A new regulatory function of the region proximal to the RGG box in the Fragile X mental retardation protein

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Summary

Fragile X mental retardation protein (FMRP) is required for normal cognition. FMRP has two autosomal paralogs, which although similar to FMRP, cannot compensate for the loss of FMRP expression in brain. The arginine- and glycine-rich region of FMRP (the RGG box) is unique; it is the high-affinity RNA-binding motif in FMRP and is encoded by exon 15. Alternative splicing occurs in the 5′ end of exon 15, which is predicted to affect the structure of the distally encoded RGG box. Here, we provide evidence that isoform 3, which removes 25 amino acids from the 5′ end of exon 15, has an altered conformation that reduces binding of a specific antibody and renders the RGG box unable to efficiently associate with polyribosomes. Isoform 3 is also compromised in its ability to form granules and to associate with a key messenger ribonucleoprotein Yb1 (also known as p50, NSEP1 and YBX1). Significantly, these functions are similarly compromised when the RGG box is absent from FMRP, suggesting an important regulatory role of the N-terminal region encoded by exon 15.

Key words: FMRP, RGG box, RNA-binding protein, Granules, Polyribosome

Introduction

Fragile X syndrome is the most common form of inherited mental retardation and is caused by loss of expression of the fragile X mental retardation protein (FMRP, encoded by *FMR1*) (Bassell and Warren, 2008). FMRP has two autosomal paralogs, FXR1P and FXR2P (encoded by *FXR1* and *FXR2*, respectively), that cannot compensate for the loss of FMRP function in brain (Coffee et al., 2010). FMRP is an RNA-binding protein with two K homology (KH) domains and a region rich in arginine and glycine residues, the RGG box (Bardoni et al., 2001). The RGG box is a high-affinity RNA-binding motif, binding G-quadruplex RNA with nanomolar affinity (Darnell et al., 2001; Schaeffer et al., 2001). Although both FXR1P and FXR2P contain an RGG box and an arginine- and glycine-rich-region, respectively, FMRP is unique in its ability to recognize G-quadruplexes, suggesting that the RGG box of FMRP plays a non-redundant role in the pathology of the disease (Darnell et al., 2009). Because of the key role of the RGG box in FMRP function, it is crucial to understand how this domain is regulated.

The *FMR1* pre-mRNA is alternatively spliced at the 3′ end to give multiple FMRP isoforms (Ashley et al., 1993; Eichler et al., 1993). Exon 15, which encodes the highly conserved phosphorylation site (Siomi et al., 2002; Ceman et al., 2003) and the RGG box, has three alternative splice acceptor sites upstream of the RGG box that maintain the RGG box but truncate the N-terminally encoded region of exon 15 (Fig. 1A). All three splice forms have been characterized at the biochemical level (Dolzhanskaya et al., 2008) but not in cells. The RGG box is part of a three-to-four strand antiparallel beta sheet that functions as a platform for nucleic acid interactions in other RNA-binding proteins (Dolzhanskaya et al., 2008). Isoform 3, the isoform with the largest N-terminal deletion in exon 15, is predicted to have a truncated first strand, significantly perturbing the side-chain conformations of the RGG box arginine residues (Dolzhanskaya et al., 2008). To understand the function of the RGG box in cells, we extended the biochemical characterization by Dolzhanskaya and colleagues by examining the cellular function of isoform 3 (Dolzhanskaya et al., 2008). Our data suggest that the N-terminal region of exon 15 is crucial for normal RGG box function, modulating translation regulation and granule formation.

Results and Discussion

The *FMR1* mRNA has multiple isoforms in brain, including isoform 3 (Ashley et al., 1993; Xie et al., 2009), but their protein products have not been visualized in the absence of the autosomal paralogs, FXR1P and FXR2P. To examine the brain isoforms of FMRP, we used a serial immunoprecipitation strategy described previously (Ceman et al., 2003). We first isolate the FMRP-containing messenger ribonucleoprotein (mRNP) complex with the FMRP-specific monoclonal antibody 7G1-1 (Brown et al., 2001). The complex is disrupted and eluted by boiling in SDS sample buffer. We then isolate free FMRP with a second immunoprecipitation as described in the Materials and Methods. We found that FMRP exists in at least three isoforms in brain (Fig. 1B, left-hand panel).

