

Molecular Insights into Mental Retardation: Multiple Functions for the Fragile X Mental Retardation Protein?

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Abstract

Mental retardation is a frequent cause of intellectual and physical impairment. Several genes associated with mental retardation have been mapped to the X chromosome, among them, there is *FMR1*. The absence of or mutation in the Fragile Mental Retardation Protein, FMRP, is responsible for the Fragile X syndrome. FMRP is an RNA binding protein that shuttles between the nucleus and the cytoplasm. FMRP binds to several mRNAs including its own mRNA at a sequence region containing a G quartet structure. Some of the candidate downstream genes recently identified encode for synaptic proteins. Neuronal studies indicate that FMRP is located at synapses and loss of FMRP affects synaptic plasticity. At the synapses, FMRP acts as a translational repressor and in particular regulates translation of specific dendritic mRNAs, some of which encode cytoskeletal proteins and signal transduction molecules. This action occurs via a ribonucleoprotein complex that includes a small dendritic non-coding neuronal RNA that determines the specificity of FMRP function via a novel mechanism of translational repression. Since local protein synthesis is required for synaptic development and function, this role of FMRP likely underlies some of the behavioural and developmental symptoms of FRAXA patients. Finally we review recent work on the *Drosophila* system that connects cytoskeleton remodelling and FMRP function.

Introduction

Mental retardation is defined as an impairment that affects brain function and that reflects functional deficits in adaptive behaviour such as interaction with people and daily living. Usually it is manifested before 18 years of age (Stevenson *et al.*, 2000). Defining features of mental retardation include an overall intelligence quotient (IQ) of less than 70. The causes of mental retardation are heterogeneous and include genetic as well as non-genetic factors like infection

during pregnancy and meningitis. The characterised genetic causes of mental retardation that have been well described to now include chromosomal abnormalities and monogenic diseases. However, a significant percentage of cases with mild retardation (50 < IQ < 70) remain unexplained. As suggested by several researchers in the field, most of the mild cases probably involve a combination of multigenic and environmental factors (Chelly and Mandel, 2001). An estimated 20-30% of mental retardation cases are thought to be due to X-linked defects (for review, see Stevenson *et al.*, 2000). The most frequent cause of inherited mental retardation is represented by the Fragile X syndrome (1 in 4000 males and 1 in 6000 females) initially discovered by Martin and Bell in 1943 (Martin and Bell, 1943).

Patients with the Fragile X syndrome exhibit mental retardation ranging from severe (IQ 20) to moderate (IQ 60), autistic behaviour and some physical features, like elongated face, large ears, joint laxity and macroorchidism (for reviews see Bardoni and Mandel, 2002; Oostra, 2002; O'Donnell and Warren, 2002). Interestingly, patients with this syndrome exhibit a quite variable social behaviour. They can learn to perform better for their daily living and with practise can improve their physical and intellectual attitude, which agrees with the positive effects of experience, including physical exercise, on the development of mammalian brains. Remarkably, some Fragile X patients have also participated to a marathon to the north pole (www.progettofilippide.cnr.it).

The syndrome results from the absence or mutation in the FMRP protein, the protein encoded by the *FMR1* gene. FMRP is widely expressed in foetal and adult tissues, with the most abundant level of expression in brain and testes (Devys *et al.*, 1993).

FMRP contains a nuclear localization and a nuclear export signal, which has led to hypothesis that it is involved in mRNA transport from the nucleus to the cytoplasm (Eberhart *et al.*, 1996). The protein contains four RNA binding domains and has the ability to bind a whole series of mRNAs/RNAs (for reviews see Kaytor and Orr, 2001; Antar and Bassell, 2003). The FMRP ribonucleoprotein particle associates with ribosomes and it is thought to be involved in cytoplasmic transport, and/or translational control of the bound mRNAs. In mammalian neurons, it has been shown that FMRP acts as a regulator of protein synthesis at synaptic sites of dendrites probably promoting maturation of dendritic spines (Zalfa *et al.* 2003) while its *Drosophila* homologues seem to be also involved in axonal growth (Zhang *et al.*, 2001; Morales *et al.*, 2002). In agreement with this function the morphological anomaly in the brain of both FRAXA post-mortem patients and *FMR1*

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knockout mice appears to be limited to the presence of abnormal dendritic spines, which morphologies resemble that of early development (Hinton *et al.*, 1991; Comery *et al.*, 1997; Nimchinsky *et al.*, 2001; Irwin *et al.*, 2001).

In the last five years, the role of FMRP has been largely elucidated thanks to the joined efforts of many outstanding laboratories in the world. In this review, we will summarise the knowledge of the fragile X syndrome and FMRP protein in mammals and review work on the *Drosophila* model system that helps to link the molecular mechanism of cytoskeleton remodelling and the Fragile X mental retardation protein. In this context we discuss our recent results concerning a new mechanism by which FMRP could regulate neuronal mRNA translation at synapses.

Molecular-genetic basis and neuronal characteristics of the fragile X syndrome

The Fragile X syndrome was initially discovered in 1943 by Martin and Bell (Martin and Bell, 1943) who reported the first pedigree of a sex-linked form of mental retardation. During the 1970s, Lubs demonstrated its co-segregation with a cytogenetic abnormality of the X chromosome (Lubs, 1969) and finally Sutherland established the nature of this anomaly as a fragile site that can be induced under certain cell culture conditions (Sutherland, 1977). Only in 1991, the syndrome was shown to be associated with a massive trinucleotide repeat expansion within the gene named *Fragile X mental retardation-1* (*FMR1*; Verkerk *et al.*, 1991, Yu *et al.*, 1991) and mapping and characterisation of the gene has brought in the last years new insights into the molecular mechanism of mental retardation.

FMR1 is a highly conserved gene that is composed of 17 exons, spans about 40 kilobases (kb) of DNA and encodes an mRNA of 3.9 kb (Figure 1). *FMR1* can be alternatively spliced, which does not appear to be tissue-specific (Ashley *et al.*, 1993; Eichler *et al.*, 1993; Verkerk *et al.*, 1993). FMRP is widely expressed, with the most abundant expression levels observed in brain and testes (Abitbol *et al.*, 1993; Devys *et al.*, 1993), the major organs affected in the Fragile X syndrome. In a majority of cases (>95% of patients), the Fragile X syndrome is caused by expansion of a polymorphic CGG repeat located in the 5' UTR of the gene. Based on the size of this expansion, it is possible to distinguish four principal types of alleles: normal (6-50 repeats), intermediate or "grey zone" (~45-60 repeats), premutation (~55-200 repeats), and full mutation (>200 repeats) alleles. The distinction between intermediate and premutation alleles is made by family history and repeat instability (Nolin *et al.* 2003; Figure 1). The full mutation-size expansions are associated with hypermethylation of the CGG repeats and the upstream CpG islands, which, most of the time, leads to transcriptional silencing of the gene and a severe physical and neurological phenotype (Verkerk *et al.*, 1991; Oberlè *et al.*, 1991; Sutcliffe *et al.*, 1992). However, rare atypical cases of Fragile X syndrome have been reported that are not associated with an amplification of the trinucleotide repeat, but with deletions or single point mutations (Gedeon *et al.*, 1992; Wohrle *et al.*, 1992; De Bouelle *et al.*, 1993; Meijer *et al.*, 1994; Hirst *et al.*, 1995; Milà *et al.*, 2000).

