, the suspension in hypo-osmotic media leads to bursting of synaptosomes and release of intact synaptic vesicles and mitochondria [6].

In conclusion, the viable synaptosome behaves similarly to the synaptic compartment *in vivo* and can react to physiological and not physiological stimulations modulating its own functions (see below).

Lower Level Processes

The synaptosomes retain the ability to release the neurotransmitters by calcium-dependent exocytosis (see ►Exocytosis) as it occurs at synapses [7]. After stimulation, the synaptic vesicles move toward the active zone and, occasionally, the fusion of the vesicles with the membrane can also be observed. The endocytotic recycling of membranes provides the replenishment of vesicles pool (see ►Synaptic vesicle recycling). The synaptosomes release different class of neurotransmitters – cathecolamines, neuropeptides and amino acid transmitters, mainly glutamate [7 and references therein].

As previously mentioned, the synaptoneurosomes retain the majority of cytoplasmic components, including the synaptic mRNAs and the protein synthesis apparatus. Active translation in these particles is shown by the incorporation of radiolabeled amido acids into proteins [5,8 and references therein]. Interestingly, protein synthesis within synaptoneurosomes is activity-regulated; after stimulation, the translation of specific subset of synaptic mRNAs, which encode for key synaptic proteins, increases. Last, but not least, the intact synaptoneurosomes possess functional neurotransmitter receptors and relative signaling complex retaining the ability to trigger events and processes occurring in the intact neuronal cell (see below).

Process Regulation

Although the synaptosome maintains the metabolic machinery of synaptic terminals, it has lost the axonal input and thus it cannot receive physiological stimuli. Nevertheless, the intact synaptosome possesses functional neurotransmitters receptors that are up to respond to pharmacological drugs. In fact, the synaptosomal membrane has a negative membrane potential whose polarity changes after receptors activation as it happens in the whole neuron [7].

The stimulation can be obtained by alteration of ionic environment increasing potassium ion, by pharmacological inhibition of ion channels such as potassium channel [7] or by administration of physiological or pharmacological receptors agonists. In particular, treatment of synaptoneurosomes with metabotropic glutamate receptor (mGluR) agonists increases the amount of ribosomes associated with mRNAs, i.e. increases general local protein synthesis [8 and references therein]. Moreover, the administration of glutamate activates the translation of specific synaptic mRNAs [5]. At last, synaptoneurosomes maintain also the ability to respond to neurotrophic factors such as brain-derived neurotrophic factor (BDNF) which activates the protein synthesis machinery [9] enhancing the translation of specific mRNAs [10].

Function (Purpose)

The synaptosomes provide a versatile model to study the ultrastructure and the physiological features of the synapses.

The synaptosomal preparations can be used as starting material to isolate synaptic elements such as synaptic vesicles, synaptic mitochondria, purified postsynaptic density and synaptic mRNAs.

The synaptosomes have been used as a model to study synaptic processes. First, the synaptosomes have been extensively exploited to investigate neurotransmitter release, especially the glutamate release, and the regulation of this process [7]. In this context, the synaptosomes also enabled the study of the molecular basis of both endocytosis and exocytosis as the synaptosome is the simplest compartment containing the endocytotic and exocytotic apparatus [7]. Second, the synaptosoneurosomes and the synaptodendrosomes have been exploited to identify the synaptically localized mRNAs and to study the regulated local protein synthesis [2,5,8 and references therein]. As the neurotransmitters receptors and the membrane maintain their functionality, the synaptosomal preparations have also been used to investigate the physiological regulation of above-mentioned processes after receptors stimulation or ion channel blockade.

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Review

Fragile X mental retardation protein control of neuronal mRNA metabolism: Insights into mRNA stability

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ABSTRACT

The fragile X mental retardation protein (FMRP) is an RNA binding protein that has an essential role in neurons. From the soma to the synapse, FMRP is associated with a specific subset of messenger RNAs and controls their posttranscriptional fates, i.e., dendritic localization and local translation. Because FMRP target mRNAs encode important neuronal proteins, the deregulation of their expression in the absence of FMRP leads to a strong impairment of synaptic function. Here, we review emerging evidence indicating a critical role for FMRP in the control of mRNA stability. To date, two mRNAs have been identified as being regulated in this manner: *PSD-95* mRNA, encoding a scaffolding protein, and *Nxf1* mRNA, encoding a general export factor. Moreover, expression studies suggest that the turnover of other neuronal mRNAs, including those encoding for the $GABA_A$ receptors subunits, could be affected by the loss of FMRP. According to the specific target and/or cellular context, FMRP could influence mRNA stability in the brain.