The crucially important RGG box is methylated on four of its arginine residues in cells, which accounts for ~90% of the FMRP methylation (Stetler et al., 2006). Isoform 3 is hypomethylated in in vitro assays, suggesting that the region upstream of the RGG box plays a role in regulating FMRP methylation (Dolzhanskaya et al., 2008). In cells, we examined the methylation state of isoform 3 FMRP, wild-type (WT) FMRP and FMRP lacking the

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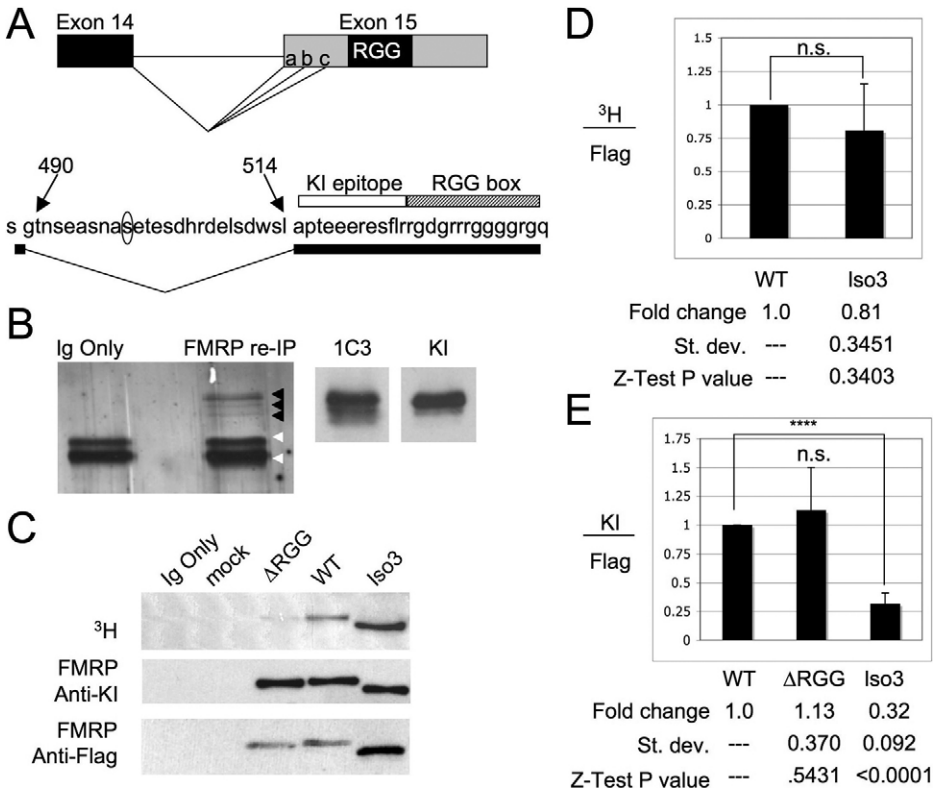