Premutation alleles are highly unstable and tend to expand to the full mutation when transmitted. The larger the premutation repeat is, the more likely it will expand to the full mutation, and this occurs only when the premutation is transmitted from a female (Malter *et al.*, 1997). The frequency of the premutation (carriers) is estimated to be 1 in 760 males and 1 in 260 female (Rousseau *et al.*, 1995). At the molecular level, individuals with premutation alleles show elevated *FMR1* mRNA levels (2-10 times higher than normal) but normal or reduced FMRP levels (Tassone *et al.*, 2000a, 2000b). This discrepancy can be explained by a reduced translational efficiency of the mRNA due to the triplet repeat in the 5'UTR (Tassone *et al.*, 2000a, 2000b; Kenneson *et al.*, 2001; Primerano *et al.*, 2002; Figure 1). Male premutation carriers have a different phenotype that has been recently discovered (Hagerman *et al.*, 2001; Greco *et al.*, 2002) and it is still mostly not understood (see below).

Since the mental retardation is the most important feature of the Fragile X patients, the brain has been most intensively studied in affected humans and mice models. Generally, the brain shows no gross defects. However, observations made using Golgi-Kopsch-stained cerebral cortices from post-mortem Fragile X brains have described dendritic spine morphologies that resemble those observed early in development. It is noteworthy that dendritic spine development is slowed in the cerebral cortex of *FMR1* KO showing a temporarily increased percentage of long thin dendritic processes. Both affected humans and *FMR1* KO mice indeed exhibit a similar dendritic characteristics (Hinton *et al.*, 1991; Comery *et al.*, 1997; Irwin *et al.*, 2001; Nimchinsky *et al.*, 2001).

How loss of FMRP affects synaptic function and leads to abnormal cognitive functions it is still an open question. In the last three years, there have been many albeit partial answers to this problem, and a picture starts to emerge from the puzzle so that in the future it will hopefully be possible to unravel the aetiology of the Fragile X syndrome. This will not only provide insights into the molecular basis of the Fragile X syndrome but also into learning and cognition in general.

Structural and functional domains of FMRP

FMRP is part of a small protein family that also includes the Fragile-X-related proteins FXR1P and FXR2P. The three proteins share the same domain structure and exhibit more than 60% amino acid identity to each other (Siomi *et al.*, 1995; Zhang *et al.*, 1995). The domain structure consists mostly of an array of four RNA-binding motifs (Figure 2): two ribonucleoprotein K homology domains (KH domains), a cluster of arginine and glycine residues (RGG box), and a new RNA-binding domain present in the N-terminal region of the protein (Adinolfi *et al.*, 1999a; Adinolfi *et al.*, 2003). Consistent with this domain structure, FMRP binds to RNA homopolymers as well as to a subset of brain transcripts (Ashley *et al.*, 1993; Adinolfi *et al.*, 1999a; Sung *et al.*, 2000; Brawn *et al.*, 2001; Darnell *et al.*, 2001; Miyashiro *et al.*, 2003). Considering the wide class of possible target mRNAs that have been recently described (see below), we think it is important to discuss the current knowledge

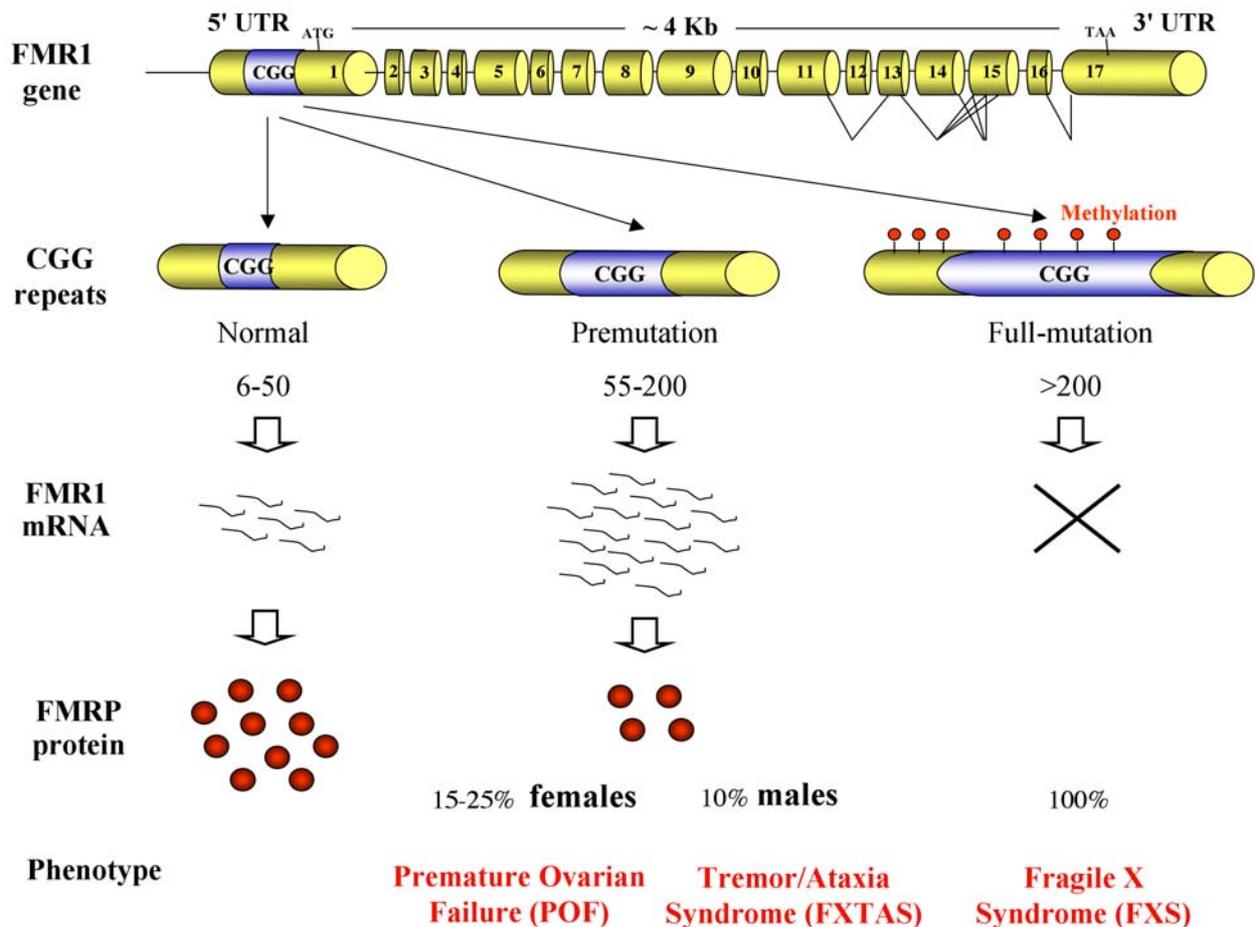


Figure 1. Structure of *FMR1* gene. *FMR1* gene has 17 exons that can undergo alternative splicing in the 3' region of the gene. The gene presents, in the 5'UTR a CGG triplet expansion. According to the number of expansions, the cell has an FMR1 mRNA level leading to different FMRP protein concentration. According to FMRP protein concentration and number of triplets, there are three possibilities, a normal individual, premutated individual who will develop with FXTAS or POF syndrome, a full mutated individual who will develop Fragile X Syndrome.

on the RNA binding domains found in FMRP, because they will determine the way FMRP binds its RNA target. General principles of RNA recognition by proteins are apparent in consensus RNA recognition motifs.

