The fragile X mental retardation protein is a molecular adaptor between the neurospecific KIF3C kinesin and dendritic RNA granules

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Fragile X mental retardation 1 protein (FMRP) is an RNA-binding protein whose absence results in the fragile X syndrome, the most common inherited form of mental retardation. FMRP contains multiple domains with apparently differential affinity to mRNA and interacts also with protein partners present in ribonucleoprotein complexes called RNA granules. In neurons, these particles travel along dendrites and axons to translocate mRNAs to specific destinations in spines and growth cones, where local synthesis of neuro-specific proteins is taking place. However, the molecular mechanisms of how RNA granules are translocated to dendrites remained unknown. We report here the identification and characterization of the motor protein KIF3C as a novel FMRP-interacting protein. In addition, using time-lapse videomicroscopy, we studied the dynamics and kinetics of FMRP-containing RNA granules in dendrites and show that a KIF3C dominant-negative impedes their distal transport. We therefore propose that, in addition to modulate the translation of its mRNA targets, FMRP acts also as a molecular adaptor between RNA granules and the neurospecific kinesin KIF3C that powers their transport along neuronal microtubules.

INTRODUCTION

The absence of the RNA-binding protein fragile X mental retardation 1 protein (FMRP) is responsible for the fragile X syndrome (FXS), the main hereditary cause of mental retardation (1). In brain, FMRP is present in cytoplasmic ribonucleoparticles (RNPs) associated with somatic and synaptic polyribosomes, strongly suggesting its involvement in the translational control of its mRNA target and particularly in synaptic localized translation (2–5). Once synapses are established, localized translation will control distinct forms of activity-dependent synaptic plasticity, associated with changes in dendritic spine morphology (6). In adult FXS patients and Fmr1 null mice, the loss of FMRP induces abnormal immature-looking dendritic spines (7,8). In addition, Fmr1 null mice display alterations of several forms of activity-dependent synaptic plasticity, cortical long-term potentiation (9), and cerebellar long-term depression (10), further substantiating the essential role played by FMRP in localized mRNA translation. FMRP-containing RNPs are also observed as RNA granules traveling in the dendritic branching (11,12). These RNA granules correspond to complex structures containing mRNA, ribosomes and different RNA-binding proteins (13–17) that achieve the transport, targeting and release of neurospecific mRNAs locally at the synapse. Interestingly, a decrease in the amount of RNA granules and of polyribosomes in dendritic spines were reported in Fmr1 null mice, pointing out a possible role for FMRP in the regulation of the formation of RNA granules (18,19).

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FMRP is one of the many components of RNP macromolecular complexes (reviewed in 20). Most of FMRP-interacting partners are RNA-binding proteins, such as its close homologues FXR1P and FXR2P (21). So far, a plethora of studies have focused on the RNA-binding properties of FMRP and several hundreds putative mRNA targets whose translation seem to be regulated by FMRP have been indexed. Among these, very few targets have been studied and validated in the neuronal context (reviewed in 22) and none has been definitely connected to the phenotype observed in patients. This suggests that the functions of FMRP in neurons are not restricted to its RNA-binding properties, a hypothesis based on the fact that FMRP interacts with cytoskeleton-linked proteins such as CYFIP1/2, Lgl and Ran-BPM (20,23), as well as with the motor proteins myosin Va, kinesin I heavy chain (KIF5) and dynein heavy chain (17,24,25). The functional significance of such interactions has been explored only in *Drosophila melanogaster* non-neural S2 cells (24), and evidence for a direct interaction between FMRP and motor proteins is lacking. In search for novel proteins that interact with FMRP in neurons, we identified the neurospecific kinesin KIF3C and we investigated the role of FMRP as a direct molecular adaptor between KIF3C and RNA granules.

**RESULTS**

**FMRP interacts with the neurospecific kinesin KIF3C in the yeast-two-hybrid system and in vitro**

In order to identify new proteins that interact with FMRP in neurons, we screened a human fetal brain cDNA library using a yeast-two-hybrid system, with the major FMRP isoform Iso7 as bait. From ~2.8 x 10⁶ clones screened, 78 positive clones displayed adenine and histidine prototrophy as well as β-galactosidase activity. PCR and restriction analysis of selected colonies showed that among these, one colony carried a 2.2 kb insert bearing a sequence 100% identical to the 3’ end of human neurospecific kinesin KIF3C cDNA (26) that is highly conserved in rodents (27). This insert corresponded to amino acids 403–792 of human KIF3C and contained also a stretch of the 3’-UTR of KIF3C cDNA. The interaction between KIF3C and FMRP was further confirmed by co-transforming the AH109 yeast strain with plasmids coding for the human KIF3C C-ter and human FMRP. Indeed, KIF3C C-ter was found to interact with FMRP, whereas no interaction was detected when plasmids bearing unrelated proteins were used (Table 1). These results showed that FMRP directly interact with KIF3C in the yeast-two-hybrid system.