Contents

The FMR1 gene and FMRP RNA binding activities

The fragile X mental retardation protein (FMRP) is an RNA binding protein (RBP). This class of molecules shuttle between the nucleus and cytoplasm and are involved in the regulation of posttranscriptional steps (splicing, nuclear export, stability, localization, and translation) that can occur in a coordinated manner—see the "RNA-operon" theory (Keene, 2007). Considering the importance of RBPs in RNA biogenesis, alterations in their functions can profoundly affect the control of gene expression and lead to a broad spectrum of human diseases that are due to both loss-of- and gain-of-function mutations (Lukong et al., 2008).

In the case of FMRP, aberrations in the trinucleotide repeat (CGG) expansion in the 5′-untranslated region (UTR) of the *FMR1* gene can result in two discrete pathologies. The fragile X syndrome (FXS), characterized by mental retardation, autistic-like behavior and anxiety, is caused by a massive expansion of the triplet (more than 200 CGG repeats); such a "full mutation" leads to transcriptional silencing of the gene and loss of FMRP expression (Jacquemont et al., 2007; Bassell and Warren 2008). On the other hand, the fragile X-associated tremor/ataxia syndrome (FXTAS), characterized by progressive cerebellar gait ataxia and intention tremor, appears to be a gain-of-function phenotype. This pathology occurs when the

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CGG repeats expansion is within the range of 55–200 copies (the so-called "premutation"), which is likely due to the toxicity of the aberrant levels of *FMR1* mRNA (Jacquemont et al., 2007; Swanson and Orr, 2007). The severe neurological phenotype exhibited in both diseases highlights the key role of FMRP in brain, where it is highly expressed (Devys et al., 1993). At the subcellular level, FMRP is mainly localized in the cytoplasm, but is also present at low levels in the nucleus (Feng et al., 1997). In neurons, FMRP is present in the cell body, along the dendrites and at the base of the synaptic spines (Antar et al., 2004; Feng et al., 1997; Ferrari et al., 2007), as well as in axonal growth cones and mature axons (Antar et al., 2006; Centonze et al., 2008; Price et al., 2006).

From the soma to the synapse, FMRP is part of large messenger ribonucleoprotein particle (mRNP) containing a number of protein partners, specific mRNAs and noncoding RNAs. These mRNPs are probably translationally silent as they travel along the dendrites. Like other mRNPs, FMRP-containing particles have a dynamic composition that undergo a series of rearrangements with its interacting proteins (Bagni and Greenough, 2005). Once the FMRP-silent granule reaches the synapse, the translational repression would be released upon neuronal stimulation thereby contributing to local neuronal plasticity (Bramham and Wells, 2007; Costa-Mattioli et al., 2009).

Biochemically, FMRP can be detected in large particles co-sedimenting with actively translating polyribosomes, in small particles co-sedimenting with silent ribosomal subunits, and with stalled (i.e., polysome associated but nontranslated) mRNP complexes (Zalfa et al., 2006). It has been estimated that ~4% of the mRNAs in the mammalian brain are associated with FMRP (Ashley et al., 1993; Brown et al., 2001). Many of the FMRP target mRNAs encode important neuronal proteins; among the best characterized are *α-CaMKII* mRNA (Dictenberg et al., 2008; Hou et al., 2006; Muddashetty et al., 2007; Napoli et al., 2008; Zalfa et al., 2003), *Arc* mRNA (Park et al., 2008; Zalfa et al., 2003), *Map1b* mRNA (Brown et al., 2001; Darnell et al., 2001; Dictenberg et al., 2008; Hou et al., 2006; Lu et al., 2004; Zalfa et al., 2003; Zhang et al., 2001), *Sapap4* mRNA (Brown et al., 2001; Dictenberg et al., 2008), and *Rac1* mRNA (Castets et al., 2005; Lee et al., 2003).