The RGG box is an RNA binding domain consisting of RGG (Arg-Gly-Gly) repeats first identified in the C-terminus of hnRNP U (Kiledjian and Dreyfuss, 1992), and also present in other hnRNP proteins, several nucleolar proteins involved in RNA metabolism and some viral proteins (Zhang and Grosse, 1997; Sandri-Goldin, 1998). So far, it has been reported that the RGG box has evolved as an interaction motif to strengthen contacts in RNA-protein complexes (Lengyel *et al.*, 2002) and is considered to have an accessory role in binding the RNA. This has been demonstrated for several RNA-binding proteins such as nucleolin (Ghisolfi *et al.*, 1992; Fouraux *et al.* 2002), fibrillarin (Lapeyre *et al.*, 1990), GAR1 in yeast (Bagni and Lapeyre, 1998) and NSR 1 (Lee *et al.*, 1991). Moreover, in hnRNP A2, RGG repeats are critical for the cellular localization of the protein (Nichols *et al.*, 2000).

The K homology (KH) module, an evolutionarily conserved sequence motif that has been originally identified

as three repeats in the human hnRNP K protein (Siomi *et al.*, 1993; for a review see Adinolfi *et al.*, 1999b), is present in a wide variety of quite diverse proteins from bacteria to human, indicating an early origin in evolution. The motif expands around a conserved VIGxxGxxI core sequence (where x is any amino acid, with a preference for positive residues). In the aligned sequences, there are no absolutely conserved residues (Gibson *et al.*, 1993; Musco *et al.*, 1996). A preference for positively charged residues on and between the proposed helices suggests a possible surface of interaction with nucleic acids. Accumulating evidence shows that KH-containing domains binds preferentially to specific RNAs (Gibson *et al.*, 1993; Lewis *et al.*, 2000). However, KH domains can also mediate binding to DNA (Duncan *et al.*, 1994; Michelotti *et al.*, 1996).

In addition to the KH domains and RGG box, the N-terminal domain of FMRP contains a significant RNA binding activity (Adinolfi *et al.*, 1999a), it does not exhibit homology to any known RNA binding motif, however, it has been recently shown an homology with proteins belonging to the "Royal Family" (Maurer-Stroh *et al.*, 2003). Further, it is not yet clear whether all RNA-binding motifs

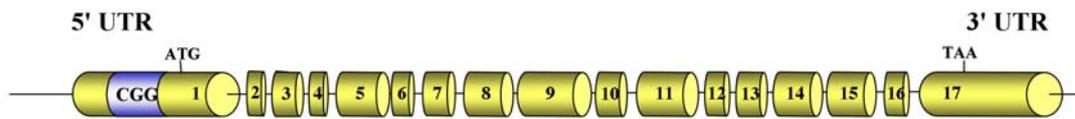
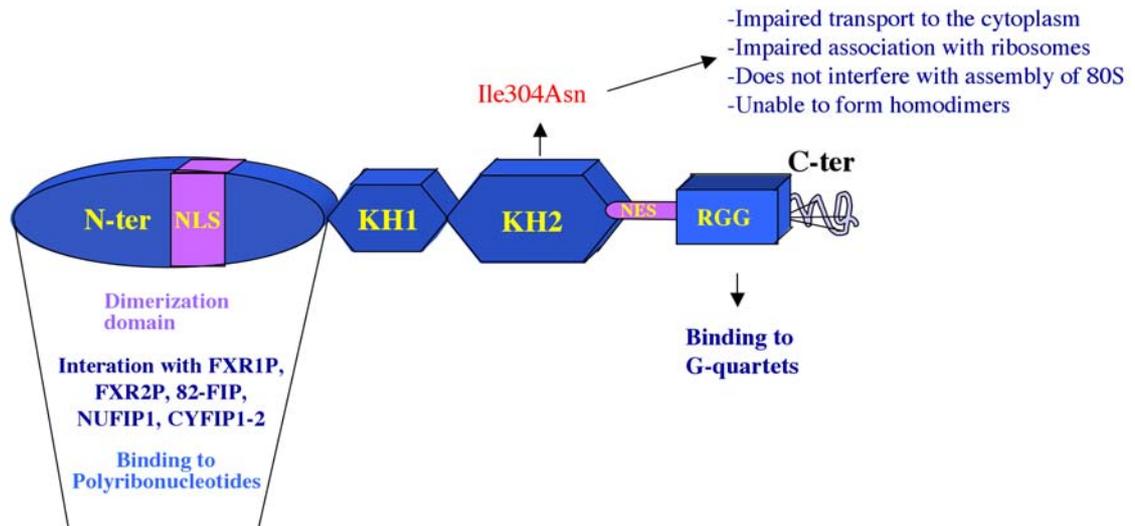
FMR1 gene**FMRP**

Figure 2. Structural and functional domains of FMRP. The protein presents four RNA binding domains, N-terminus, KH1, KH2, RGG box. A functional nuclear localization and export signals are also present. Each protein domain has also been shown to assolve one or more functions like interaction with RNAs and other proteins.

present in FMRP collaborate to bind to one RNA, or whether they can simultaneously accommodate several RNAs. Finally, the RNA binding motifs may also serve other roles. Interestingly, an Ile304–Asn missense mutation was identified in the second KH module of *FMR1* in a particularly severe Fragile X patient (DeBouille *et al.*, 1993; Figure 2). The mutation lies at the core of the hydrophobic RNA binding pocket of the KH domain (Lewis *et al.* 2000) and partially alters the RNA binding activity of FMRP (Siomi *et al.*, 1994; Feng *et al.*, 1997a; Brown *et al.*, 1998). Significantly, the mutated FMRP is unable to form homodimers (Feng *et al.*, 1997b), to inhibit the translation of various mRNA in vitro and to inhibit the formation of the initiation complex 80S (Laggerbauer *et al.*, 2001; Li *et al.*, 2001). Finally, FMRP Ile304–Asn missense mutation does not shuttle rapidly between the nucleus and the cytoplasm (Tamanini *et al.*, 1999).

In summary, FMRP shares a domain structure typical of hnRNP proteins, binds mRNA in vitro, and association with various RNAs has been demonstrated. The real targets of FMRP binding, however, remain elusive. To elucidate these targets, animal models are necessary.

The biological model systems to study the human syndrome

The FMRP protein family is highly conserved in evolution and at least one *FMR1*-homologous protein has been characterised in *Xenopus*, *Drosophila*, chicken, mouse and human (Verkerk *et al.*, 1991; Price *et al.*, 1996; Wan *et al.*, 2000). No *FMR1* gene is present in yeast and *C. elegans* (Shtang *et al.* 1999). *FMR1*, *FXR1* and *FXR2* genes have all been identified in several species, while the *Drosophila* genome encodes only one member of the family. Major insights into the function of the FXR family proteins have been obtained by studies on mouse and *Drosophila*.

