Localization of FMRP-associated mRNA granules and requirement of microtubules for activity-dependent trafficking in hippocampal neurons

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Fragile X syndrome is caused by the absence of the fragile X mental-retardation protein (FMRP), an mRNA-binding protein, which may play important roles in the regulation of dendritic mRNA localization and/or synaptic protein synthesis. We have recently applied high-resolution fluorescence imaging methods to document the presence, motility and activity-dependent regulation of FMRP granule trafficking in dendrites and spines of cultured hippocampal neurons. In this study, we show that FMRP granules distribute to F-actin-rich compartments, including filopodia, spines and growth cones during the staged development of hippocampal neurons in culture. Fragile X mental-retardation protein granules were shown to colocalize with ribosomes, ribosomal RNA and MAP1B mRNA, a known FMRP target, which encodes a protein important for microtubule and actin stabilization. The levels of FMRP within dendrites were reduced by disruption of microtubule dynamics, but not by disruption of F-actin. Direct measurements of FMRP transport kinetics using fluorescence recovery after photobleaching in living neurons showed that microtubules were required to induce the mGluR-dependent translocation into dendrites. This study provides further characterization of the composition and regulated trafficking of FMRP granules in dendrites of hippocampal neurons.

Keywords: Dendritic mRNA localization, fragile X mental-retardation protein, fragile X syndrome, metabotropic glutamate receptor, microtubule, microtubule-associated protein 1B

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The fragile X mental-retardation protein (FMRP) is an mRNA-binding protein with diverse functions in varied regions of the neuron (Antar & Bassell 2003). Absence of the FMRP causes fragile X syndrome (FXS), the most common heritable form of mental retardation. With four RNA-binding domains, a nuclear localization sequence and a nuclear export sequence (Bardoni et al. 1997; Eberhart et al. 1996), FMRP may play a role in the nucleocytoplasmic shuttling of mRNA (Feng et al. 1997b), dendritic mRNA localization (Miyashiro et al. 2003) or synaptic protein synthesis (Weiler et al. 1997; Zalfa et al. 2003). Fragile X mental retardation protein is also tightly associated with polyribosomes in neurons (Khandjian et al. 2004; Stefani et al. 2004). Many studies on FMRP use synaptosomal preparations (Cornell et al. 1997). While these studies have provided critical insight into understanding FMRP synthesis, these techniques do not precisely reveal the spatial compartmentalization of FMRP and dynamic aspects of its regulated trafficking to various subcellular compartments. In addition, the use of sectioned material from brain has been important for documenting the dendritic localization of FMRP (Feng et al. 1997a); yet these immunocytochemical approaches are limited in their spatial resolution.

We have previously used cultured hippocampal neurons as a model system to study aspects of FMRP localization, with high spatial resolution, and defined physiological signals involved in regulated trafficking (Antar et al. 2004). Fragile X mental-retardation protein was observed in granules within dendrites and spines. Depolarization of neurons using KCl resulted in the rapid localization of FMRP to dendrites. The increased levels of FMRP in dendrites, in response to depolarization, were not dependent on protein synthesis. The rapid, activity-dependent trafficking of FMRP, fused to enhanced green fluorescent protein (EGFP), was also observed in live neurons. The KCl-induced trafficking was blocked by antagonists of metabotropic glutamate receptors, including MPEP, which is an antagonist of mGluR5. Previous work has shown that a particular type of LTD is enhanced in hippocampal slices from FMR1-knockout mice (Huber et al. 2002). As this type of LTD requires mGluR5 activation and protein synthesis (Huber et al. 2001), these studies taken together suggest that FMRP trafficking may be involved in mediating a type of synaptic plasticity that is altered in FXS. Further work is needed to elucidate the mechanism of regulated FMRP transport and to identify the associated mRNAs that are cotransported, as this would provide important insight into the role of FMRP in protein synthesis-dependent plasticity.
mRNAs are frequently transported in granules, self-contained functional packets, that carry most, but not all, of the molecular machinery necessary for localization and local synthesis (Krichevsky & Kosik 2001). This may include mRNA-binding proteins, bound mRNAs, ribosomes, adapter proteins, molecular motors and other machinery that the granule will utilize at a distal target for local translation (Willemsen et al. 2004). A recent study has shown that RNA granules, which contain FMRP and many other mRNA-binding proteins, can be coprecipitated with conventional kinesin heavy chain in the form of a large complex (Kanai et al. 2004). However, this study did not identify any direct molecular interactions between mRNA-binding proteins contained in the granules and kinesin. Previous work has shown that EGFP–FMRP was localized in granules within neurites of PC12 cells (De Diego Otero et al. 2002). Motile granules displayed both oscillatory and unidirectional transport, the latter of which moved at average rates of 0.2 μm/second which were microtubule dependent.