FMRP has been implicated in both mRNA transport and regulation of local protein synthesis in neurons (Bassell and Warren, 2008). Both FMRP and associated mRNAs travel along dendrites, a dynamic process that is promoted by synaptic stimulation (Antar et al., 2004; Bassell and Warren, 2008; Ferrari et al., 2007). The transport of FMRP and associated mRNAs can occur along microtubule tracks through the interactions with the motor protein kinesin (Antar et al., 2005; Davidovic et al., 2007; Dictenberg et al., 2008; Kanai et al., 2004). While some studies did not detect gross alterations in mRNA targeting in the absence of FMRP (Muddashetty et al., 2007; Steward et al., 1998; Zalfa et al., 2007), others showed that the dendritic localization of *RGS5* mRNA was impaired (Miyashiro et al., 2003). More recently, some investigations demonstrated that FMRP is involved in activity-dependent dendritic transport of several target mRNAs such as those encoding *Map1b*, *α-CaMKII*, *Sapap4* (Dictenberg et al., 2008). From these data, we can conclude that FMRP regulates mainly activity-dependent dendritic transport with the exception – so far – of *RGS5* mRNA that seems to be mislocalized also in basal conditions.

In neurons, FMRP is thought to be a repressor of translation (Lu et al., 2004; Muddashetty et al., 2007; Napoli et al., 2008; Zalfa et al., 2003; Zhang et al., 2001). FMRP also cosediments with both polysomes and mRNPs and, for this reason, possibly repress translation at multiple steps (i.e., initiation and elongation) (Zalfa et al., 2006). Ceman et al. (2003) reported that exogenous FMRP associates to apparently stalled polysomes, and mutants that cannot be phosphorylated run-off from those fractions (Ceman et al., 2003). It is tempting to hypothesize that FMRP could shuttle between the two fractions depending on whether it is posttranslationally modified or not, although this mechanism still needs to be elucidated. However, the change in phosphorylation does not affect FMRP association to RNA (Ceman et al., 2003). Two recent reports indicate that the endogenous phosphorylation of FMRP through the PP2A/S6K1 pathway would modulate the translational repression of FMRP mRNA targets in response to mGluRs signaling (Narayanan et al., 2007, 2008).

Recent data support a model in which FMRP represses the activity-dependent translation at synapses via the previously identified interacting factor CYFIP1 (*C*ytoplasmic *F*MRP *I*nteracting *P*rotein 1); CYFIP1 in turn interacts with the cap-binding factor eIF4E (Napoli et al., 2008). The FMRP–CYFIP1–eIF4E complex inhibits the translation of several FMRP target mRNAs; after synaptic stimulation, the FMRP–CYFIP1 complex partially dissociates from eIF4E and protein synthesis takes place (Napoli et al., 2008). This model closely resembles the mechanism described for two regulatory complexes: vertebrate Maskin/Neuroguidin-CPEB and *Drosophila* Cup-Bruno. In such cases, a protein (CYFIP1, Maskin or Neuroguidin, Cup) sequesters the initiation factor eIF4E and simultaneously binds an RNA binding protein (i.e., FMRP, CPEB, and Bruno, respectively); this configuration tethers the repression complex to a specific subset of mRNAs (Richter and Klann, 2009). In addition to the regulation of mRNA localization and synaptic protein synthesis, emerging evidence supports a role of FMRP in regulating another step of posttranscriptional control – mRNA stability.

The control of mRNA stability and degradation

mRNA stability is a highly regulated posttranscriptional step tightly coordinated with mRNA translation. A specific RNA surveillance mechanism, nonsense-mediated decay (NMD), removes mRNAs harboring premature stop codons to prevent the accumulation of aberrant, possibly dominant-negative, proteins (Behm-Ansmant and Izaurralde, 2006). Once mRNAs overcome NMD control, they can be translated or sequestered such that they can undergo translation or degradation at a later time. In the latter case, several factors can modulate the decay rate of mRNAs and mediate the communication between translation and degradation. Moreover, the interplay between translation and degradation may take place in cytoplasmic foci referred as P bodies (PB), where mRNAs can be degraded or stored to later re-enter the translating pool of mRNAs (Parker and Sheth, 2007). These bodies are functionally related to other cytoplasmic aggregates, namely stress granules (SG), which are composed by stalled translational preinitiation complexed (Kedersha et al., 2005). FMRP has been detected in both PB and SG (Zalfa et al., 2006). The complete nature and function of these bodies remain to be elucidated.