Structurally, KIF3C is composed of three domains, which are common to all kinesin family members (Fig. 1A). Human (792 amino acids) and mouse (796 amino acids) KIF3C are evolutionary conserved since their amino acid sequences are 94.9% homologous. Therefore, in the present study, we used the available mouse KIF3C cDNA constructs. The kinesin motor region lies within the N-terminal 380 amino acids, while the central stalk domain contains an α-helix from residues 455 to 633, probably involved in homo- or heterodimerization (27). The C-terminal domain of KIF3C delineates a globular proline-rich tail domain (amino acids 634–796), which contains the motor domain that has been proposed to interact with microtubules but not with the cargoes. The ability of KIF3C to interact with FXR1P and FXR2P, the two homologues of FMRP, was also tested. The GST-KIF3C 71 C-ter or GST-KIF3C N-ter immobilized on glutathione-Sepharose beads were incubated with either in vitro translated FMRP, FXR1P, FXR2P or luciferase as a negative control. The three members of the FXR family were shown to bind to the immobilized GST-KIF3C 71 C-ter, whereas luciferase did not (Fig. 1B). No binding to GST-KIF3C N-ter, or GST alone, was observed, confirming the specificity of the interaction. Interestingly, no binding of FMRP to the 71 last amino acids of KIF3B, another member of the KIF3 family was observed (not shown). To verify that the interaction between FMRP and KIF3C was direct and not RNA-dependent, GST-pulldowns were also performed in the presence of RNases A/T1. This treatment did not alter the binding of FMRP to KIF3C, indicating that the interaction occurs at the protein-protein level. To assess the robustness of the interaction between FMRP and GST-KIF3C 71 C-ter, another series of GST-pulldowns was conducted in the presence of different salt concentrations (Fig. 1C). In the absence of salts, the amount of FMRP bound to GST-KIF3C C-ter was three times higher than in the presence of 150 mM NaCl. At 500 mM NaCl and above, only trace amounts of FMRP could be detected. These results suggest that KIF3C binds FMRP, FXR1P and FXR2P through its cargo docking domain corresponding to its last 71 amino acids and that this interaction is labile in high-salt conditions.

**KIF3C co-localizes with FMRP in the neuronal soma and is present in FMRP containing granules throughout the dendritic branching**

To validate in vivo the potential interaction between KIF3C and FMRP, we performed immunofluorescence double labeling on rat brain cerebellar sections using mAb1C3 against FMRP and the affinity-purified anti-KIF3C antibody. In agreement with Yang and Goldstein (27), strong anti-KIF3C staining was detected in the Purkinje cell layer of the cerebellum (Fig. 2). Staining was strong in the cytoplasm of the soma and the proximal dendrites, becoming weaker distally. On the other hand, staining for FMRP was predominant in the soma, and faint signals were detected in the dendritic branching, whereas it appeared below detection levels in distal dendrites, as previously reported (30,31). Analyses of merged
images reveal that KIF3C and FMRP are both present in the somatic compartment.

In order to assess the co-localization of KIF3C and FMRP in the dendritic branching of neurons, we double-stained primary cultures of rat hippocampal neurons with mAb1C3 and anti-KIF3C antibodies. Confocal microscopy analyses revealed strong staining of KIF3C in the soma, as well as in the dendritic arborization. As previously reported for KIF5 (32), most KIF3C freely diffuses in the cytoplasm in a cargo-unbound form. However, at higher magnification, a granular-like structure is observed in the dendritic compartment (Fig. 3A), and at higher magnification, KIF3C staining appears as granules in the dendritic arborization. Omission of GTP or incubation at 4°C in vivo together with FMRP and L7, suggests that a population of FMRP-containing RNPs remained attached to polymerized microtubules. Omission of GTP or incubation at 37°C polymerization of microtubules could have induced artefactual re-association of FMRP with microtubules, analyses were performed using different experimental conditions that prevent microtubule polymerization (Fig. 5). Using the standard microtubule buffer devoid of non-ionic detergent, considerable amounts of FMRP were recovered in the first pellet (P1) obtained at 12 000 g that contains a subset of FMRP-RNPs associated with polyribosomes attached to the ER (2). The 12 000 g supernatant was then subjected to a microtubule-polymerization step by incubation at 37°C, in the presence of GTP, and polymerized microtubules were obtained after centrifugation at 25 000 g. At this step, the presence of tubulin, together with FMRP and L7, suggests that a population of FMRP-containing RNPs remained attached to polymerized microtubules. Omission of GTP or incubation at 4°C, both regimes known to prevent microtubules-polymerization, yielded trace amounts of β-tubulin as well as FMRP and the ribosomal L7 marker in the P2 fraction, indicating that these preparations contain a selective subsets of proteins.