In this study, we use quantitative and high-resolution fluorescence methods in fixed and live cultured hippocampal neurons to further characterize the composition of the FMRP granule and the role of microtubules in mGluR-dependent trafficking in dendrites. We show that FMRP granules colocalized with MAP1B mRNA and ribosomal RNA (rRNA) in dendrites, suggesting a large complex that may need to be transported for local translation at synapses. Microtubules were shown to be required for the mGluR-dependent transport of EGFP–FMRP in live neurons.

Materials and methods

Hippocampal culture and drug treatments
Rat hippocampi were dissected and cultured on embryonic day 18 (E18) as described (Goslin & Banker 1998). Coverslips were coated with poly-l-lysine (1.0 mg/ml) and then plated (60,000–90,000 cells/cm² for immunofluorescence (IF) and 350,000 cells/cm² for live cell imaging) in minimal essential medium (MEM) with fetal bovine serum (10%) for 2 h. Coverslips were then inverted onto dishes growing astroglia and cultured in defined Neurobasal (NB) medium (Gibco, Grand Island, NY, USA) supplemented with glutamax and B-27 growth supplement (Gibco). For cytoskeletal disruption studies, cells were treated 1 h. prior to fixation with either colchicine (10 μg/ml) or cytochalasin-D (5 μg/ml). Cells were then fixed with 4% paraformaldehyde in 1x phosphate-buffered saline (PBS) at room temperature for 18 min and then washed and stored in PBS–MgCl₂.

Immunofluorescence
Immunofluorescence was performed as described (Tiruchinapalli et al. 2003). Primary antibodies were incubated for 1 h, room temperature. Fragile X mental-retardation protein was detected with a mouse monoclonal antibody (1C3; 1:1000, Chemicon, Temecula, CA, USA) and synapsin with a rabbit polyclonal antibody (Sigma, St Louis, MO; 1:500). Secondary antibody incubations (1 h, room temperature), included Cy3 and Cy5 fluorochrome-conjugated antimouse and rabbit antibodies (1:1500, 1:500, respectively; Jackson ImmunoResearch, West Grove, PA, USA). Alexa 488-conjugated phalloidin was used to stain F-actin (1:500; Molecular Probes, Eugene, OR, USA).

Fluorescence in situ hybridization (FISH) with digoxigenin probes
Amino-modified oligonucleotide probes were synthesized to the coding region of mouse MAP1B mRNA and 18S rRNA and labeled with digoxigenin succinamide ester as described previously (Bassell et al. 1998). Fixed cells were subjected to FISH with the digoxigenin-labeled probes, blocked with bovine serum albumin and subjected to immunofluorescence with antidigoxigenin antibodies (Bassell et al. 1998). For Cy3 visualization, a Cy3-conjugated mouse antidigoxigenin antibody (1:1500) was followed by a Cy3-conjugated antimouse IgG antibody (Jackson ImmunoResearch). For Cy2 detection, blocking was done in normal donkey serum. Dig-labeled probes were detected using sheep antidigoxigenin antibody (1:10; Roche Molecular Biochemicals, Indianapolis, IN) and a Cy2-conjugated antisheep antibody (1:500; Roche Molecular Biochemicals).

Data analysis, digital imaging and image reconstruction
Fluorescence images were detected using a Nikon Eclipse inverted microscope equipped with a 60x Plan-Neofluar objective, 100 W mercury arc lamp and HiQ bandpass filters (Chroma Tech, Brattleboro, VT). Images were captured with a cooled CCD camera (Quantix, Photometrics, Tucson, AZ) running IP Laboratory software (Scanalytics, Fairfax, VA). Z-stacks were acquired (11 sections at 0.2 μm each) and deconvolved using Power Microtome (Vaytek, Fairfield, IA). Volume rendering and three-dimensional reconstruction was executed with Imaris software (Bitplane, Zurich, Switzerland).