FMR1 is highly conserved between human and mouse with a nucleotide and amino acid identity of 95% and 97% respectively (Ashley *et al.*, 1993). The expression pattern of murine *FMR1* is similar to its human counterpart in both tissue specificity and timing of expression (Hinds *et al.*, 1993). The *FMR1* knockout mouse was generated by homologous recombination disrupting exon 5. This murine model for the fragile X syndrome lacks normal FMRP protein and shows macroorchidism, deficit in spatial learning ability, hyperactivity (Bakker *et al.*, 1994), and dendritic spine abnormalities (Comery *et al.*, 1997; Irwin *et al.*, 2001; Nimchinsky *et al.* 2001). With these features,

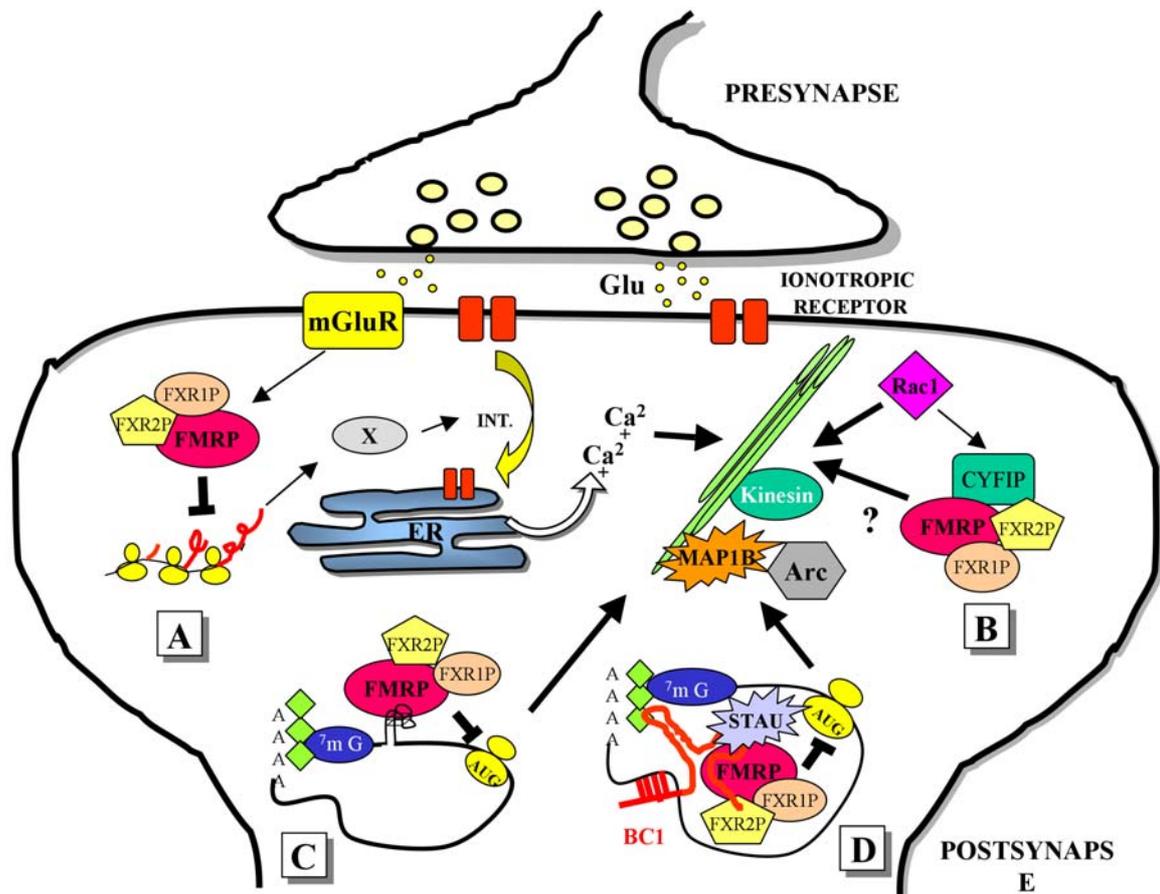


Figure 3. FMRP working model at synapses. The FMRP-ribonucleoproteins are probably transported to synapses via microtubules and motor proteins. At synapses, protein synthesis occurs according to the different cellular stimuli leading to an independent response of the single synapse influencing synaptic plasticity. (A) The stimulation of mGluRs enhances the FMRP synthesis, which act as a negative regulator of translation of proteins (X) involved in ionotropic receptor internalization during the long-term depression (LTD) in hippocampus. This process could bring to an increase of mGluR-dependent LTD that could effect on the lengthening of dendritic spines via a local increase of calcium from internal stores. (B) Cytoskeleton and synaptic remodelling occurs also via the Rac1 pathway that is modulated by the action of CYFIP-FMRP complex. To inhibit the translation of mRNA encoding key synaptic proteins, FMRP can probably bind mRNAs via the G-quartet present on the selected mRNAs (C) or via adaptor/bridge molecules like BC1/BC200 (D).

the *FMR1* mouse is at least phenotypically a fair model for the Fragile X syndrome. Molecularly, the ideal model is a knock in mouse with a CGG expansion higher than 100 that, due to technical reasons is difficult to generate. Only recently instability of CGG repeats has been observed in transgenic mice but in this case the endogenous gene has not been replaced (Baskaran *et al.*, 2002). Underlining the poor performance in memory and learning, the *FMR1* mouse shows impaired synaptic plasticity in the brain centres responsible for learning, in particular reduced long-term potentiation (LTP) in the cortex and enhanced mGluR-dependent long-term depression (LTD) in the hippocampus (Huber *et al.*, 2002; Li *et al.*, 2002), both which further show changes in synaptic plasticity processes. Thus, this mouse model is highly suitable for a molecular analysis of the target mRNAs/RNAs and of interacting proteins, as has been presented by several groups (see below). Hopefully, this analysis will lead in the future to a better understanding of the human disease.

Recently, a *FMR1* gene homologue has been identified and characterised in *Drosophila* and named *dfxr* (Wan *et al.*, 2000). Other than in mammals, the fly has only one gene encoding an FMRP-related gene. Either, *dfxr* fulfils the role of all three mammalian counterparts, or mammals have three related pathways each of which needs one specialised *fxr* family member. Interestingly, *dfxr* gene is most similar to *FXR2*, and both dFXR and mouse FXR2P have a role in the circadian clock (Dockendorff *et al.*, 2002; Morales *et al.*, 2002; Bontekoe *et al.*, 2002). Thus, this might be the function from which the FMRP family evolved.

dFXR is expressed at high levels in the central nervous system and in the muscle where it is localised to the cytoplasm. The expression pattern of dFXR during *Drosophila* embryogenesis reflects a combination of the tissue distributions of *FMR1* and the FXR proteins observed in mouse and human embryos (Wan *et al.*, 2000) and a splice variant is expressed differentially during embryogenesis (Schenck *et al.*, 2002). Overexpression of

Table 1. Summary of the FMRP interactors.