Since the experimental conditions used here to induce in vitro polymerization of microtubules could have induced artefactual re-association of FMRP with microtubules, analyses were performed using different experimental conditions that prevent microtubule polymerization (Fig. 5). Using the standard microtubule buffer devoid of non-ionic detergent, considerable amounts of FMRP were recovered in the first pellet (P1) obtained at 12 000 g that contains a subset of FMRP-RNPs associated with polyribosomes attached to the ER (2). The 12 000 g supernatant was then subjected to a microtubule-polymerization step by incubation at 37°C, in the presence of GTP, and polymerized microtubules were obtained after centrifugation at 25 000 g. At this step, the presence of tubulin, together with FMRP and L7, suggests that a population of FMRP-containing RNPs remained attached to polymerized microtubules. Omission of GTP or incubation at 4°C, both regimes known to prevent microtubules-polymerization, yielded trace amounts of β-tubulin as well as FMRP and the ribosomal L7 marker in the P2 fraction, indicating that these proteins are recovered only in the polymerized microtubule fraction. The 25 000 g supernatant was further centrifuged at 105 000 g to yield a P3 fractions that contain polyribosomes and RNA granules. As expected, this fraction contains the homologue FXR1P in mouse testis microtubule extracts (34).

Coomassie-blue staining of the proteins present in the final microtubule-enriched fraction (Fig. 4A.) and total homogenate (Fig. 4B) clearly show the enrichment in a 55 kDa protein, while a drastic reduction of total proteins was observed. This 55 kDa protein was identified as β-tubulin (Fig. 4A). Densitometric analyses of the Coomassie-stained proteins indicated a ~15-fold enrichment of β-tubulin, while immunoblot analyses to reveal the presence of FMRP, KIF3C and L7 did not show a clear increase of the proteins, indicating that only a fraction of these proteins is recovered with polymerized microtubules (see also below). The presence of the ribosomal protein L7 is indicative of the presence of ribosomes attached to the microtubules. On the other hand, synaptophysin, an integral membrane glycoprotein present in presynaptic vesicles in neurons, as well as the 78 kDa glucose-regulated/immunoglobulin heavy chain binding protein (GRP78/BiP), an endoplasmic reticulum (ER)-localized protein, were not detected in the microtubules-enriched pellet, suggesting that these preparations contain a selective subsets of proteins.

As a motor protein, KIF3C binds to microtubule structures in order to power the transport of its cargo. To validate the possibility that FMRP and KIF3C interact biochemically with microtubules, we searched for the presence of both proteins on such isolated structures. A fraction highly enriched in microtubules was obtained from mouse brain extracts through two cycles of temperature-dependant polymerization and depolymerization in the presence of GTP, using a standard procedure that has been applied to reveal the presence of FMRP and its homologue FXR1P in mouse testis microtubule extracts (34).

Table 1. FMRP, FXR1P and FXR2P interact with the C-terminal domain of kinesin KIF3C in Yeast

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<th>Partner B</th>
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AH109 yeast strain was co-transformed with the indicated plasmids. Tests for interactions were performed by plating the transformants on a medium depleted in adenine and histidine, to check for activation of the reporter genes ADE2 and HIS3. All transformants positive for interaction on medium depleted in adenine and histidine showed a blue color in the presence of X-Gal, confirming the Lac Z activation. Interaction could be detected between KIF3C C-ter and FMRP, FXR1P and FXR2P, respectively. Growth was comparable to strains transformed with FMRP, FXR1P, FXR2P as well as with p53 and the SV40 T-antigen known to interact with each other, whereas no growth was observed when pACT2-KIF3C C-ter was transformed together with the empty pGBK7 vector or with pGBK7-p53 coding for an unrelated protein.