Fig. 1. Splice acceptor site 15c is used in the brain, and isoform 3 is normally methylated but exists in an altered conformation in cells. (**A**) Exon 15 contains three splice acceptor sites (a–c) at the N-terminal end of the RGG box: the splice site c, referred to as 15c (Dolzhanskaya et al., 2006), removes amino acids 490–514, which includes the phosphorylated serine residue (circled). Numbering is based on GenBank accession number NP_032057. The KI epitope is identified in this paper and previously (Reis et al., 2004). (**B**) FMRP was purified from mouse brains and visualized on a silver-stained 4–20% gradient gel (BioRad) (left-hand panel). The Ig Only column used the immunoprecipitating antibody 7G1-1; black arrowheads indicate FMRP isoforms and white arrowheads Ig bands. Purified FMRP was probed with either 1C3 (middle panel) or KI (right-hand panel). (**C**) L-M (TK–) VC cells were mock-transfected (mock), or transfected with EGFP-expression vectors for WT FMRP (WT), ΔRGG or isoform 3 (Iso3) and metabolically labeled with [^{3}H]methyl-methionine and resolved on a 7.5% gel. Top panel: autoradiography of Flag-immunoprecipitated FMRP. Middle and bottom panels: immunoblots with KI and anti-Flag antibodies. (**D**) Results of three independent experiments analyzed by densitometry using ImageJ. Densitometry values from [^{3}H]methyl-methionine autoradiographs were divided by those with anti-Flag antibody to generate a ratio of ^{3}H to Flag. The fold change was determined based on the WT ratio being set to 1.0. A value for the ΔRGG construct was not calculated (the signal was below detection). (**E**) Densitometry values with KI were divided by densitometry values with anti-Flag antibody to generate a ratio of KI to Flag. The fold change was determined based on the WT ratio being set to 1.0. ****$P<0.0001$ using a Z-test. n.s., not statistically significant.

RGG box (ΔRGG), which is unmethylatable (Stetler et al., 2006). WT FMRP is encoded by the longest isoform, containing all 17 exons, as described previously (Stetler et al., 2006). In contrast to the in vitro studies, isoform 3 appeared to be hypermethylated compared with the methylation of WT FMRP (Fig. 1C, top panel). To examine the amount of transgene-encoded protein, we probed with the anti-FMRP antibody, KI (Fig. 1C, middle panel). Although this result suggested equal loading, we also probed with an antibody to the N-terminal Flag epitope. Surprisingly, the Flag immunoblot showed that more isoform 3 protein had been loaded (Fig. 1C, bottom panel). These results led to two conclusions: first, that there was no change in the methylation state of isoform 3 in cells (Fig. 1C,D); and second, that the 25 amino acids absent in isoform 3 are crucial for recognition by KI (Fig. 1A).

Comparisons of the ratio of KI reactivity to Flag antibody reactivity demonstrated that these antibodies do not have equal recognition and that isoform 3 has lost epitope(s) recognized by approximately two-thirds of the polyclonal antisera KI but retains an epitope recognized by the remaining antibody. Thus, isoform 3 is recognized approximately one-third as well as WT FMRP or ΔRGG (Fig. 1E). We used this specific recognition by KI to determine whether isoform 3 was present in the brain, as mass spectrometry analysis of brain-derived FMRP did not result in sufficient coverage of exon-15-encoded protein to be informative (personal communication by Benjamin Garcia, Princeton University, Princeton, NJ). Probing of the brain-derived FMRP with an antibody specific to the N-terminus of FMRP (1C3) (Khandjian et al., 1998) detected all FMRP isoforms. By contrast, KI was unable to detect the smallest FMRP isoforms in a comparable exposure (Fig. 1B, right-hand panels), suggesting that the smaller FMRP isoforms are missing the first 25 amino acids of exon 15.

KI was generated using amino acids 516–632 of human FMRP (515–631 in mouse) (Fig. 1A) (Reis et al., 2004) and effectively recognizes ΔRGG (Fig. 1C), which lacks amino acids 526–547. However, KI has a substantially reduced recognition of isoform 3, which is missing amino acids 490–514. Our results suggest that the epitope required for the majority of the KI recognition lies between amino acids 516 and 525, comprising, in part, the antiparallel beta sheet perturbed in isoform 3 (Dolzhanska et al., 2008).

Although isoform 3 has an altered conformation N-terminal to the RGG box, we found that it was normally methylated in both the mouse fibroblast cell line L-M (TK–) (Fig. 1C,D) and the mouse STEK *FMR1*-knockout fibroblast cell line (data not shown), probably reflecting the ability of protein arginine methyl transferases (PRMTs) to methylate substrate arginine-glycine-bearing peptides without a need for protein context (Gary and Clarke, 1998). Our observation was in contrast to the in vitro studies, which showed that isoform 3 was hypomethylated in reticulocyte extract (Dolzhanskaya et al., 2006). This apparent disparity might be because we examined isoform 3 in cells, which probably express a wider number of PRMTs than do reticulocytes.