To determine mean IF intensity for FMRP, a defined region of interest (ROI) was traced along a dendrite, selected at least 10 μm from the cell body. To normalize for differences in dendritic area, total IF intensity of the ROI was divided by the area of the ROI. Each experiment was analysed using a paired Student’s t-test.

EGFP–FMRP transfection
pEGFP–FMRP was generated by inserting polymerase chain reaction products of the open-reading frame of murine FMRP into the SacI and EcoRI sites of the pEGFP-C1 vector containing a CMV promoter (BD Biosciences, San Diego, CA, USA). Cultured neurons were transfected using CaPO₄ as described (Kohrmann et al. 1999a) and EGFP–FMRP was expressed overnight.

Electron microscopy
Neurons were washed briefly in MEM and then fixed for 15 min (4% paraformaldehyde and 0.1% glutaraldehyde in
0.1 M sodium cacodylate). Coverslips were incubated with primary antibody diluted 1:1000 in blocking buffer (TBS, 2% BSA) for 15 h at 4 °C and washed several times in buffer. Gold-conjugated (1 nm) secondary antibodies (Amersham Biosciences, Arlington Heights, IL, USA) were applied for 3.5 h incubation and washed several times. Cells were postfixed (2.5% glutaraldehyde) and stained with 1% osmium tetroxide and 1.5% potassium ferricyanide in buffer for 20 min. Ultrasmall gold particles were enhanced (GoldenHance-EM; Nanoprobes, Yaphank, NY) for 10 seconds. Cells were stained with 1% aqueous uranyl acetate (pH 4.2) for 20 min and dehydrated completely. Samples were embedded and sectioned by standard procedures. Sections were stained with 4% uranyl acetate and 0.2% lead citrate (3 min, room temperature). A Jeol 1200 EX TEM was used at 60 kV to image the immunogold labeling.

**Fluorescence recovery after photobleaching (FRAP)**

Rat hippocampal neurons (E18) were cultured on 40 mm coverslips (Bioptechs, Butler, PA, USA) and transfected with EGFP–FMRP. Transfected cells were imaged within 12 h of transfection in MEM medium (Gibco) containing 20 mM Na-HEPES and B27 supplements. Images were captured on an Olympus Fluoview 500 confocal microscope using an argon blue (488 nm) laser. Capture and analysis was previously described (Zhang et al. 2001b) with these modifications: each FRAP time point (1–5 min) reflects the timed average fluorescence intensity of three sequential frames (10 seconds apart) to minimize noise. Briefly, 9–10 DIV-transfected neurons were identified by GFP fluorescence and a dendritic segment containing GFP-FMRP was subjected to FRAP to establish a baseline recovery rate. After a 5-min measured recovery, media containing DHPG (20 μM) was transfused into the chamber. After 15 min of exposure to DHPG, the same cell was again measured using FRAP. After a 5-min measured recovery, media containing both DHPG and nocodazole (10 μg/ml) was transfused into the chamber. After 30-min exposure to nocodazole, the same cell was again measured using FRAP. To determine the 50% recovery rates (at which 50% of the signal is recovered), a best-fit logarithmic line plot for the data of each cell was drawn using Excel (Microsoft, Redmond, WA) and the corresponding regression analysis equation was used to determine the 50% recovery by setting $y = 0.5$.

**Results**

**FMRP localizes to sites of F-actin dynamics during development of hippocampal neurons in culture**

We have previously used IF to describe the localization of FMRP to dendritic spines of hippocampal neurons, which were cultured for over 2 weeks. In this study, we have also examined FMRP distribution at earlier stages in culture. At 3–5 days in vitro, early neurites and the axonal growth cone