Normally, housekeeping genes produce invariantly stable transcripts while the turnover of some mRNAs undergoes a tight regulation to rearrange gene expression to certain cellular and/or developmental stimuli. Many mRNAs have a fast decay rate, resulting in a low steady-state level of protein under basal conditions. After stimulation, the mRNAs are rapidly induced by transcriptional activation and a modest increase in the amount leads to a significant variation in their expression (Khabar, 2007). In neurons, among the mRNAs regulated at the stability level are those encoding proteins related to neuronal growth (GAP-43, NGF, Tau), enzymes or enzyme inhibitors (acetylcholinesterase, neuroserpin), receptors (D_2 dopamine receptor, m4 muscarinic receptor, β_1-adrenergic receptor), and transcription factors (c-Fos, N-Myc/c-Myc) (Bolognani and Perrone-Bizzozero, 2008).

The decay rate of mRNAs depends on *cis*-acting elements frequently located in their 3′UTRs as well as on their associated *trans*-acting factors. A well-characterized sequence involved in mRNA stability is a 50- to 150-nucleotide sequence rich in adenosine and uridine, the so-called AU-rich element (ARE). These sequences are

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located in the 3′ UTRs of the mRNAs that are regulated by the AU-rich RNA binding proteins (AUBPs). In some cases, these sequences are rich in different residues such as GU or C (Kim and Gorospe, 2008; Vlasova et al., 2008). The importance of AREs in regulating gene expression is highlighted by the fact that 5–8% of human genes encode ARE-containing transcripts (Bakheet et al., 2001). Although AREs were originally defined as an AUUUA core associated with instability (Shaw and Kamen, 1986), it became clear over the years that ARE motifs can vary somewhat and regulate mRNA stability in both directions (Barreau et al., 2005). In fact, the interaction between ARE sequences and ARE-binding proteins can block or enhance the recruitment of the mRNA decay machinery and lead to a rapid modification of gene expression in response to environmental and developmental conditions.

Among the RNA binding proteins that associate with these RNA elements there are AU-binding factor 1 (AUF1) (Zhang et al., 1993), tristetraprolin (TTP) (Carballo et al., 1998), ELAV/Hu (Dalmau et al., 1990), CUG triplet RNA binding protein 1 (CUG-BP1) (Vlasova et al., 2008), and butyrate response factor-1 (BRF1) (Stoecklin et al., 2002). While the first two proteins are detected in all tissues, some of the Hu protein are neurospecific (Hambardzumyan et al., 2009). In general, AUF1, TTP, and CUG-BP are destabilizing factors that decrease the half-life of an mRNA, while the Hu proteins are stabilizing factors that promote stability and translation. AUF-1 might mediate stability and degradation depending on the mRNA and the cell type (Sela-Brown et al., 2000; Xu et al., 2001). In addition, microRNAs also affect mRNA stability. In a genome-wide microarray analysis, it has been shown that some microRNAs downregulate many target mRNAs (Lim et al., 2005). Further studies have also identified the molecular mechanism and the protein complex(es) involved (Bagga et al., 2005; Behm-Ansmant et al., 2006; Wu et al., 2006).

In mammals, two major pathways for mRNA degradation have been described. The first step is the removal of the poly(A) tail, which opens both 5′ and 3′ ends for exonucleolytic attack.

In fact, the interaction of the poly(A) and the 5′ end of the mRNA, via the PABP–eIF4G complex, forms a closed-loop state of the mRNA that is not accessible to the exonucleases (Mazumder et al., 2003).

In the first pathway, after deadenylation, the decapping enzymes Dcp1 and Dcp2 eliminate the 5′ cap and the mRNA body is degraded by the 5′→3′ exonuclease Xrn (Wilusz and Wilusz, 2004). Alternatively, the decay occurs in 3′→5′ direction catalyzed by the exosome, a large exonucleolytic complex. The residual cap is degraded by the scavenger enzyme DcpS (Wilusz and Wilusz, 2004).

One of the most well-described neuronal mRNAs regulated at the stability level is that encoding growth-associated protein 43 (GAP-43), a developmentally regulated protein involved in axon elongation in both developing and regenerating neurons (Benowitz and Routtenberg, 1997). The expression of GAP-43 is posttranscriptionally controlled by HuD, a neuronal ARE-binding protein belonging to the family of ELAV/Hu proteins. HuD recognizes an U-rich element in the 3′UTR of *GAP-43* mRNA and stabilizes it by interfering with the removal of the poly(A) tail (Beckel-Mitchener et al., 2002; Chung et al., 1997). Indeed, increased levels of HuD correspond to higher GAP-43 expression (Anderson et al., 2001; Bolognani et al., 2006; Pascale et al., 2004), leading to changes in neurite outgrowth (Anderson et al., 2001) and in synaptic plasticity (Bolognani et al., 2007b; Pascale et al., 2004).