The RGG box is required for normal polyribosome association: its removal causes loss of FMRP from the translating polyribosomes (heavier fractions) and a subsequent accumulation of ΔRGG in lighter fractions (Blackwell et al., 2010). To determine whether isoform 3 has compromised RGG box function in cells, we examined polyribosome association in WT FMRP- and isoform 3-expressing cells and found a significant loss of isoform 3 from polyribosomes (Fig. 2, fractions 5 and 6). Accordingly, we found an increase in isoform 3 in fraction 1 compared with WT FMRP (Fig. 2). Our results suggest that the region upstream of the RGG box is important for normal FMRP function, probably through regulation of the RGG box. We suspect that ΔRGG still binds RNAs, given that there are other RNA-binding domains in FMRP (Bassell and Warren, 2008). Isoform 3 also binds RNA in in vitro studies, although with specificities distinct from WT FMRP (Denman and Sung, 2002). Thus, the loss of isoform 3 and ΔRGG from polyribosomes might be due to either less RNA binding by the RGG box or translation suppression of bound RNAs.

It has been reported that expression of ΔRGG in STEK cells results in the formation of fewer FMRP-positive granules compared with upon expression of WT FMRP, although this observation was not quantified (Mazroui et al., 2003). In addition, FMRP isoform 12, which lacks exons 12, 14 and the RGG box, is markedly absent from dendritic granules (Levenga et al., 2009). We hypothesized that the RGG box is required for normal association with granules. Because the presence of a wild-type copy of *FMR1* rescues the isoform 12 phenotype, we performed this experiment in STEK cells (Mazroui et al., 2002). To quantify the change in RGG-box-dependent FMRP-containing granules, we used an assay previously described to quantify granules in cultured cells [(Ling et al., 2004); described in the Materials and Methods]. As expected, we found that loss of the RGG box resulted in fewer FMRP-containing granules throughout the cell (Fig. 3A,B, compare WT with ΔRGG). Similarly, expression of isoform 3 also resulted in significantly less FMRP-containing granules compared with WT FMRP. There was no statistically significant difference in granule size, and the granules had an average area of ~77 pixels, equivalent to a diameter of less than 1 μm (Fig. 3C). There was also no statistically significant difference in average granule intensity (data not shown). This decrease in granules containing isoform 3 was not significantly different from the reduction observed with ΔRGG (Fig. 3B). Thus, similar to the polyribosome studies, loss of the region N-terminal to the RGG box has the same effect on function as losing the RGG box.

In addition to the proposed RGG box regulatory domain, isoform 3 also lacks the phosphorylation site at serine 499