FMRP and KIF3C co-fractionate in GTP-induced mouse brain microtubule preparations

As a motor protein, KIF3C binds to microtubule structures in order to power the transport of its cargo. To validate the possibility that FMRP and KIF3C interact biochemically with microtubules, we searched for the presence of both proteins on such isolated structures. A fraction highly enriched in microtubules was obtained from mouse brain extracts through two cycles of temperature-dependant polymerization and depolymerization in the presence of GTP, using a standard procedure that has been applied to reveal the presence of FMRP and its homologue FXR1P in mouse testis microtubule extracts (34).
great majority of FMRP (3,4), but low levels of tubulin. Analyses of the final supernatant (Sup) reveal that considerable amounts of soluble tubulin are recovered in the final supernatant in conditions that prevent microtubule-polymerization, while FMRP and L7 are not detected. These data indicate that, in the conditions used to polymerize microtubules, a subset of RNPs containing FMRP and L7 can be recovered associated with these in vitro polymerized structures. Comparing the amount of FMRP recovered in the P2 fraction (polymerized microtubules) with that of P3 containing polyribosomes, we estimate that in the experimental conditions used here, approximately 5–15% of total brain FMRP is recovered in structures associated with microtubules while the great majority remains associated with polyribosomes.

KIF3C co-immunoprecipitates with FMRP in mouse brain microtubule preparations

To further document the in vivo interaction between FMRP and KIF3C, we performed immunoprecipitation studies using the microtubule enriched fraction. As a negative control, we used the mAb1C3 directed against FMRP that fails to immunoprecipitate FMRP (35 and our unpublished observation) and a fortiori KIF3C (Fig. 6, top panels). In contrast, when using mAb7G1-1 antibody directed against FMRP, substantial amounts of FMRP were recovered, especially at high salts concentrations, in agreement with Ceman et al. (35), and KIF3C could be detected in association with FMRP only in buffer containing NaCl concentrations lower than 150 mM in agreement with the GST-pulldown experiments (Fig. 1C). These results indicate that both, in vitro and in vivo, the interaction between FMRP and KIF3C is salt labile. Finally, we used the anti-KIF3C antibody to immunoprecipitate KIF3C (Fig. 6, bottom panels) and could reveal the presence of FMRP, however only at low salt concentrations.

These analyses confirm the protein–protein interaction between the motor protein KIF3C and FMRP and suggest that the two proteins are present in complexes associated with polymerized microtubules.
In vivo dynamics and kinetics of neuronal granules containing GFP-FMRP

To investigate in vivo the dynamics and kinetics of particles containing FMRP, we followed the movements of GFP-FMRP using time-lapse video-microscopy in primary cultured neurons (Fig. 7). Dendritic granules containing exogenous GFP-FMRP had previously been shown to contain Fmr1 mRNA and ribosomal RNA (11,12,36), and we therefore hypothesized that this experimental system would reflect endogenous transport of FMRP-containing granules. In addition, since overexpression of FMRP is known to induce the formation of cytoplasmic stress-granules-like structures (37), cultures were analyzed at early times after transfection to minimize accumulation of GFP-FMRP (see Material and Methods section). In these conditions, we observed that GFP-FMRP displayed a granule-like pattern in the somato-dendritic compartment (Fig. 7), in agreement with previous studies (11,38). GFP-FMRP granules were heterogenous in size, with large granules often encountered in the soma, whereas smaller granules were distributed throughout the dendritic arborization (Fig. 7A). As already reported for neuronal RNA granules (13,16), the majority of the large GFP-FMRP particles remained static, corresponding most likely to either stress granules or mega-granules. In contrast, small granules displayed processive movements in both anterograde and retrograde directions, whereas others displayed back-and-forth oscillatory movements. Time-lapse videomicroscopy was performed only on small GFP-FMRP granules which most likely reflect endogenous FMRP RNA granules. Examples of the decomposition of the movements of two small granules during 140 s are illustrated in Figure 7B, while the distance moved by each granule along the x- and y-axis versus time is presented in Figure 7C. To estimate the speed of the back-and-forth oscillatory movements, the mean speed of granules that mainly reflects processive movements were...
calculated over a period of 15 min. The average rate was 0.0736 ± 0.0017 μm/s, whereas oscillatory movements displayed an average rate of 0.0156 ± 0.0015 μm/s.

Finally, we calculated the maximal distance covered by GFP-FMRP granules in 104 individual neurons. Five sub-populations of granules were defined by their traveled distance (0–99, 100–199, 200–299, 300–399 and ≥400 μm, respectively, Fig. 7D). More than 70% FMRP granules were found between 0 and 300 μm from the cell body, with 40% of granules traveling between 100 and 199 μm. Since the minimal traveled distance was estimated to be 74 μm, this indicates that GFP-FMRP granules transport does not result from passive diffusion from the soma, but are rather actively transported in the dendrites. The maximal distance was estimated to be 750 μm, indicating that granules are able to travel long-range distances.