FMRP interactor	Cellular localization	Binding to other FMRP interactors	RNA binding activity	Isolation method
FXR1P	nucleus and cytoplasm	FXR2P, CYFIP2	+	yeast two-hybrid system (Zhang <i>et al.</i> , 1995)
FXR2P	nucleus and cytoplasm	CYFIP2	+	yeast two-hybrid system (Zhang <i>et al.</i> , 1995)
NUFIP1	nucleus	/	+	yeast two-hybrid system (Bardoni <i>et al.</i> , 1999)
CYFIP1	cytoplasm	/	+	yeast two-hybrid system (Schenck <i>et al.</i> , 2000)
CYFIP2	cytoplasm	FXR1P, FXR2P	+	high sequence homology with CYFIP1 (Schenck <i>et al.</i> , 2000)
82-FIP	nucleus and cytoplasm	/	+	yeast two-hybrid system (Bardoni <i>et al.</i> , 2003)
Nucleolin	nucleus	/	+	Co-immunoprecipitation (Ceman <i>et al.</i> , 1999)
YB1/p50	nucleus	/	+	Co-immunoprecipitation (Ceman <i>et al.</i> , 2000)
Staufen	cytoplasm	Pur- α	+	Co-immunoprecipitation (Ohashi <i>et al.</i> , 2002)
Pur- α	cytoplasm	Myosin Va mStaufen FXR1P-FXR2P	+	Co-immunoprecipitation (Ohashi <i>et al.</i> , 2002)
Myosin Va	cytoplasm	Pur- α	+	Co-immunoprecipitation (Ohashi <i>et al.</i> , 2002)

dfxr leads to cell death by apoptosis (Wan *et al.*, 2000). *dfxr* null flies display enlarged synaptic terminals, whereas neuronal overexpression results in fewer and larger synaptic buttons (Zhang *et al.*, 2001). Synaptic structural defects are accompanied by altered neurotransmission. The synaptic dFXR phenotypes mimic defects observed in mutants with altered levels of Futsch, a microtubule-associated protein with homology to mammalian MAP1B (Hummel *et al.*, 2000; Roos *et al.*, 2000). Interestingly, it was demonstrated that dFXR associates with *futsch* mRNA and negatively regulates Futsch expression. Moreover, a *dfxr/futsch* double mutant restores the *dfxr* synaptic structural and functional defects in the eye and neuromuscular junctions, suggesting that dFXR is acting as a translational repressor of Futsch to regulate microtubule-dependent synaptic growth and function (Zhang *et al.*, 2001).

Finally, Schenck *et al.* presented data suggesting that the *Drosophila* homolog CYFIP could be the missing link between FMRP activity and cytoskeleton remodelling at synapses (see below) (Schenck *et al.*, 2003). In summary, studies of the two animal models have significantly advanced our understanding of the molecular basis underlying the observed macroscopic and microscopic defects in the Fragile X patients.

FMRP-interacting proteins

Many, if not most, proteins do not function alone but as a complex with other biological macromolecules, proteins or nucleic acid. Knowledge of the identity and contribution of interacting proteins is therefore essential to understand the function of FMRP. Using two-hybrid assay in yeast and the N-terminal portion of FMRP as a bait, Mandel, Bardoni and colleagues have isolated and characterised a significant number of putative FMRP-interacting proteins (for a review Bardoni and Mandel, 2002).

Among them, FXR1P and FXR2P have already before been shown to interact with FMRP in the nucleus and in the cytoplasm (Zhang *et al.*, 1995). These proteins interact with the N-terminal portion of FMRP that indeed is a homodimerisation/heterodimerisation domain (Siomi *et al.*, 1996; Adinolfi *et al.*, 2003. Figure 2).

Moreover, the two-hybrid screens identified several murine and human proteins capable of interacting with

FMRP, which have been named for their sub-cellular localisation. The nuclear FMRP-interacting protein NUFIP1 is an RNA binding protein localised in the nucleus in a punctuate pattern, capable of nucleo-cytoplasmic shuttling. It does not interact with FXR1P and FXR2P, and only recently, via a multiple sequence alignment, Maurer-Stroh *et al.* found that NUFIP1 is a homologue of *Saccharomyces cerevisiae* Rsa1p, a protein involved in a nucleoplasmic assembly step of 60S ribosomal subunits (Bardoni and Mandel, 2002; Maurer-Stroh *et al.*, 2003).

The cytoplasmic FMRP-interacting proteins CYFIP1 and 2 are highly homologous to each other. CYFIP2 interacts with all the FXR family members, while CYFIP1 is specific for FMRP. CYFIP1 and 2 are localised at synapses (Schenk *et al.*, 2001) and CYFIP1 was previously identified as an interactor of the small GTPases Rac1 (Kobayashi *et al.*, 1998). As already mentioned, very recent data using the *Drosophila* system have linked *dfxr* to the Rho-GTPase pathway and synapsis formation (Schenck *et al.*, 2003) (Figure 3B).

The 82 kDa FMRP-interacting protein, 82-FIP, appears to be localised both in the nucleus and cytoplasm. It shows no homology to proteins of known function or to any known functional domain and, similarly to NUFIP1 and CYFIP1, interacts with FMRP but not with FXR1P and FXR2P. The protein is found in most neurons and is cell-cycle dependent in culture cells (Bardoni *et al.*, 2003).

Immunoprecipitation experiments identified further FMRP-interacting proteins, including nucleolin, YB1/p50, Pur α , and Staufen (Ceman *et al.*, 1999, 2000; Ohashi *et al.*, 2002). Pur proteins and Staufen are implicated in regulation of mRNA transport and translation (Li *et al.*, 2001; Kohrmann *et al.*, 1999; Duchaine *et al.*, 2002) via a possible interaction with a kinesin motor (Ohashi *et al.*, 2000). Consistent with a synaptic function, both Pur proteins and Staufen are associated in a complex containing the small dendritic non messenger RNA *BC1* (Ohashi *et al.*, 2002; Mallardo *et al.*, 2003).

Further, Willemsen and colleagues have shown that FMRP moves in neurites of PC12 cells as a particle in a microtubule dependent manner (De Diego Otero *et al.*, 2002). Together, these data strongly suggest a role in dendritic/axonal transport of the respective FMRP complex(es).

The functional significance of most of these interactions, summarized in Table 1, remains to be firmly established. The upcoming picture, however, suggests that FMRP may function as a shuttle protein between the nucleus and the cytoplasm that interacts with different protein and RNA components mediating processes of DNA regulation, RNA transport and translation. Thus, it can be conceived that FMRP associates with an RNA starting from its transcription, during transport, and up to its translation, possibly fine-tuning several of these steps. Interestingly, even if FMRP is not present, some dendritic mRNAs that have been shown to bind to FMRP, including *Arc* and α -*CaMKII*, are apparently normally localised in *FMR1* KO mice (Steward *et al.*, 1998). We should expect to find different ribonucleocomplexes containing FMRP, FXR1P and FXR2P that can be dynamically changed according to the various steps of FMRP function and the physiological needs of the cell. Since FMRP in particular is not an essential protein, we expect it to play a role only for a subset of the neuronal mRNPs and to fine-tune aspects of neuronal function rather than being a housekeeping gene that is necessary for expression of every mRNA.

FMRP and its targets mRNAs

Towards unravelling the pathogenesis of the Fragile X syndrome, it is important to elucidate the mRNA targets of FMRP in healthy cells. Several approaches have been successfully used to isolate FMRP target RNAs. These studies have identified a series of putative *in vivo* ligands and one structural RNA motif that mediates FMRP binding.