![Figure 1: Localization of fragile X mental-retardation protein (FMRP) to actin-rich structures during development of hippocampal neurons in culture.](a) Double-label immunofluorescence, deconvolved images detect FMRP (red) and F-actin (green) at 3 DIV. F-actin (green) fills the incipient axon and minor neurites and is concentrated in their terminal growth cones (arrows). Fragile X mental-retardation protein (red) was enriched at the leading edge of growth cones (arrows) and filopodia. (b) 10 DIV hippocampal neurons show FMRP in long, thin filopodia emerging from the dendritic shaft. (c) At 25 DIV, most filopodia have been replaced by dendritic spines, rounded protrusions with bulbous, actin-rich heads. Fragile X mental-retardation protein is highly abundant in dendritic spines. Scale bar is 5 μm.
extend from the soma (Fig. 1a). The beginning of synapse formation occurs between 5 and 10 days, and intense filopodial outgrowth (Fig. 1b) accompanies this process. In this culture system, 10–12 days begins dendritic spine formation, which then peaks at 14–25 days (Fig. 1c). From 3 DIV on, FMRP granules localized into processes, concentrated in the distal tips of both axonal and neuritic growth cones (Fig. 1a), the sites of most active morphologic change at this stage. At 5 DIV, FMRP localized to about 25% of dendritic filopodia. This same level of filopodial distribution continued throughout 10–21 DIV (Fig. 1b). Fragile X mental-retardation protein was also present in most dendritic spines (Antar et al. 2004; Feng et al. 1997b) beginning at 12 DIV, and remaining in these structures through 25 DIV (Fig. 1c). Again, FMRP was present in highly motile, actin-rich structures. Interestingly, total FMRP levels in dendritic shafts do not fluctuate during the critical period of development, remaining constant from 8 to 19 DIV, after normalization for dendritic area (Antar et al. 2004).

**FMRP colocalizes with the machinery of translation**

Combining IF analysis with in situ hybridization, FMRP dendritically colocalized with rRNA, and clusters of colocalization occurred especially at branch points (Fig. 2a, inset). To further evaluate colocalization, a Z-series was acquired, deconvolved to remove out-of-focus light and subjected to volume rendering in a three-dimensional analysis (Imaris, Fig. 2b). More than 50% of the FMRP signal (red) colocalized with rRNA (green; colocalization in yellow). Analysis by electron microscopy also showed FMRP colocalized with polyribosomes (Fig. 2c, arrows) along microtubules (arrowheads) even within a very narrow region of dendrite.

**FMRP localizes in dendrites and at synapses with MAP1B mRNA, a regulator of cytoskeletal morphology**

A few studies have indicated that one of the FMRP targets is microtubule-associated protein 1B (MAP1B) mRNA (Brown et al. 2001; Darnell et al. 2001; Zhang et al. 2001a). Altered translational repression of MAP1B mRNA in FMR1-knockout mice leads to hyperstabilized microtubules, which may compromise aspects of neuronal development and synaptogenesis (Lu et al. 2004). It is unknown whether MAP1B mRNA is localized to neuronal processes and associated with FMRP in granules. To examine the spatial distribution of FMRP and MAP1B mRNA, oligonucleotide probes to MAP1B mRNA were designed, and in situ hybridization combined with double-label immunofluorescence was performed. MAP1B mRNA [Fig. 3(a), green] was dendritically localized in granules and exhibited abundant colocalization with FMRP (red, inset). A Z-series followed by deconvolution and volume rendering of the boxed dendrite in Fig. 3(a) depicts FMRP and MAP1B mRNA that colocalized in granules (yellow) at synapses (synapsin staining, blue) [Fig. 3(b), insets a, b]. MAP1B mRNA and FMRP are shown in a granule abutting synapsin,

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**Figure 2: Fragile X mental-retardation protein (FMRP) localizes in distal dendrites with rRNA.** (a) In situ hybridization combined with immunofluorescence shows FMRP (red) colocalization with rRNA [(green), arrows, inset]. Inset shows FMRP and rRNA clusters at branch points. (b) Three-dimensional reconstruction of top dendrite, from second branch point (a) after Z-series and deconvolution. Yellow granules show colocalization of FMRP and rRNA. Blue shadow represents cell outline from phase image. (c) Immuno-EM staining of 4 DIV cortical neuron depicts FMRP localization on microtubules (arrowheads, inset) and FMRP colocalization with polyribosomal aggregates (arrows, inset).

the presynaptic marker [Fig. 3(b), inset a]. Four such synaptic interactions with the colocalized mRNA–protein complex are depicted (inset b). Abundant dendritic proteins are not necessarily colocalized. For example, the highly abundant and dendritically localized mRNA-binding protein Staufen did not colocalize with FMRP [Fig. 3(c)].
Microtubules are required for FMRP localization and mGluR-dependent transport in live neurons