**KIF3C drives the transport of FMRP-containing granules in vivo**

To validate the functional involvement of KIF3C in the transport of FMRP granules, we expressed GFP-FMRP alone or in the presence of RFP-KIF3C and of its dominant-negative form lacking the N-terminal motor domain (RFP-KIF3CΔN). We hypothesized that this truncated version of KIF3C that still contains the stalk (dimerization domain) and tail domain (cargo binding domain) could be incorporated into traveling complexes. However, since this truncated version lacks a motor domain, it was expected that it was unable to power the transport of its cargo along microtubules, presumably by disrupting endogenous KIF3C motor activity.

RFP-KIF3C and RFP-KIF3CΔN were expressed, together with GFP-FMRP, in cortical neurons. As shown in Figure 8A, RFP-KIF3C and RFP-KIF3CΔN displayed a cytoplasmic granular pattern comparable to that observed for endogenous KIF3C (Fig. 3). Since time-lapse videomicroscopy experiments revealed that accumulation of KIF3C or KIF3CΔN did not affect significantly the movements and speeds of FMRP granules (not shown), we therefore further analyzed the maximal distance traveled by FMRP granules (Fig. 8).

First, we calculated that the presence of the dominant-negative KIF3CΔN significantly decreased the average maximal distance of granules to 191 μm (n = 112), when compared to GFP-FMRP either alone (250 μm, n = 104, P < 0.0001) or together with KIF3C (215 μm, n = 106, P < 0.036). The main sub-populations of GFP-FMRP granules that were affected by over-expression of KIF3C or its dominant negative KIF3CΔN corresponded to granules traveling from 0 to 299 μm (Fig. 8B), representing 70% of GFP-FMRP granules (see above). Accumulation of KIF3C increased the sub-populations of granules between 100 and 199 μm (Fig. 8), while those present at 300 μm were reduced. On the other hand, the dominant-negative
KIF3CΔN strongly increased the number of FMRP granules proximal to the cell body (0–100 μm range) from 1 to 20%, while those present between 200–299 μm were reduced from 28 to 14% (Fig. 8B). In addition, FMRP granules in the 300–399 μm range and above were also diminished in the presence of KIF3CΔN, indicating that this mutant alters long-range transport of FMRP granules (Fig. 8B). These data demonstrate in vivo the functional implication of the neurospecific kinesin KIF3C in the transport of RNA granules containing FMRP.

DISCUSSION

Translation repression and transport of mRNA within neuronal RNA granules are thought to be tightly coupled processes, probably to prevent ectopic translation of dendritic mRNAs while ‘en route’ to the synapse. Several RNA-binding proteins present in RNA granules are thought to play a dual role in translation repression and transport. This dual role has been proposed for the RNA-binding protein CPEB that binds and regulates the synaptic translation of CamKIIa mRNA, while facilitating at the same time its dendritic transport, apparently via interaction with motor proteins (39), and also for the zipcode-binding protein ZPB1 that targets β-actin mRNA into dendrites and axons (40). However, no evidence has been provided so far for a direct interaction between RNA-binding proteins with a motor protein. In a similar way, FMRP has been described as a translation repressor in vitro (41,42) and in vivo (37). Moreover, a role for FMRP in the dendritic localization of several mRNA has been suggested (12,43), but its direct role as an adaptor molecule between RNA granules and motor proteins has not been experimentally documented. In the present study, we provide evidence that FMRP and a member of the kinesin II family, the neurospecific kinesin KIF3C, interact directly in vitro and co-localize in granule-like structures in the somato-dendritic compartment of neurons in vivo. Moreover, we show that FMRP and KIF3C can be specifically recovered and co-immunoprecipitated from in vitro polymerized brain microtubules fractions. This direct interaction allows us to speculate that, in addition to regulate translation repression in RNA granules, FMRP behaves also as a molecular adaptor between RNA granules and KIF3C. We estimate that under the experimental conditions used, while the vast majority of FMRP-containing RNP remains associated with polyribosomes, 5–15% of FMRP-RNPs are associated with microtubule structures, probably representing the fraction of FMRP traveling in RNA granules in the dendritic arborization. Whether one single FMRP molecule can bind its target mRNA and simultaneously interact with KIF3C is not known. Alternatively, we propose that a certain class of FMRP binds only mRNA, while other species behave as molecular adaptors with KIF3C. It should be recalled that FMRP forms homo- and heteromers throughout

Figure 7. GFP-FMRP granules movements in primary cultured neurons. (A) GFP-FMRP forms granules in 6 DIV transfected primary cultures of telencephalon neurons. (B) Two sets of granules highlighted in the insets (1 and 2) were studied and their movements in dendrites recorded during 140 s with 20 s intervals. Note that the time-lapse series corresponding to the inset 2 was taken from 300 s up to 440 s; therefore, the initial image of the time-lapse series presented in (B) is different from (A). (C) Distances traveled of the two granules from the start point traced by the arrowheads in panel (B) and their final moved distances along the x-axis (white circles) and the y-axis (black circles). (D) Histogram plotting maximal distances traveled by GFP-FMRP granules in 104 analyzed neurons.
It is therefore possible that post-translational modifications, such as phosphorylation, are the factors that govern these functions.