The first approach made use of precipitated RNA from human brain extracts using biotinylated-FMRP affinity resins. Few RNAs were identified and did not point to a certain cellular function. Most remarkable is the identification of the *FMR1* mRNA as it suggests that FMRP binds to its own messenger (Sung *et al.*, 2000). Further, this study identified several repetitive elements, the significance of which is not clear. In this case, the authors claim that only the KH2 domain is required for the binding. Warren and colleagues had previously shown that FMRP binds *in vitro* its own mRNA as well as to *MBP* mRNA (Brown *et al.*, 1998). Recently, the same authors (Brown *et al.*, 2001) have used immunoprecipitation from mouse brain and a microarray technique to identify co-precipitating RNAs. 432 mRNAs have been identified that are associated with FMRP, and 5% of which encode proteins involved in synaptic function, where the most pronounced defect in Fragile X is located. Further analysis using lymphoblastoid cells from Fragile X patients compared to healthy controls showed that 251 human mRNAs are both up- and down-regulated at the translational level. For three mRNAs, the change in translational activity was verified by checking the distribution on a polysome gradient, even though in this case no quantification has been reported. Finally, the 80 mRNAs that were most significantly precipitated with FMRP were compared with the up- or down-regulated mRNAs. 11 mRNAs are found in both sets, suggesting a direct regulation by FMRP. It should be noted that lymphoblastoids are non-neuronal cells and therefore, important co-factors of FMRP regulation may be missing

in these cells. Among the 11 mRNAs, there is the *MAP1B* mRNA that has also been found to be translationally repressed using *Drosophila* mutants and *FMR1* KO mice (Zhang *et al.*, 2001; Zalfa *et al.*, 2003).

In another microarray approach, Cavallaro and colleagues analysed how the presence or absence of FMRP influences the abundance of mRNAs, usually assumed to reflect strength of transcription. They compared mRNA levels from control wildtype and *FMR1* KO mice with high-density cDNA microarrays containing 8731 cDNA clones (D'Agata *et al.*, 2002). A change of more than twofold was observed for the mRNA levels of 73 genes. 41 of these did not have homology to any known gene, while the remaining 32 genes have similarity to known genes and could be grouped into four different classes: signal transduction, metabolism, structural proteins and nucleic acid synthesis and modification. Among the known mRNAs that showed more than 10fold change, they found Ubiquitin specific protease7 and Microtubule-associated protein 2 (*MAP2*). None of these mRNAs was common to the ones found in the previous work from Warren and colleagues (Brown *et al.*, 2001) even if they belong to the same functional groups.

Darnell and collaborators (Darnell *et al.*, 2001) have shown, using *in vitro* selection of random RNA sequences (SELEX) that the RGG box binds a class of mRNAs with a characteristic tertiary structure, namely the G-quartets. Mapping of an FMRP-binding element on the *FMR1* mRNA had independently demonstrated that FMRP binds to G quartets (Schaeffer *et al.*, 2001). This finding introduces a new vision in terms of RNA-protein interaction because the RGG box has been considered to be a non-specific RNA binding domain that unfolds RNA secondary structure and is unable of sequence-specific RNA binding (for a general reading Dandekar, 2002). 31 candidate mRNAs containing a G quartet motif were identified in the database. Twelve of these were assayed for FMRP binding and six RNAs bound to FMRP with considerable affinities having a K_d ranging from 75 nM to 467 nM. All of them are associated with synaptic function and included *MAP1B* mRNA. The other 6 did not interact with FMRP.

In collaboration with Warren's lab, the authors then analysed the distribution of these mRNAs on polysome gradients to gauge the efficiency with which they are translated. This joined work thus yielded a list of 6 RNA targets containing G-quartets that directly bind to FMRP and change their translational efficiency in Fragile X cell lines (Darnell *et al.*, 2001, Figure 3C)

The Eberwine and Greenough laboratories describe, using the sophisticated technique of Antibody Positioned RNA Amplification technique, APRA, around 80 new molecules that are associated to FMRP (Miyashiro *et al.*, 2003). They found RNAs encoding proteins involved in multiple functional pathways, such as receptors, signalling, cell structure, secretory pathways, and gene expression. The mRNA targets identified did not overlap with the previous study (Brown *et al.*, 2001) probably due to different microarrays used and/or due to different starting material (cultured neurons, whole brain) in the two studies. Finally, only a minority of the RNAs that bind to FMRP (23%) contain a G quartet, suggesting that this motif is not the

sole determinant of FMRP binding. These varying data clearly show on one side that it is extremely difficult to obtain an absolute result using the chip/array technology, and on the other side provide a broad range of putative molecules that can be further studied using a more functional approach.

Finally, we have shown that FMRP binds strongly to the small non-messenger cytoplasmic RNA *BC1*, and *BC1* simultaneously associates with some mRNAs regulated by FMRP, strongly suggesting that *BC1* RNA recruits FMRP to the targeted mRNAs, thereby determining the specificity of FMRP action (Zalfa *et al.*, 2003). To support this hypothesis, we searched for regions of complementarity between *BC1* and the regulated mRNAs. We found that *BC1* RNA is predicted to basepair to *MAP1B*, α -*CaMKII* and *Arc* mRNAs (Figure 3D). Interestingly, the sequence complementarity is found at the base of the longer stem-loop, according to a stable predicted secondary structure of *BC1* RNA (Rozhdetsvenski *et al.*, 2001). Thus, *BC1* is an important factor in FMRP-mediated translational repression. Consistently, it has been recently shown that *BC1* participates in translational repression *in vitro* by inhibiting the formation of the 48S preinitiation complex (Wang *et al.*, 2002). Further substantiating the role in translational control, *BC1* has also been shown to associate with poly(A) binding protein and eIF4A (Muddashetty *et al.*, 2002). Thus, *BC1* RNA acts as a bridge between FMRP and specific target mRNAs forming the translational inhibition complex.

In this sense, *BC1* acts similarly to the micro RNAs (miRNAs). Although much smaller (22 nt on average), miRNAs form imperfect base-pairings with their target RNAs and inhibit, in an yet poorly understood manner, translation of these RNAs. Up to now, several hundred miRNAs have been identified in *C.elegans*, *Drosophila* and mammals, but the corresponding target mRNAs have proven difficult to identify, not least because the base pairing interactions are short and imperfect. Only recently target genes have been identified in mammals (Kawasaki and Taira, 2003). Similar difficulties are to be expected when looking for the additional *BC1* targets. And the analogies may even stretch further: lately, dFXR has been shown to associate with microRNAs as well as to the exonuclease DICER, a key enzyme in the biogenesis of miRNPs (Caudy *et al.*, 2002; Ishizuka *et al.*, 2002). Therefore, members of the FXR family appear to be part of the functional miRNP complexes, and it is conceivable that they actually provide the translational silencing activity of the miRNP complex.

It is possible that various mRNAs interact with FMRP according to their sequence signals, for example, G-quartet structure binds to the RGG box of FMRP (Darnell *et al.*, 2001), while other mRNAs bind to different RNA binding domains of FMRP, or associate via *BC1* RNA. Possibly, the individual binding mode may also determine whether the FMRP-mRNA complex participates in nuclear processes, nucleo-cytoplasmic shuttling, dendritic/axonal mRNA transport, or translational control (see also above).

It becomes clear that, thanks to different experimental approaches, we now have a wide collection of candidate targets for the Fragile X protein. This will be the starting point for developing functional studies and pharmacological treatments.

FMRP as a new regulator of translation

Based on its association to polysomes and mRNPs, it has been hypothesised that FMRP has a role in translational regulation of gene expression in neurons and probably in testis. Two different groups have indeed shown that FMRP functions *in vitro* as an unspecific repressor of translation (Li *et al.*, 2001; Laggerbauer *et al.*, 2001) and lately the same result has been extended to cell culture using co-transfection experiments (Mazroui *et al.*, 2002). To define how translation is inhibited, it is extremely important to define whether FMRP co-associates on a polysomal gradient with the smaller complexes including mRNPs, ribosomes, and ribosomal subunits (ribonucleoparticle or mRNP fraction), or with the larger complexes containing predominantly actively translating ribosomes (polysome fraction). The first would indicate a role as repressor in translational initiation, the latter in translational elongation and/or termination.