FMRP and the regulation of mRNA stability

In addition to its role in mRNA transport and translation, recent evidence shows that FMRP may have an activity related to mRNA decay. Three studies have shown that mRNA abundance is affected when FMRP expression is abolished. Although such reports do not directly demonstrate a role of FMRP in mRNA stability, they provided a first clue that mRNA levels are altered in the absence of FMRP. First, in a high-throughput screen to identify FMRP target mRNAs, Brown et al. (2001) found that the expression levels of 144 genes were changed in lymphoblastoid cells from fragile X patients. In another analysis, Miyashiro et al. (2003) identified in brain some of the same mRNAs as Brown et al. (2001) and described their expression pattern; 3 out of 11 mRNAs were reduced in the *FMR1* KO versus wild type mouse hippocampus. For two of these mRNAs, those encoding the ribosomal component p40/LRP and the G-protein-coupled receptor kinase 4 (GRK4), the dendritic localization was unaffected, while for the one encoding the dystroglycan-associated glycoprotein 1 (DAG1), both localization and abundance were reduced. Moreover, FMRP loss may also lead to an impaired expression of $GABA_A$ receptors mRNAs. The *δ subunit* mRNA, previously identified by Miyashiro et al. (2003), has been found to be downregulated in *FMR1* KO neurons in a genome-wide expression profiling study (Gantois et al., 2006) as well as in other studies addressing its localization (Dictenberg et al., 2008). Consistent with these results, El Idrissi et al. (2005) reported a decreased expression of the $GABA_A$ β3 subunit in several brain areas from *FMR1* KO mice (El Idrissi et al., 2005). Interestingly, a further indication of FMRP as neuronal mRNA-stabilizing factor came from D'Hulst et al. (2006) who reported that the mRNAs encoding 8 out of the 18 known GABA subunits (α_1, α_3, α_4, β_1, β_2, γ_1, γ_2, as well as the above mentioned δ) were significantly reduced in the cortex, but not in the cerebellum, of FMRP-lacking mice. In addition, the expression of all the three subunits conserved in *Drosophila* appears to be compromised as well (D'Hulst et al., 2006).

Finally, two recent reports implicate FMRP as a direct modulator of mRNA turnover. First, FMRP has been shown to be involved in the regulation of *PSD-95* mRNA stability in hippocampus (Zalfa et al., 2007). PSD-95 encodes a key scaffolding protein of the postsynaptic density (PSD), the signal transduction machinery at glutamatergic synapses (Kim and Sheng, 2004). Because PSD-95 loss compromises both the structure and the function of dendritic spines (Migaud et al., 1998; Vickers et al., 2006), alterations in PSD-95 expression could contribute to the cognitive impairment caused by the absence of FMRP. *PSD-95* mRNA is part of the FMRP–mRNP *in vivo*; the C-terminal domain of FMRP binds a G-rich structure in its 3′UTR (shown not to be structured as a G-quartet) (Zalfa et al., 2007). By inhibiting transcription in primary neurons with actinomycin D, it has been shown that FMRP protects *PSD-95* mRNA from decay specifically in the hippocampus (Fig. 1A). The stabilizing effect of FMRP can also be enhanced upon neuronal activity, such as the stimulation of metabotropic glutamate receptors (mGluRs) (Zalfa et al., 2007). Furthermore, the region-specific effect of FMRP on *PSD-95* mRNA stability is consistent with other reports indicating that PSD-95 synaptic translation is affected in the cortex of FMRP-lacking mice (Muddashetty et al., 2007; Todd et al., 2003). Thus, it is possible that FMRP has multiple independent functions in the regulation of posttranscriptional control of gene expression (i.e., stability, mRNA translation) depending on the cellular context and developmental timing.

The FMRP complex regulating *PSD-95* mRNA turnover has not yet been identified. The FMRP-binding site in the 3′UTR is close to three U-rich tracts; two of them contain AREs. These motifs could be crucial for *PSD-95* mRNA half-life and the function of FMRP could prevent the action/binding of other destabilizing factors (Fig. 1A). Moreover, FMRP is not a general regulator of neuronal mRNA decay. The stability assay revealed that only 2 out of 12 known FMRP targets (*PSD-95*, *Map1b*, *α-CaMKII*, *Fxr1*, *G3bp*, *App*, *RhoA*, *Ef-1α*, *Vdac1*, *HnRNPA2*, *Sapap4*, and *Mbp*) have a compromised half-life in the absence of FMRP (Zalfa et al., 2007). The observation that *Myelin basic protein* (*Mbp*) mRNA has also a