Fragile X mental-retardation protein localization in dendrites of hippocampal neurons in response to mGluR activation and KCl depolarization was shown to be rapid and dynamic (Antar et al. 2004), suggesting a role of cytoskeletal based motors. To ascertain whether microtubules were necessary for FMRP localization in dendrites, cytoskeletal disrupting drugs were bath applied to mature cultures, immunostained and analysed for alterations in FMRP localization (Fig. 4a–j). Phallolidin staining (Fig.4a–c) showed that the actin cytoskeleton was unaffected by colchicine (Fig.4b), but actin filaments became disrupted upon 1 h incubation with cytochalasin-D (Fig.4c). Fragile X mental-retardation protein localization, however, was not significantly blocked by the actin-disrupting agent, as reflected by an insignificant change in mean fluorescence intensity as determined by a two-tailed Student’s t-test \( P = 0.42 \) (no drug: 928.00 ± 151.32 versus cytochalasin-D: 752.50 ± 52.70; Fig. 4f,j). Fragile X mental-retardation protein localization in dendrites was significantly reduced after perturbation of microtubules with colchicine (Fig. 4e,j) (no drug: 928.00 ± 151.32 versus colchicine: 430.11 ± 52.70). Normal FMRP distribution and FMRP distribution after treatment with the actin and microtubule-disrupting drugs in an F-actin-FMRP merge are depicted in Fig. 4(g–i).

Stimulation-induced transport kinetics of EGFP–FMRP are altered upon microtubule disruption as determined by FRAP

While many studies have suggested a requirement for microtubules in the active transport of mRNPs into dendrites, few studies, if any, have measured this requirement directly in living cells. To directly investigate the requirement of microtubules for the transport of FMRP mRNPs, hippocampal neurons (10 DIV) were transfected with EGFP–FMRP and transport kinetics were measured using fluorescent recovery after photobleaching (FRAP). Dendrites of cells expressing the fusion protein were identified and subjected to the following paradigm: first the basal recovery rate was measured over a 5-min time interval; then the type I mGluR agonist, DHPG, was added, a known inducer of FMRP transport (Antar et al. 2004) and FRAP was measured after 15 min; finally, the cell was exposed to the microtubule-depolymerizing agent nocodazole (with DHPG present) for 30 min and FRAP was again measured. Since it was previously found that FMRP abundance in dendrites of neurons exposed to depolarizing stimulation was the highest after 45 min, this paradigm would be predicted to show a peak of FMRP FRAP rates at the 30 min postnocodazole time point (45 min DHPG overall) unless microtubules were required for the transport of FMRP into the bleached zone.

The measured FRAP rates of three different cells subjected to the above paradigm were quantitatively graphed (Fig. 5g–i) and a representative series of images showing the recovery of
one of those cells was generated (Fig. 5a–f). The relative intensities of FMRP fluorescence were displayed using a color-coded intensity mapping profile, and the look-up bar is shown in the bottom right corner of image (Fig. 5c). The upper panel of images shows FRAP in the presence of DHPG, where FMRP recovery appears quite robust after 5 min (Fig. 5c) in comparison to the postbleach image (Fig. 5b). This is in contrast to the recovery observed after 30 min in nocodazole (Fig. 5f), which looks negligible compared to the corresponding postbleach image (Fig. 5e). The yellow arrows point to areas that showed abundant FMRP signal before FRAP, highlighting the differences in recovery observed in DHPG and after 30 min in nocodazole. Since fluorescence recovery requires active transport of protein into

**Figure 4: Fragile X mental-retardation protein (FMRP) traffics on microtubules and its localization is disrupted by microtubule-disrupting drugs.** (a) Fragile X mental-retardation protein localization is blocked by depolymerization of microtubules, but not of actin filaments. Panels a–c show phalloidin staining of F-actin. Note normal actin morphology in panels a, b and disrupted actin structure (panel c) caused by bath application of actin depolymerizing agent, cytochalasin-D. Panels d–f: FMRP localizes into distal dendrites; however, this localization is disrupted (e) by bath application of microtubule-disrupting drug, colchicine. Panels g,h: Merge of FMRP and actin from above channels reveals FMRP distribution with respect to the actin cytoskeleton. (j) Quantification shows that FMRP localization in dendrites is dependent on microtubules, but not the actin cytoskeleton. $P = 0.002$. Micron bar is 5 μM.
bleached zones, the recovery rate is directly correlated to FMRP transport kinetics. Panels g–i show a graphical representation of measured recovery rates of three cells, and while the exact rates of recovery for each cell differed slightly, the overall trend between treatments was consistent: 15 min of DHPG treatment (red squares) significantly enhanced the rate of FMRP transport over the unstimulated basal rate (blue diamonds), while nocodazole caused a significant impairment of FMRP transport. The recovery rates were extrapolated from the graphs of each cell for each treatment, and the combined averages were calculated and compared to the rates for the basal state (‘no addition’) in panel j (Fig. 5). On average, DHPG increased the transport rate of FMRP by 7.8-fold, while nocodazole caused an average decrease of 60-fold compared to unstimulated rates. Taken together, these data clearly demonstrate that microtubules are required for the active transport of FMRP into dendrites of hippocampal neurons.