Since 1996, a long debate has arisen concerning the FMRP distribution/co-localization with the ribosomes/polysomes. At the beginning, Kandjian and colleagues (Kandjian *et al.*, 1996) described, using non-denaturing detergents in extract preparation (0.5% NP-40), the association of FMRP to actively translating polyribosomes. In the same year, Dreyfuss and colleagues (Siomi *et al.*, 1996) published that FMRP is associated to ribonucleoparticles, in particular to the 40S-60S and 80S fractions. These last results were obtained using a different protocol that did not include detergent. Since then, several laboratories have tried to detect the association of FMRP with the polysomes-mRNPs. Interestingly, Warren and colleagues showed that FMRP is equally distributed between polysomes and mRNPs using conditions similar to the ones used by Kandjian and colleagues (0.3% NP-40; Feng *et al.*, 1997b; Brown *et al.*, 2001). Finally, Siomi and collaborators in *Drosophila* have shown that dFXR is associated to the 80S and ribonucleoproteins when the cytoplasmic extracts are treated with a similar detergent (0.5% Triton; Ishizuka *et al.*, 2002). Given the above background, we wished to characterize the distribution of FMRP on a polysomal gradient using a new specific antibody for FMRP against the C-terminus of the protein. Using different chemical conditions (in the presence or absence of detergents like triton 0.2-0.5%), our data clearly point out that FMRP is part of the ribonucleoprotein fraction, in agreement with its role as repressor of translational initiation (Zalfa *et al.*, 2003, and unpublished observations). Indeed, using rabbit reticulocyte extracts it was shown that FMRP inhibits the assembly of 80S ribosomes on the target RNAs (Laggenbauer *et al.*, 2001).

Thus, it is still disputed how FMRP inhibits translation. The picture of how FMRP selects its target mRNAs, in contrast, starts to become clearer.

FMRP involvement in synaptic plasticity

Spine dysmorphogenesis: cause or effects?

Dysmorphogenesis of the spines associated with mental retardation was initially described by Purpura (Purpura,

1974). The common characteristics found within the Fragile X patients by Rudelli and Greenough's labs are longer and thinner spines compared to control individuals (Rudelli *et al.*, 1985; Hinton *et al.*, 1991; Irwin *et al.*, 2001). Considering that this investigation has been performed on post-mortem patients, it becomes clear that the number of individuals analysed is limited. A total of 6 human brains have been so far studied and the detailed familiar histories are not known for each patient implying that other brain pathologies can not be excluded. Nevertheless the three independent studies showed long and thin prominent spine heads resembling immature spine-like structures called filipodia (Rudelli *et al.*, 1985).

Impaired spine morphology in the barrel region of somatosensory cortex as well as in hippocampal neurons has also been reported in mice (Comery *et al.*, 1997; Braun and Segal, 2000; Nimchinsky *et al.*, 2001). While in the animal model this phenotype/morphology seems to be transitory, in human this morphology persists as it is described in the post-mortem patients (Greenough *et al.*, 2001).

In the mouse model, FMRP has been shown to localize to spines (Feng *et al.*, 1997a) and to be regulated in an activity-dependent manner (its levels can increase after barrel cortex stimulation and rearing mice in complex environments) both in cortex and hippocampus, two structures important in learning and memory (Irwin *et al.*, 2000). It has been shown that *FMR1* mRNA is present in synaptoneuroosomes and the synaptic level of FMRP increases following administration of specific mGluR-specific agonists (Weiler *et al.*, 1997). We have shown that FMRP regulates local mRNA translation that is considered to be a regulation step involved in long-term plasticity. Activity-dependent potentiation of synaptic transmission is expressed through multiple parallel mechanisms and requires new protein synthesis. Localised synaptic protein synthesis is considered an important component of synaptic plasticity. In this way, neurons may translate new proteins exactly where they are needed to modify synapses in response to potentiating stimuli (for a review Steward and Schuman 2001). It has been shown that polyribosomes redistribute from dendritic shafts into spines with enlarged synapses during LTP (long-term depression) in developing rat hippocampal slides. Moreover, after stimulation the spines had enlarged synapses, which may serve to sustain the potential response (Ostroff *et al.*, 2002). This indicates that stimulation of synapses coincides with an enlargement of the structure, which serves at least in part to accommodate local protein synthesis machinery. Thus, local protein synthesis is an important part of synaptic plasticity.

Electrophysiological studies on the Fragile X mouse model

Synaptic plasticity, indeed the ability to change the synaptic structure is thought to be directly correlated to learning and memory, which are the major impairment in the Fragile X patients. There are two major in vitro models for the study of synaptic plasticity: the electrophysiological long-term potentiation (LTP) and the long-term depression (LTD). A major contribution to both effects comes from the

glutamergic receptor system. Its receptors fall into two general classes, the ionotropic and the metabotropic receptors. The ionotropic AMPA and NMDA receptors at glutamergic synapses are heteromeric complexes of homologous subunits (GluR1-4 for AMPA receptors and NR1, NR2A-D for NMDA receptors) that differentially combine to form various receptor subtypes (Hollmann and Heinemann, 1994). Metabotropic glutamate receptors (mGluR) are made of eight different members that can be subdivided into three groups on the basis of sequence homologies and their capacity to couple to specific enzyme systems. In the KO mouse for *FMR1*, the mGluR-dependent LTD was found to be increased in hippocampal slides (Huber *et al.*, 2002). This form of LTD depends on the postsynaptic protein synthesis and involves internalisation of AMPA and NMDA receptors (Synder *et al.*, 2001; Figure 3A). Bear and collaborators suggest that FMRP may negatively regulate mRNAs encoding for proteins involved in mGluR dependent LTD affecting hippocampal LTD (Huber *et al.*, 2002). According to this model, a link between FMRP, the glutamatergic system and synaptic plasticity could be the local increase of calcium from internal stores affecting at the end the lengthening of dendritic spines (Vanderklisch and Edelman, 2002; Figure 3A).

Interestingly, it has been shown that GluR1 is down-regulated in the cortex in the *FMR1* KO mice accompanied by a reduction in NMDA-AMPA mediated LTP, another process dependent on local protein synthesis (Li *et al.*, 2002) while no effect has been observed on hippocampal LTP (Godfraind *et al.*, 1996). Although the GluR1 subunit of the AMPA receptor does not appear to be directly controlled by FMRP, the mRNA encoding for the α -Ca⁺⁺/calmodulin-dependent protein kinase II it is regulated (Zalfa *et al.*, 2003). AMPA receptors have been shown to be phosphorylated by several protein kinases including PKC, cAMP-dependent protein kinase (PKA) and CaMKII (Tan *et al.*, 1994; Roche *et al.*, 1996). In 2002 Poncer *et al.* have shown that expression of a recombinant constitutively active form of α -CaMKII specifically potentiates AMPA- but not NMDA-mediated signal transmission in infected neurons, suggesting that the increased conductance seen in their system likely involves direct phosphorylation of GluR1 (Poncer *et al.*, 2002). Furthermore, investigations of activity-dependent development of neurons within several regions of the nervous system have demonstrated the involvement of the ionotropic glutamate receptors, both NMDA and AMPA (Inglis *et al.*, 2002; Song and Huganir, 2002; Sala *et al.*, 2003) in controlling dendrite morphogenesis.