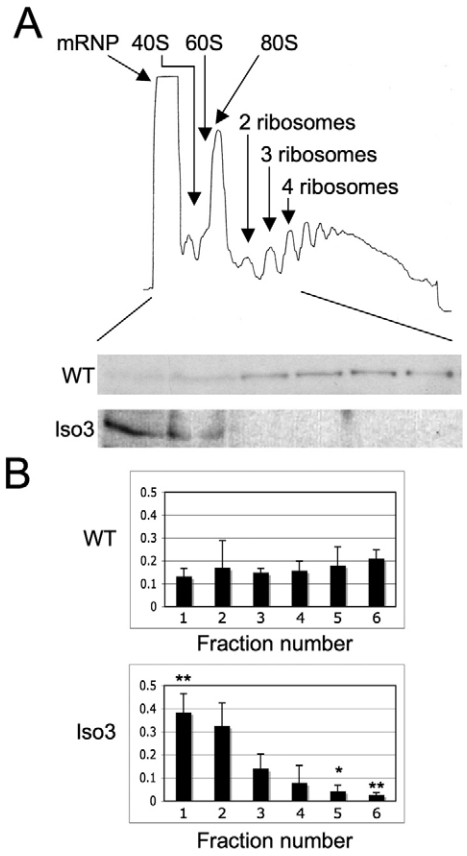

Fig. 2. Isoform 3 has reduced polyribosome association compared with that of WT FMRP. (**A**) Representative polyribosome profile of transfected STEK cells probed with monoclonal antibody 1C3 for transgene-encoded FMRP as shown in the immunoblots below (Iso3, isoform 3). (**B**) In three independent experiments, the distribution of WT or isoform 3 in the first six fractions was calculated as the amount of FMRP in each fraction divided by the total amount of FMRP using ImageJ and Microsoft Excel. Histograms represent means ± s.d. *$P<0.05$; **$P<0.01$ compared with WT using unpaired Student's *t*-test ($n=3$).

(Fig. 1A). To determine whether changes in granule formation were caused by loss of phosphorylation, we measured granule formation of an unphosphorylatable form of FMRP (S499A) (Ceman et al., 2003). Consistent with that previous study, we found no significant difference in granule number compared with WT FMRP (Fig. 3, compare WT with S499A), and there was no significant difference in granule size (Fig. 3C) or intensity (data not shown). To determine whether FMRP loading onto granules requires synthesis of new mRNA, we blocked transcription with actinomycin D and found a significant decrease in FMRP granule formation (Fig. 3, compare WT with WT+act.) but no significant difference in granule size (Fig. 3C) or intensity (data not shown). This result suggests that formation of FMRP-containing granules requires mRNA synthesis and that mRNA is a crucial factor in determining whether FMRP is present on granules.

The results of both the polyribosome and the granule assay suggest that RNA binding by the RGG box is impaired in isoform 3; however, it is also possible that a protein component is affected. In fact, FMRP-containing granules consist of many different proteins (Kanai et al., 2004). The region absent in isoform 3 contains a binding site for microspherule protein 58 (MSP58, also