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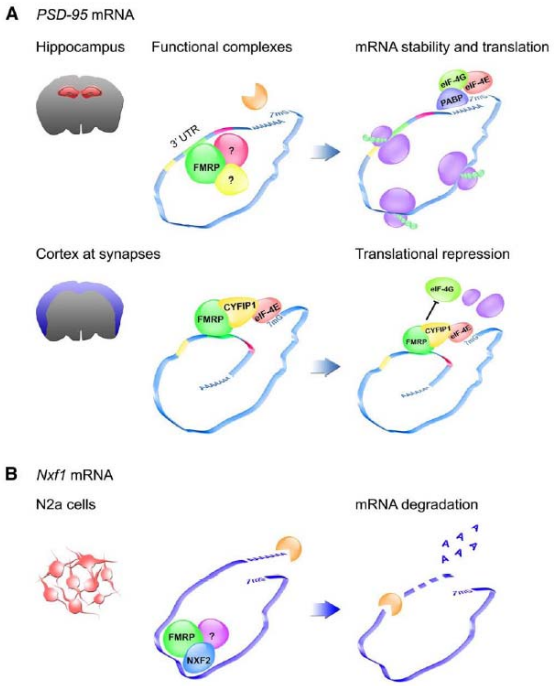

Fig. 1. FMRP in the control of mRNA stability. (A) Regulatory effect of FMRP on *PSD-95* mRNA in the hippocampus (upper panel, Zalfa et al., 2007) and in the cortex (lower panel, Muddashetty et al., 2007, Napoli et al., 2008). FMRP (in green) directly interacts with the 3'UTR of *PSD-95* mRNA. The FMRP-binding site (G-rich region, in green) is close to U-rich regions (in yellow) and AU-rich tracts (in purple). In the hippocampus (upper panel), the FMRP complex, which could include other factors involved in the control of mRNA turnover (in yellow and purple), would block the entry of the exonucleases (in orange) and consequently mRNA degradation. Consequently, the stable mRNA can undergo translation. In the cortex, at synapses (lower panel), the FMRP complex would tether *PSD-95* mRNA to a repression complex. One possibility is via CYFIP1. In this case, CYFIP1 would prevent eIF4E (in red) interacting with eIF4G (in light green), therefore inhibiting the initiation of translation. PSD-95 could also be possibly repressed via other mechanisms, i.e., microRNAs (not shown here). (B) Role of FMRP in the regulation of *Nxf1* mRNA half-life in a neuronal cell line (Zhang et al., 2007). FMRP (in green) directly binds NXF2 (in blue) and the complex associate to the messenger. It is still unknown which component of the complex mediates the recognition of the mRNA. The FMRP–NXF2 complex and possibly other proteins (purple) would facilitate the degradation of *Nxf1* mRNA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

decreased stability extends this function to oligodendrocytes (Zalfa et al., 2007).

FMRP has also been reported to contribute to *Nxf1* mRNA stability (Zhang et al., 2007). *Nxf1* mRNA encodes the large subunit of the mRNP export receptor involved in the transport of mature transcripts from the nucleus to the cytoplasm. NXF1 is the predominant component of the nuclear export factor family, which also includes NXF2, previously identified as a direct partner of FMRP in both testis and brain. The authors initially proposed that FMRP could act as an adaptor protein recruiting a specific subclass of mRNPs to NXF2 and to facilitate the nuclear export (Lai et al., 2006). More recently, using a mouse neuroblastoma cell line (N2a cells), they showed that upon NXF2 overexpression, the turnover of the messenger increased and consequently the mRNA levels were reduced. Because this effect was abolished by silencing FMRP in the neuroblastoma cell line, the authors proposed that FMRP could contribute to the degradation induced by NXF2 (Zhang et al., 2007) (Fig. 1B).