Discussion

Subcellular localization and developmental functions for FMRP

We have used high-resolution fluorescence imaging methods to visualize the subcellular localization of FMRP in cultured hippocampal neurons. This information provides important clues about the possible function of FMRP during neuronal development. Our previous study has localized FMRP granules to synapses and dendritic spines of 21 DIV-cultured hippocampal neurons (Antar et al. 2004). A quantitative morphometric analysis indicated that 56% of synapses colocalized with FMRP, whereas 76% of spines colocalized...
with FMRP. These data are consistent with original immuno-EM studies that first documented the presence of FMRP postsynaptically (Feng et al. 1997b). Our quantitative analysis of a strong prevalence of FMRP in spines is consistent with its tight association with polyribosomes (Feng et al. 1997b; Stefani et al. 2004) and the purported role of FMRP in protein synthesis-dependent synaptic plasticity (Huber et al. 2002; Bear et al. 2004).

Fragile X mental-retardation protein may have important developmental functions and the observation of impaired synaptic plasticity may be due, in part, to defects in the formation of synapses. At earlier stages of development in cultured neurons, FMRP was detected in neuritic growth cones (3 DIV) and then later in dendritic filopodia (10 DIV). Together, these findings suggest a role for FMRP in regulation of dynamic F-actin-rich structures that are involved in neurite growth and cell–cell interactions that underlie synaptogenesis. In that dendritic filopodia are believed (by some) to be spine precursors (Yuste & Bonhoeffer 2004), the presence of FMRP in these structures suggests its possible function in filopodial conversion and/or spine maturation. This idea is relevant in light of the findings of an excess or hyperabudance of long, thin immature spines in Golgi-stained brain sections from FMR1-knockout mice and human patients with FXS (Comery et al. 1997; Irwin et al. 2000; Rudelli et al. 1985). It will be important to perform further experiments in the mouse FMR1-knockout model to more precisely distinguish dendritic filopodia from innervated spines and assess alterations in their density. It will also be important to learn how such structural defects in filopodial and spine regulation lead to altered protein synthesis-dependent plasticity in FXS.

With regard to our observations of FMRP in growth cones, this finding is relevant in light of the recent literature on a role for local protein synthesis in growth cone motility and path finding (Martin 2004). It will be of interest if there are defects in filopodial regulation in growth cones, as there appears to be in dendrites. The idea of axonal defects, in the absence of FMRP granules and regulation of microtubule-dependent trafficking of FMRP in the regulation of neuronal morphology during development.

**FMRP granules and regulation of microtubule-dependent trafficking**

Here, we show that the localization of FMRP granules in dendrites of cultured hippocampal neurons is dependent on microtubules, similar to that previously described for PC12 cells (De Diego Otero et al. 2002). In addition, we have shown EM images that further suggest an interaction between FMRP and microtubules. In neurites of PC12 cells, EGFP–FMRP granules moved bidirectionally at an average rate of 0.2 μm/second (De Diego Otero et al. 2002). It will be interesting to see whether similar rates are observed in dendrites of hippocampal neurons. Dynamic, bidirectional and microtubule-dependent movements have also been described for other mRNA-binding proteins such as Staufen (Kohrmann et al. 1999b), zipcode-binding protein 1 (Zhang et al. 2001b) and cytoplasmic polyadenylation element-binding protein (CPEB) (Huang et al. 2003).