Using the *FMR1* KO mouse model, we have shown that translational repression in vivo by FMRP is strongest in the dendritic/synaptic fraction, where it regulates in particular specific dendritic mRNAs, including those encoding key synaptic proteins like Arc/Arg3.1, MAP1B and α -CaMKII (Zalfa *et al.*, 2003) (Figure 3D).

Finally, another link between spine morphology and FMRP activity was recently reported above by Schenck *et al.* Initially, Mandel and colleagues (Schenck *et al.*, 2001) showed that one of the cytoplasmic interactors of FMRP, Cytoplasmic FMRP Interacting Protein 1 (CYFIP1) binds

also to Rac1, a member of the Ras superfamily of GTPases (Kobayashi *et al.*, 1998). The Ras family members regulate actin cytoskeleton and are implicated in dendritic modelling (Nakayama *et al.*, 2000, Figure 3). We have shown, in collaboration with Mandel's group, that CYFIP1 is also present at synapses (Schenck *et al.*, 2001). The role of dFXR/FMRP and Rac1 in cytoskeleton modelling of the synapses is conclusively demonstrated by elegant *Drosophila* studies by Schenck *et al.* (2003). They clearly show that *dCYFIP* mutant neurons exhibit impaired axonal pathfinding and growth and defects in motor axon terminals. Finally, they show that biochemical interactions between dCYFIP and dfxr or dRac1 are mutually exclusive and they establish that these molecules act in a common genetic pathway controlling the neuronal and synaptic morphology (Figure 3B).

Undiscovered roles of FMRP

Although FMRP by immunocytochemistry is localized in the cytoplasm, both a functional nuclear localization signal (NLS) and a nuclear export signal (NES) have been identified within FMRP, suggesting that FMRP might shuttle between the nucleus and cytoplasm (Eberhart *et al.*, 1996). A small amount of FMRP has been revealed in the nucleus by both light and electron microscopy (Verheij *et al.*, 1993; Eberhart *et al.*, 1996; Feng *et al.*, 1997a). Cells treated with Leptomycin B, which blocks mRNA export, partially retains FMRP and the FXRs in the nucleoplasm indicating that the nuclear export of these proteins is dependent on mRNA export. An even more striking nuclear retention is observed for the mutated FMRP containing the Ile304Asn amino acid substitution in the second KH domain (Tamanini *et al.*, 1999). Even if a domain similar to an NLS has been identified in exon 14, overexpressed FMRP lacking exon 14 can indeed enter the nucleus (Fridell *et al.*, 1996; Bardoni *et al.*, 1997). Interestingly, this exon is alternatively spliced, and certain FMRP isoforms are therefore excluded from the nucleus (Sittler *et al.*, 1996). FMRP appears to serve a role in the nucleus that is independent from its role at the synapses, since it associates with a distinct set of proteins in the nucleus, including nucleolin, YB1, and NUFIP (Ceman *et al.*, 1999; 2000, Bardoni *et al.*, 2002). Interestingly, a core particle containing FMRP, FXR1P and FXR2P is both present in the nucleus and cytoplasm. The nuclear function, however, still awaits further studies.

So far, work on FMRP has greatly focused and advanced on the mental retardation aspect. Patients with the Fragile X Syndrome, however, exhibit a series of physical characteristics that are very heterogeneous. They include macroorchidism (Turner *et al.*, 1980), long face and large ears (Opitz *et al.*, 1984; Hagerman *et al.*, 1984a, 1984b). Of these, the macroorchidism is particularly striking and consistently observed. Furthermore, Fragile X patients often exhibit connective tissue disorders, including velvet-like skin, finger-joint hyperextensibility, recurrent otitis media, aortic root dilatation, and mitral valve prolapse (Loehr *et al.*, 1986, Hagerman and Cronister, 1996). Finally, there are behavioural abnormalities, frequently including autistic features ranging from shyness, poor eye contact, and social anxiety in less affected individuals to

hyperactivity, hand flapping, hand biting, and perseverative speech in the severely affected (Merenstein *et al.*, 1996), as well as seizures and EEG findings consistent with epilepsy (Musumeci *et al.*, 1999, Sabaratnam *et al.*, 2001). How and why lack of FMRP causes all these phenotypes is completely unknown. An intriguing idea is that modifier genes, common to other pathologies (heart or connective pathologies) could also be playing a role in the Fragile X Syndrome.

In this context, it is also interesting to note that until few years ago the premutation carriers were considered not affected, just recently, it has been reported that premutation carriers exhibit a clinical phenotype with particular and unique features: premature ovarian failure (POF) in females (16-24% of total females with premutation alleles; Allingham-Hawkins *et al.*, 1999) and a new neurological syndrome, recently identified in some males with premutations older than 50 years, called Fragile X Tremor/Ataxia Syndrome (FXTAS), involving progressive action tremor with ataxia, cognitive decline and global reduction of brain volume (Hagerman *et al.*, 2001). Both POF and FXTAS are absent in Fragile X patients, therefore these phenotypes are considered unique to the premutation (Allingham-Hawkins *et al.*, 1999) (Figure 1).

Some patients with FXTAS show also eosinophilic and ubiquitin-positive intranuclear inclusions in both neuronal and astrocytic nuclei of the cortex, cerebellum, brainstem and hippocampal formation (Greco *et al.*, 2002). The origin of this inclusion is unknown, although elevated *FMR1* mRNA levels in these premutation carriers may lead to the neuropathological changes (Greco *et al.*, 2002). Interestingly, engineered mice with premutation alleles also show elevated *FMR1* mRNA levels and ubiquitin-positive intranuclear inclusions in neuronal and glial cells (Willemsen *et al.*, 2003).

Future perspectives

It is becoming clear that FMRP is involved in synaptic plasticity through at least two different ways, first the regulation of mRNA transport and translation, second the response to glutamate receptor mediated signalling. FMRP contains four different RNA binding domains that could in part cooperate and/or select a subpopulation of RNA according to the cellular need (nuclear function, transport from the nucleus, delivery to the synapses and translational regulation in the cell body as well as at synapses). While mRNA transport and translation seems to be not only limited to dendrites (Giuditta *et al.*, 2002) from recent studies in *Drosophila* the function of FMRP in axons becomes more intriguing.

On one side, the future goal is now to understand how the different mechanisms reported as a scheme in Figure 3 can be linked one to the other to allow FMRP function in the cell. Deciphering how FMRP works will help to unravel the molecular and cellular bases of many neurological disorders as well as to gain insight into basic brain functioning.

On the other side, it is important to find a therapeutical strategy for the syndrome, since transcriptional silencing due to hypermethylation is responsible for the FMRP defect,

it is conceivable that FMR1 promoter can be reactivated. Neri and collaborators have shown promising preliminary results in this direction (Chiurazzi *et al.*, 1998).

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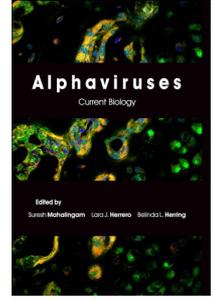
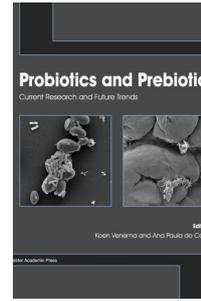
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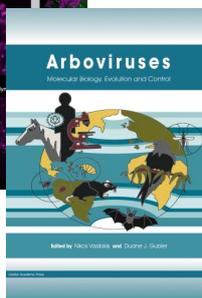
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