FMRP: similarities and differences with other neuronal regulators of mRNA stability

Hu/ELAV proteins

One of the few specific proteins that have been described as key regulators of mRNA turnover in brain are those belonging to the Hu family of proteins. The Hu proteins were first described as

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autoantigens in patients affected by paraneoplastic encephalomyelitis (Dalmau et al., 1990). The human proteins were then recognized to be orthologous of the *Drosophila* embryonic lethal abnormal vision (ELAV) protein, a splicing regulator important for neural development (Simionato et al., 2007). In mammals, four ELAV/Hu proteins have been described: HuR (alias HuA), HuB, HuC, and HuD (Brennan and Steitz, 2001). While HuR is ubiquitous and HuB is present in the brain and in germ cells, the expression of HuC and HuD is restricted to neurons. The neuronal ELAV/Hu proteins (nELAV) are essential for development of the nervous system. In fact, Hu proteins are reported as early markers of neuronal commitment and show a specific timing of expression in the developing brain (Okano and Darnell, 1997; Pascale et al., 2008). Studies *in vivo* or in primary neurons in which the nELAV/Hu expression has been perturbed by either overexpression or downregulation directly implicate these proteins in neuronal differentiation as well as in learning and memory (Akamatsu et al., 2005; Anderson et al., 2001; Bolognani et al., 2004, 2007a; Pascale et al., 2004; Quattrone et al., 2001). In neurons, the nELAV/Hu proteins are expressed in the cell body and along the dendrites (Bolognani et al., 2004; Tiruchinapalli et al., 2008a,b); HuD has been also found in the neurites of PC12 cells (Aranda-Abreu et al., 1999; Smith et al., 2004). Like many other RNA binding proteins, the Hu proteins are detected both in the nucleus and in the cytoplasm, suggesting different roles in different compartments. Although a role in splicing and nuclear polyadenylation is possible, the best characterized functions are the control of mRNA decay and the regulation of protein synthesis (Hinman and Lou, 2008). Focusing on the mRNA stability process, the Hu proteins can positively modulate the half-life of a subset of mRNAs critical for neuronal differentiation and maintenance. This group includes mRNAs encoding key transcription factors (c-Fos, c-Myc), molecules involved in neurite outgrowth and synapse functionality (the above mentioned GAP-43, Tau, acetylcholinesterase, neuroserpin) and determinants of neural differentiation (Musashi-1) (Pascale et al., 2008).

AUF1 and KSRP

While the Hu proteins are mostly implicated in the stabilization of mRNAs, other neuronal decay-promoting factors have also been described. One of the first identified is the AU-binding factor 1 (AUF1), also known as hnRNP D (Zhang et al., 1993). AUF1, which shuttles between the nucleus and cytoplasm, consists of four isoforms generated by alternative splicing of a single transcript (Sarkar et al., 2003a; Wagner et al., 1998). The different isoforms are expressed in the brain and show specific activities in binding and modulating the decay of ARE-containing transcripts (Dobi et al., 2006; Raineri et al., 2004; Sarkar et al., 2003b). Although the mRNAs associated with AUF1 are well characterized in nonneuronal cells (Bhattacharya et al., 1999; Mazan-Mamczarz et al., 2009), few target mRNAs have been identified in the brain. One example is the mRNA encoding the α_2 subunit of the nitric oxide-sensitive guanylyl cyclase in the cerebellar granule cells; the messenger is bound by AUF1 but upon the activation of the *N*-methyl-D-aspartate (NMDA) glutamate receptors, AUF1 is downregulated and the α_2 mRNA is stabilized (Jurado et al., 2006). In this case, there is an activity-dependent regulation mediated by AUF1. It has also been suggested that AUF1 is involved in integrating genetic and epigenetic signals during cortical development. It is specifically expressed in subsets of proliferating neural precursors and differentiating postmitotic neurons of the developing cortex (Lee et al., 2008). Recently, AUF-1 has been shown to promote the degradation of some target mRNAs but increase the stability and translation of other mRNAs; this duality may be due to relative abundance of AUF1 (Mazan-Mamczarz et al., 2009).

Another destabilizing factor present in neurons is the human K homology splicing regulatory protein (KSRP), a protein originally described as a splicing regulator (Min et al., 1997). KSRP is homologous to the murine zipcode binding protein 2 (ZBP2), involved in *β-actin* mRNA localization in neurons (Gu et al., 2002). The protein is expressed in neurons and in glia and it is distributed in both the nucleus and the cytoplasm where it can interact with mRNAs and enhance their turnover (Chou et al., 2006; Gherzi et al., 2004; Snee et al., 2002). Despite the fact that KSRP is neuronal, no specific target mRNAs in the brain have been characterized so far.