KIF3C belongs to the family of anterograde motors kinesin II that contains also KIF3A and B. KIF3C is the neurospecific member, being mainly expressed in brain and retina (27,28). On the other hand, KIF3A and KIF3B are the most studied and are thought to function in fast axonal transport of several cargoes via interaction with the kinesin-associated protein KAP3, a non-motor accessory subunit that recognizes the cargoes (45,46). The heterodimer KIF3A/B complex transports vesicles containing fodrin (29) and Kv1 channels (47), as well as axonal RNA granules containing Tau mRNA (48). KIF3A/C complex is associated with axonal vesicles; however, a fraction of KIF3C does not associate with KIF3A, evoking that it could function as a homodimer (27,28). Our study show that, in contrast to KIF3A/B or KIF3A/C complexes which function in axonal targeting of vesicles via the adapter KAP3, the homodimer KIF3C actually transports dendritic RNA granules via direct interaction with FMRP that serves as a direct molecular adaptor between KIF3C and the RNA granules. Indeed, direct binding of cargoes to kinesin heavy chain is thought to drive the transport towards dendrites, whereas binding to kinesins via the accessory protein KAP or kinesin light chain KLC targets cargoes towards the axon (17,49).

Using videomicroscopy approaches, we observed that in primary cultured neurons, GFP-FMRP granules display oscillatory bidirectional movements over short distances as well as processive movements over longer distance. Oscillatory movements of GFP-FMRP granules have a mean speed of 0.0156 ± 0.0015 m/s, in line with the average speed calculated for GFP-Puro granules (0.034 ± 0.025 m/s; 17). Processive movements have an average speed of 0.0736 ± 0.0017 m/s, compatible for granules labeled for BCI untranslated RNA (0.07 ± 0.01 m/s; 50), Arc mRNA (0.08 ± 0.01 m/s; 51) or CaMKII mRNA (0.05 ± 0.03 m/s; 52). Moreover, the velocity of these RNA granules is comparable to that observed for GFP-Stau (0.1 m/s; 13) and GFP-Puro granules (0.10–0.12 m/s; 17), both RNA-binding proteins also detected in RNA granules. These processive movements correspond to long-range dendritic transport of GFP-FMRP RNA granules that is disrupted by overexpression of the dominant negative of KIF3C lacking its N-terminal motor domain, similarly to the dominant-negative KIF5C transporting GFP-Puro granules (17). Accumulation of KIF3CΔN had no effect on granules traveling from 0 to 299 m away from the soma; however, it tended to impede transport more distally, probably due to interference with other motors required for this longer-range transport. The fact that this dominant-negative does not abrogate totally the distal transport of GFP-FMRP granules, suggests the involvement of additional different motors in the long-range transport of FMRP granules. This is in line with our immunofluorescence data showing that not all granules containing FMRP harbor KIF3C. Neuronal mRNA granules being highly heterogenous multimolecular complexes (13,15–17), it can be proposed that different motor proteins could transport specific subpopulations of FMRP-containing granules. Indeed, selective association of RNA granules with specific kinesin

Figure 8. Functional impact of a KIF3C dominant-negative mutant on the transport of GFP-FMRP granules. (A) Neurons (6 DIV) were transfected with both GFP-FMRP and RFP-KIF3C or GFP-FMRP and RFP-KIF3CΔN, or GFP-FMRP alone. (B) The percentage of GFP-FMRP granules transported at different distances was plotted in each condition. Confocal analysis was performed on 104 (GFP-FMRP alone), 106 (GFP-FMRP plus RFP-KIF3C) and 112 (GFP-FMRP plus RFP-KIF3CΔN) transfected neurons, respectively.
motors might provide a mechanism to target mRNAs selectively to axons or dendrites (49). The nature of these motors remains elusive, even though myosin Va, dynein (25), as well as KIF5 (17) were reported to co-immunoprecipitate with FMRP, Staufen and Purα.