A role of FMRP in the dendritic localization of a few target mRNAs has been suggested by in situ hybridization studies on sections from FMR1-knockout brain (Miyashiro et al. 2003). Rapid movements, such as seen for FMRP, are consistent with kinesin- and dynein-directed movements of RNA granules (Kanai et al. 2004). mRNA-binding proteins, like FMRP, may function as adapter molecules, and link the mRNA to the motor, as has been shown for the CPEB facilitated transport of CaMKIIα mRNA (Huang et al. 2003). Recently, RNA granules containing FMRP and many other mRNA-binding proteins were isolated by association with kinesin heavy chain (Kanai et al. 2004). However, a direct interaction between any of the many RNA-binding proteins isolated and kinesin has yet to be established, and remains a problem to be resolved in the field. Future work is needed to elucidate the molecular interactions between FMRP and kinesin motors to test the hypothesis that it acts as an essential adapter for mRNA transport. An alternative possibility is that FMRP plays a role only in translational regulation, and some other mRNA-binding proteins are needed for mRNA transport. However, since the acts of mRNA transport and translational regulation are often coupled (Bassell & Kehic 2004), this remains an attractive model for FMRP function as well. Given the importance of local mRNA translation at the synapse, the role of FMRP in delivery of mRNAs and the consequence of its loss in FXS on the intracellular distribution and dendritic abundance of mRNAs remains an important area of investigation.

We have investigated whether the dynamic and the microtubule-dependent trafficking of FMRP could be regulated by glutamatergic signaling pathways that are involved in long-term synaptic plasticity. Our previous study showed that metabotropic glutamate receptor activation could rapidly stimulate FMRP localization to dendrites, through a mechanism that requires a rise in internal calcium and protein kinase C (Antar et al. 2004). The group I mGluR agonist, DHPG, rapidly induced FMRP. Here, we show using FRAP analysis of EGFP–FMRP that the DHPG-induced trafficking of FMRP in live neurons is dependent on microtubules. It will be interesting to learn how PKC-dependent phosphorylation events may regulate, for example, the interaction between FMRP and motors.

It will also be important to elucidate the significance of retrograde movements of FMRP, which may include dynein-dependent interactions. We have shown that synaptic stimulation, via either AMPA or mGluR activation, can trigger loss of FMRP staining from synapses (Antar et al. 2004). It will be interesting...
if FMRP acts as a signaling molecule in the retrograde direction, perhaps returning to the nucleus to pick up new mRNA cargoes destined for translation at the synapse.

**Molecular and spatial interactions between FMRP and MAP1B mRNA**

Fragile X mental-retardation protein has been shown to be tightly associated with actively translating polyribosomes using in vitro assays (Feng et al. 1997b; Khandjian et al. 2004; Stefani et al. 2004). Our electron microscopy reveals that FMRP localizes on dendritic microtubules near sites of polyribosomes. Fluorescence in situ hybridization coupled with FMRP immuno-fluorescence also shows that rRNA colocalized with FMRP in proximal, medial and distal dendrites, and especially at branch points, suggesting that FMRP is localized to distal sites with translational components, providing the means for local protein synthesis. These findings are consistent with the idea that FMRP may regulate translation at localized sites within the neuron.

It remains to be determined which FMRP-target mRNAs are regulated locally. Here, we show that MAP1B mRNA is dendritically localized and colocalizes with FMRP granules in dendrites and developing synapses of cultured hippocampal neurons. Previous work has shown that MAP1B mRNA is bound by FMRP in a G-quartet-dependent manner (Brown et al. 2001; Darnell et al. 2001). In Drosophila, dFMR represses the translation of Futsch mRNA, a MAP1B homolog (Zhang et al. 2001a). The absence of dFMR1 resulted in increased Futsch expression, which correlated with synaptic defects. In a recent paper, FMRP-dependent repression was increased Futsch expression, which correlated with synaptic skeletal organization during development. It will be interesting to explore FMRP-mediated translational regulation in controlling cytoskeletal dynamics.

In addition, it is possible that in the absence of FMRP, regulation of MAP1B mRNA translation is impaired, causing altered local regulation of cytoskeletal dynamics involved in synapse development and morphology. Our data localizing FMRP with MAP1B mRNA at the synapse provide a possible link between MAP1B mRNA localization and mGluR-mediated changes in protein synthesis and morphologic plasticity. It is relevant that MAP1B can also bind actin in addition to microtubules and affect their cytoskeletal interface in dendritic spines (Dehmelt & Halpain 2004). Future work on the mechanism and local regulation of FMRP interactions with MAP1B, and other target mRNAs, will provide new insight into the function of mRNA localization and local protein synthesis in neurons during development and the underlying defects in FXS.

**References**


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