Compared to other neuronal proteins involved in the control of mRNA turnover, FMRP emerges as a novel regulatory factor with unique features. FMRP shares many characteristics with the other modulators of mRNA stability such as subcellular distribution and its ability to "multi-task". In fact, all the RBPs involved in mRNA decay shuttle between nucleus and cytoplasm; in this way, the posttranscriptional fate of mRNA can be modulated from birth to death by a common set of factors. That is, many proteins that regulate stability also take part in the translational control, resulting in the coupling of the two processes. Since it has been shown that mRNA stability could also be translation-dependent (Moore and Proudfoot, 2009), further investigations are necessary to verify whether the effect of lack of FMRP on the decay of *PSD-95* mRNA could be partially translation-dependent.

In addition to these shared features, FMRP exhibits unique properties in the context of mRNA decay. First, most of the stability regulators are recruited on the target mRNA by AU-rich elements (AREs); FMRP binds a variety of recognition motifs on the associated mRNAs. Interestingly, at least in the case of *PSD-95* mRNA stability, the binding site (G-rich) is included in a region containing two AREs. Second, the above mentioned RBPs have a specific role in mRNA decay, namely stabilization by the Hu proteins and the degradation of AUF1 and KSRP. Thus far, FMRP can protect *PSD-95* mRNA or enhance the decay of *Nxf1* mRNA, indicating a double role of FMRP in regulating the mRNAs stability in neurons. This dual function is reminiscent of the effect of AUF1 on different sets of mRNAs (Mazan-Mamczarz et al., 2009).

How can we reconcile FMRP's apparent opposite functions? Several not mutually exclusive possibilities should be considered. First, the stabilizing activity of FMRP could be dictated by a certain cellular context (neuron or glia) or subcellular compartment (cell body or synapse). In addition, there may be influences by the concentration of FMRP as well as the proteins with which it interacts (e.g., FMRP is differentially expressed in cortex and hippocampus; see Ferrari et al., 2007). In this case, the protein level in each component would determine the effect (stability or instability) and shift the equilibrium in one or the other direction. This would suggest that FMRP interacts with mRNAs as a multimeric complex, which might define its function and its sequence specificity. Second, FMRP posttranslational modifications may account for different functions of the protein. FMRP cosedimentation with polysomes change depending on its state of phosphorylation (Ceman et al., 2003). Third, sequences in the mRNA may determine its response to FMRP activity. That is, G-rich sequences close to ARE sequences could define a stable mRNA while other sequences on the mRNA, for example, those recognized by a FMRP–microRNA complex would instead lead to mRNA destruction. Fourth but not least, the *FMR1* gene is alternatively spliced (Zalfa and Bagni, 2004) and more than 10 different isoforms are generated. While their precise pattern of expression and function is unknown, it is easy to imagine that different isoforms could have opposite effects on mRNA metabolism.

The characterization of the molecular complexes that, together with FMRP, regulate *PSD-95* and *Nxf1* mRNAs in brain may provide insights into the dual molecular mechanism of FMRP activity. It is becoming evident that FMRP plays a key role in shaping the temporal and subcellular pattern of mRNA regulation during development by determining the fate of mRNAs in a tissue and disease-dependent manner. Further studies on the FMRP–mRNP complexes involved in

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mRNA stability will open new avenues for the regulation of neuronal mRNA stability by FMRP and its impact on the fragile X syndrome.

Acronyms

α-CaMKII	Calcium/calmodulin-dependent protein Kinase kinase type II alpha chain
Arc	Activity-regulated cytoskeleton-associated protein
APP	Amyloid Precursor Protein
AUF1	AU-binding Factor 1
CYFIP1	Cytoplasmic FMRP Interacting Protein 1
DAG1	Dystroglycan Associated Glycoprotein 1
EF-1A	Elongation Factor 1-alpha
FMRP	Fragile X Mental Retardation Protein
FXR1	Fragile X mental retardation syndrome-Related protein 1
G3BP	GAP SH3 domain-Binding Protein 1
GAP-43	Growth-Associated Protein 43
HnRNP A2	Heterogeneous nuclear RiboNucleoProtein A2
KSRP	K homology Splicing Regulatory Protein
MAP1B	Microtubule Associated Protein 1B
MBP	Myelin Basic Protein
NXF1	Nuclear eXport Factor 1
PSD-95	Postsynaptic Density Protein 95
Rac1	Ras-related C3 botulinum toxin substrate 1
RGS5	Regulator of G-protein Signaling 5
RhoA	Ras homology gene family, member A
SAPAP4	SAP90/PSD-95-Associated Protein 4
VDAC1	Voltage-Dependent Anion-selective Channel protein 1
ZBP2	Zipcode Binding Protein 2

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