A large-scale analysis of the mRNA associated with FMRP in dendritic complexes (43) revealed that KIF3C mRNA is a potential target of FMRP. This raises the possibility that FMRP could address and modulate the local translation of KIF3C mRNA in dendrites and at the synapse, thereby controlling the movement of mRNA granules locally. Similar to Fmr1, the expression of KIF3C is strongly upregulated during embryonic development and in differentiating neuroblastomal cells (53), suggesting an important role for this motor protein at the onset of differentiation and throughout all the process of neurite elongation, perhaps in concert with FMRP which is essential for neuronal spines development and maturation (20,23). The KIF3C null mouse is apparently viable, reproduces and develops normally, without noticeable abnormalities (54), probably owing to the compensation of KIF3A and KIF3B. However, no complete anatomical and behavioral study has been performed yet on this mouse strain, and it would be worth searching whether KIF3C null mice display immature-looking abnormal dendritic spines such as Fmr1 null mice (7).

Our study provides evidence that, in certain sub-populations of RNA granules, FMRP interacts with the motor protein KIF3C that controls their targeting towards dendrites. These results provide new insights on FMRP’s functions and raise the possibility that the absence of FMRP in Fragile X patients might therefore not only impede the translation of neurospecific mRNA targets, but also alter the targeting and transport of dendritic mRNA. In the absence of FMRP, classes of RNA granules might remain in the soma, ‘orphan’ of the right adapter to link them to the motor KIF3C required for their dendritic transport. The discovery of an RNA-binding protein as a molecular adapter between RNA granules and a member of the kinesin family opens new perspectives. This would provide a mechanism by which dendritic mRNA are sorted via specific interaction with RNA-binding proteins and the choice of a motor that targets the granules to specific distal locations. A great challenge resides in the characterization of RNA granules transported in dendrites, both at the RNA and protein level, which will help understanding part of the mechanisms underlying the regulation of dendritic RNA granules transport and local synaptic translation.

**MATERIALS AND METHODS**

**Yeast two-hybrid screen and GST-pulldowns**

Yeast-two-hybrid screening was performed as described previously (33). From ~2.8 x 10⁶ clones screened, 78 positive colonies showed adenine and histidine prototrophy and β-galactosidase activity. Among these, one colony carried a 2.3 kb insert. This insert corresponded to the C-terminal portion (amino acids 403–792) as well as a portion of the 3′-UTR of the cDNA of human neurospecific kinesin KIF3C.

**Purification of recombinant fusion proteins and GST-pulldown assays**

The pBSK(+) construct containing mouse KIF3C full-length cDNA and pGex-KG construct encoding GST-KIF3C 71 C-ter were provided by Zhaohuai Yang and Lawrence S. B. Goldstein (Howard Hughes Medical Institute, University of California, San Diego, CA, USA). The pGex-4T-1/KIF3C N-ter plasmid encoding GST-KIF3C amino acids 1–383 was generated by ligation of the PCR product amplified from the pBSK(+)/mouse KIF3C full-length cDNA (primers: 5′-GCC GGA TCC GCC AGT AAG ACC AAG GCC AG-3′, 5′-TTC CTG GAA TTC CCT CAG CAG-3′) into the BamHI/EcoRI site of pGex-4T-1 (Amersham, GE Healthcare). Expression and purification of recombinant proteins and GST-pulldown assays were performed as described (27,33).

**Immunofluorescence on rat cerebellar sections and primary cultured neurons**

Adult rat brain sections and 14 days in vitro (DIV) primary cultured rat hippocampal neurons were prepared and fixed as described (33,55). Affinity-purified anti-KIF3C antibody (27) was used at 1:100 dilution (overnight at 4°C). Immunodetection of FMRP with mAb1C3 (1:500, Chemicon) and β-tubulin with mAbE7 (1:500, Developmental Studies Hybridoma Bank, University of Iowa) was performed as described previously (33,37). Samples were analyzed in epifluorescence or confocal microscopy with a Nikon Eclipse E800 microscope equipped with a Hamamatsu CCD camera.

**Mouse brain microtubule preparation and subcellular fractionation**

Microtubules were prepared from the 2-week-old mouse brain through two cycles of temperature-dependant cycles of microtubules polymerization/depolymerization in the presence of GTP, following a protocol adapted from Valle (56), previously used to study the association of FXR1P to testis microtubules (34). For subcellular fractionation studies, mice brains were homogenized in standard PEM buffer and clarified at 12 000 g to lead P1. The resulting supernatant was then incubated with or without 1 mM GTP (Sigma), at 4°C or at 37°C for 30 min, to induce polymerization of the microtubules. Polymerized microtubules were collected by centrifugation (30 min, 4°C) at 25 000 g to lead P2. The supernatant containing unpolymerized microtubules and soluble proteins was further centrifuged at 105 000 g for 2 h, to lead P3 and the final supernatant (Sup). All pellet fractions were re-suspended in a final volume of denaturing SDS–PAGE buffer equivalent to the starting volume used to homogenize the brain. Equal volumes of each fraction were loaded on a 11% SDS–PAGE and analyzed by western-blotting using mAb1C3 against FMRP, mAbE7 against β-tubulin, anti-L7 antibody as described (34), SYP(D-4) against the synaptic vesicle marker synaptophysin (1:2500; Santa Cruz Biotechnology) and the ER marker GRP78/BiP (1:10000; Stressgen).
Immunoprecipitations using mouse brain microtubule-enriched preparations