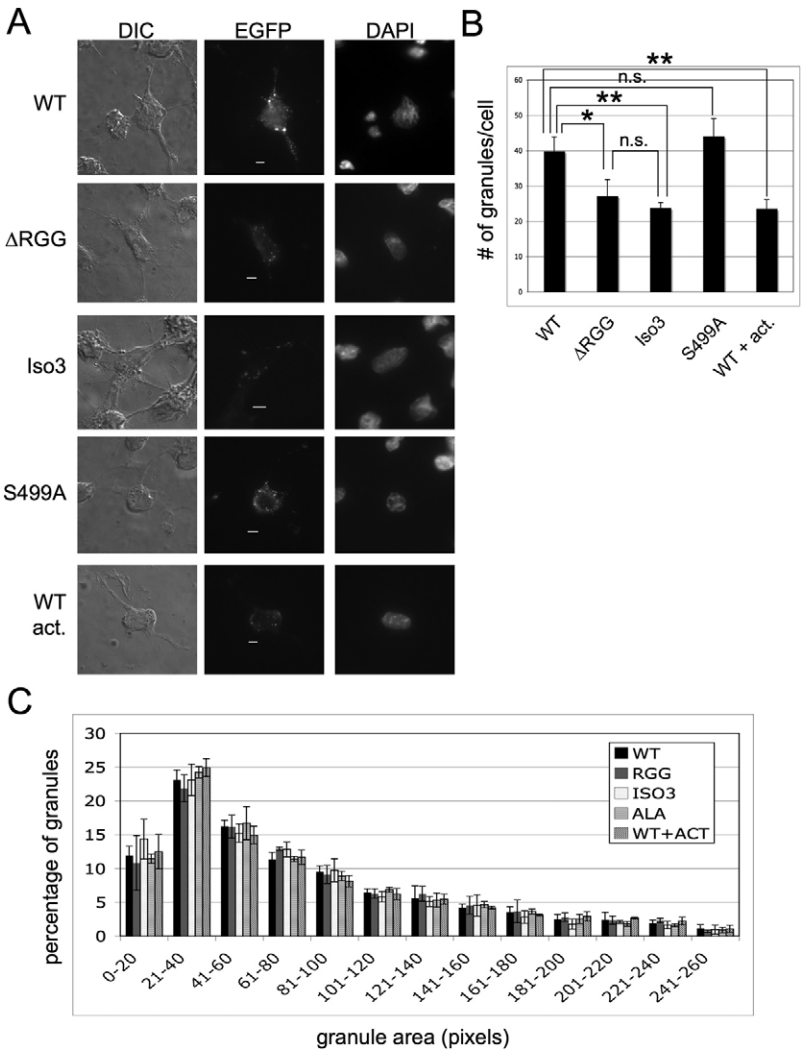

Fig. 3. The region N-terminal to the RGG box, the RGG box and RNA synthesis are required for normal granule formation. (**A**) DIC, fluorescence (EGFP) and DAPI images of STEK cells expressing EGFP-tagged WT FMRP, FMRP ΔRGG, FMRP isoform 3 (Iso3), FMRP (S499A) or WT FMRP after transcription was blocked with 0.05 μg/ml actinomycin D (WT act.), as indicated. Scale bars: 5 μm. (**B**) The histogram represents the mean ± s.d. of the number of granules counted per cell over three independent trials. n.s., not statistically significant; *$P<0.05$; **$P<0.01$, using ANOVA. (**C**) The histogram represents the distribution of granules as determined by pixel size for each experimental condition as a percentage of the total number of granules counted. Each experiment was performed in triplicate and no statistically significant differences were found using ANOVA.

known as microspherule protein 1) (Davidovic et al., 2006) and the C-terminus of FMRP interacts with the Ran-binding protein in the microtubule-organizing center (RanBPM, also known as RANBP9) (Menon et al., 2004). To assess whether any changes in protein association occur upon removal of the N-terminal region of the RGG box or the RGG box itself, we visualized associated proteins by silver staining. We found one distinct band that was reproducibly present for WT FMRP but that was absent for both ΔRGG and isoform 3 (Fig. 4A). This protein migrated at ~50 kDa, similar to a known FMRP-associated protein, Yb1 (also known as p50, NSEP1 and YBX1) (Ceman et al., 2000). Probing an independent immunoprecipitation revealed that the protein absent in both the ΔRGG and isoform 3 samples was Yb1. Like FMRP, Yb1 is an RNA-binding protein found on polyribosomes (Evdokimova et al., 1995; Funakoshi et al., 2003). It is also a core granule protein (Minich et al., 1993; Skabkin et al., 2004) whose association with FMRP is believed to be independent of RNA, suggesting a protein–protein interaction. Yb1 might associate with the C-terminus of FMRP, such that perturbation of the RGG box disrupts association with Yb1, affecting granule formation. Alternatively, Yb1-containing granules might be unable to efficiently recruit isoforms of FMRP in which the RGG box is compromised.

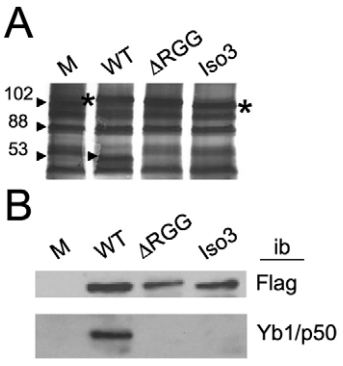

Fig. 4. Core mRNP protein Yb1 (p50) associates with WT FMRP but not with ΔRGG FMRP or isoform 3 FMRP. (**A**) L-M (TK–) mock-transfected VC cells (M) or those expressing EGFP-tagged WT, ΔRGG or isoform 3 (Iso3) FMRP were immunoprecipitated. Anti-Flag peptide elutions were resolved on a 10% gel and silver stained. FMRP proteins are indicated with asterisks. A 50-kDa band associated with WT FMRP is indicated by indicated with an arrowhead. The position of molecular mass markers in kDa is shown on the left-hand side. (**B**) An independent Flag purification immunoblotted (ib) for Flag or Yb1 (Yb1/p50), as indicated.