Immunoprecipitations were performed using freshly polymerized brain microtubules re-suspended in cold NE-PEM buffer (PEM, supplemented with 0.5% NP40, 10 mM EDTA) and homogenized using a Teflon-glass potter. Homogenates were centrifuged at 15,000 g for 10 min to sediment insoluble material and the resulting supernatant was used for immunoprecipitation. The concentration of salts was then adjusted to 0.15, 0.3, or 0.4 M NaCl. Antibodies [5 μg of mAb1C3 and mAb7G-1 (35; obtained from the Developmental Studies Hybridoma Bank, University of Iowa) or 50 μl of affinity-purified anti-KIF3C antibody] were pre-incubated (overnight at 4°C) with 60 μl protein A/G beads (Calbiochem) in the presence of 0.1 mg/ml of each BSA, yeast tRNA, glycogen (Sigma) in NE-PEM buffer. Beads with bound antibodies were then washed twice with NE-PEM. Proteins were eluted from the beads with 60 μl SDS-buffer and heat denatured for 5 min. Approximately one third of the eluate was loaded on a 7.5% SDS–PAGE and proteins transferred onto nitrocellulose membrane were revealed using the appropriate antibodies.

Time-lapse video microscopy

For direct visualization of KIF3C in primary cultured neurons, mouse KIF3C was fused to the red fluorescent protein (RFP) by cloning into PCR (primers: 5'-GCC CTC GAG CGC CAG TAA GAC CAA GGC CAG-3', 5'-CGG GGA TCC GTC ATG GTC TAC CAC TGT GTC AG-3') its cDNA in the Xhol-BamHI sites of the CMV-promotor driven plasmid peRFP (BD Biosciences). A dominant negative form of KIF3C tagged with RFP (RFP-KIF3CΔN) lacking its motor domain (amino acids 1–380) was also generated. For this purpose, the cDNA corresponding to its C-terminal domain (amino acids 383–796) was excised from peRFP/KIF3C using KIF3C cDNA EcoRI site and peRFP BamHI site, and subcloned into peRFP. To construct peGF/FMRP, FMRP iso7 cDNA was subcloned from pGBKTK7/FMRP iso7 (33) and transferred to the EcoRI/PstI sites of peGF (BD Biosciences). Primary cultures of mouse telencephalon were carried out as described (57). At DIV5, neurons were transfected with the peGF/FMRP, peRFP-KIF3C or peRFP-KIF3CΔN vectors using Lipofectamine reagent according to the manufacturer’s instructions (Invitrogen). After 5 h post-transfection, the medium was changed and neurons were allowed to recover overnight before proceeding for analyses. To minimize accumulation of GFP-FMRP, we chose early time after transfection (16–20 h). This allowed detection of individual particles in dendrites and to study of their subsequent intracellular transport.

For time-lapse videomicroscopy, cells were maintained at 37°C in a 5% CO₂ live-cell incubation chamber (Saur, Reutlingen, Germany). Images were taken with a cooled CCD camera (Cool Snap HQ, Roper Scientific) mounted on the Leica DM IRE2 microscope and a 100 × oil immersion objective lens (N.A. 1.4, Zeiss). The cells were illuminated with a DG4 shutter. The intensities of fluorescence along the processes of each neuron were measured with a user-defined threshold with the MetaMorph software (Universal Imaging). Fluorescent images were captured every 5 s for 15 min. The mean movements of granules were measured on a 15 min scale using the SpotTracker plugging of the ImageJ software (Wayne Rasband, NIH, Bethesda, NY, USA). For analysis of the maximal transport distances of granules, neurons were fixed (see above) and a minimum of n = 100 transfected cells for each condition were observed under a Leica TCS SP2 AOBS confocal microscope. The maximal distance was calculated from the root of the dendrite to the farthest traveling granule of each cell, using the ImageJ software.

All animal procedures conformed to the guidelines of the Canadian or French Council for the care and use of laboratory animals, and all efforts were made to minimize animal suffering.

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