FMRP is a dynamic protein, associating with a large collection of mRNAs that it regulates the expression of [by either facilitating their intracellular localization on transport granules (Antar et al., 2004) or by activating or suppressing their translation (Brown et al., 2001)]. That FMRP has different isoforms might underlie how it has so many intracellular functions and can be found on both translating and non-translating granules (Beaulieu, 2000; Mazroui et al., 2002; Aschrafi et al., 2005). Furthermore, the state of a cell might dictate which FMRP isoforms are present, leading to the execution of a specific function. When P19 cells were induced to differentiate, splice site usage was shifted to 15c, suggesting that isoform 3 is required for neurite production or function (Xie et al., 2009). In conclusion, understanding how the RGG box is regulated will give insight into how FMRP binds its target mRNAs, assembles with granules and regulates translation.

Materials and Methods

Cell lines and assays, DNA constructs and transfection studies

VC (vector control) is the RSV.gpt vector transfected fibroblast L-M (TK–) cell line described by Ceman et al. (Ceman et al., 1999). Cells were grown as described previously (Blackwell et al., 2010). Transient transfections were performed using polyethylenimine (Sigma) at the same concentration as the DNA. Isoform 3 was constructed from WT FMRP using the deletion primers: 5′-CCTGGATATA-CTTCAGCTCCAACAGAGGAAG-3′ and 5′-CTTCCTCTGTTGGAGCTGAAG-TATATCCAGG-3′.

Metabolic labeling was performed as previously described (Stetler et al., 2006) and immunoprecipitations were performed using Flag-M2–Sepharose beads (Sigma). Polyribosome analysis was performed as described previously (Blackwell et al., 2010).

Antibodies and western blotting

The KI antibody was provided by Andre Hoogeveen (Erasmus University, Rotterdam, The Netherlands), the anti-Flag (Sigma) and anti-Yb1 antibodies were obtained from Valentina Evdokimova (University of British Columbia, Canada), the 7G1-1 antibody is as described previously (Brown et al., 2001), the 1C3 antibody was obtained from Jean-Louis Mandel (Institute of Genetics Research, Ilkirch, France). Antibody reactivity was visualized using an anti-mouse-IgG horseradish peroxidase (HRP)-conjugated antibody (KPL, Gaithersburg, MD) or anti-rabbit-IgG HRP-conjugated antibody (Amersham) and developed with ECL (Amersham).

FMRP purification from mouse brain

CBA/CaJ adult male mice were provided by Albert Feng at the University of Illinois at Urbana-Champaign (Institutional Animal Care and Use Committee protocol number 09098). Brains were prepared as described previously (Winograd et al., 2008). To isolate FMRP from the FMRP-containing mRNP complex, a sequential immunoprecipitation strategy was utilized, which was as described previously (Ceman et al., 2003). Briefly, after immunoprecipitation overnight with 7G1-1, FMRP-containing complex was disrupted and eluted by boiling in SDS sample buffer. The elution volume was then increased with Triton X-100-containing buffer (0.5%) to sequester the SDS and then re-immunoprecipitated with 7G1-1. FMRP was resolved by SDS-PAGE (4–20% gels; BioRad) and analyzed with silver stain (BioRad).

Microscopy

Transfected STEK cells were treated with cytochalasin-D (20 μM), to induce projections in S2 cells as described previously (Ling et al., 2004), fixed, stained with DAPI (Kim et al., 2009) and examined by fluorescence microscopy (Zeiss Axiovert 200 M inverted microscope, 63×). GFP-positive cells that displayed projections but also had no nuclear deformities suggestive of cell death were used for granule counting. Granules were counted using Cell Profiler 2.0 (Carpenter et al., 2006). GFP foci were enhanced using the enhance or suppress features module and the speckles setting. To measure primary objects (granules), the signal intensity threshold was determined using the robust background adaptive method and objects above threshold were identified. A total of 23–26 cells were counted per trial and all experiments were performed in triplicate.

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