Published in final edited form as:

Dev Neurobiol. 2017 June ; 77(6): 738–752. doi:10.1002/dneu.22453.

Splice form-dependent regulation of axonal arbor complexity by FMRP

Stephanie E. Zimmer¹, Steven G. Doll¹, A. Denise R. Garcia^{1,2}, and Michael R. Akins^{1,2,*}

¹Department of Biology, Drexel University, Philadelphia, PA

²Department of Neurobiology and Anatomy, Drexel University, Philadelphia, PA

Abstract

The autism-related protein FMRP (Fragile X mental retardation protein) is an RNA binding protein that plays important roles during both nervous system development and experience dependent plasticity. Alternative splicing of the *Fmr1* locus gives rise to twelve different FMRP splice forms that differ in the functional and regulatory domains they contain as well as in their expression profile among brain regions and across development. Complete loss of FMRP leads to morphological and functional changes in neurons, including an increase in the size and complexity of the axonal arbor. To investigate the relative contribution of the FMRP splice forms to the regulation of axon morphology, we overexpressed individual splice forms in cultured wild type rat cortical neurons. FMRP overexpression led to a decrease in axonal arbor complexity that suggests that FMRP regulates axon branching. This reduction in complexity was specific to three splice forms – the full-length splice form 1, the most highly expressed splice form 7, and splice form 9. A focused analysis of splice form 7 revealed that this regulation is independent of RNA binding. Instead this regulation is disrupted by mutations affecting phosphorylation of a conserved serine as well as by mutating the nuclear export sequence. Surprisingly, this mutation in the nuclear export sequence also led to increased localization to the distal axonal arbor. Together, these findings reveal domain-specific functions of FMRP in the regulation of axonal complexity that may be controlled by differential expression of FMRP splice forms.

Keywords

Fragile X syndrome; local translation; axon branching; neurite outgrowth; RNA granule

INTRODUCTION

FMRP (Fragile X mental retardation protein) plays diverse roles in the regulation of neuronal structure and function. Most notably, FMRP is a component of RNA granules in which it functions as a critical regulator of neuronal protein synthesis during both development and experience dependent plasticity (Bassell and Warren, 2008). However, FMRP also has translation-independent functions that include modulating the gating properties and subcellular distribution of ion channels (Brown et al., 2010; Deng et al., 2013;

*Correspondence to: Michael R. Akins, Ph.D., Department of Biology, Drexel University, PISB 319; 3245 Chestnut, Philadelphia, PA 19104, Phone: 215-571-4453, Fax: 215-895-1273, michael.r.akins@drexel.edu.

Ferron et al., 2014). Mutations in the *FMR1* gene in humans lead to loss of all these FMRP functions and result in Fragile X syndrome (FXS), the most common form of inherited autism and intellectual disability (Penagarikano et al., 2007). Neuronal development occurs largely normally in the absence of FMRP, as neither FXS patients nor animal models exhibit large-scale changes in brain morphology. Mutations in FMRP instead result predominantly in deficits at the level of connectivity between neurons (Bhakar et al., 2012).

Changes in neuronal connectivity that occur in the absence of FMRP include alterations in the morphology of the axonal arbor. For example, stereotyped developmental pruning of hippocampal mossy fibers in mice requires FMRP (Ivanco and Greenough, 2002). In cultured rodent neurons, loss of FMRP leads to an increase in the elaboration of the axonal arbor through misregulation of Semaphorin-3A-mediated repression of MAP1b translation (Li et al., 2009). Genetic experiments in *Drosophila* have revealed that loss of the FMRP homologue dFMR can lead to axonal defects including both pathfinding errors and abnormal extension (Dockendorff et al., 2002; Morales et al., 2002; Michel et al., 2004). Further, *Drosophila* neurons that overexpress dFMR have a simplified axonal arbor (Pan et al., 2004). FMRP therefore plays a conserved role in regulating the structural complexity of the axonal arbor.

Analysis of neurons that completely lack FMRP does not allow an assessment of the relative contributions of its splice variants. FMRP is extensively alternatively spliced, giving rise to 12 isoforms that contain different functional and regulatory domains. The full length (isoform 1) FMRP contains multiple characterized domains that are excluded from particular splice forms (Figure 1). These domains include RNA binding motifs (hnRNP-K homology (KH) domains and an RGG box), nuclear export and localization sequences, and sites for posttranslational modification via phosphorylation and methylation. All of the splice forms are expressed in the rodent brain, with the specific pattern of expression varying both across the brain and during development (Verkerk et al., 1993; Denman and Sung, 2002; Dolzhanskaya et al., 2006; Xie et al., 2009; Brackett et al., 2013). FMRP localizes to axons in the nervous system as well as in cultured neurons (Antar et al., 2006; Price et al., 2006; Christie et al., 2009; Akins et al., 2012). Whether all FMRP splice forms can localize to the axon and/or regulate the complexity of the axonal arbor is unknown.

To investigate these questions, we used a systematic approach to ask whether individual FMRP splice forms localized to axons or resulted in a simplification of the axonal arbor when overexpressed in wild type neurons. The use of wild type neurons allowed us to analyze the effect of manipulating the levels of a single splice form while still maintaining endogenous expression of the other splice variants. Consistent with studies conducted in *Drosophila*, we found that overexpression of FMRP led to a reduction in the axonal arbor complexity. However, not all splice forms exhibited this property. Instead, this effect was limited to three splice forms: the full-length isoform 1, isoform 7, and isoform 9. Isoform 7 is the most highly expressed splice form in rodent brains, while isoform 9 lacks a phosphorylated serine and a methylation signal. These differences between splice forms did not reflect differences among the isoforms in expression levels or axonal localization. Instead, the findings were consistent with a model in which the presence or absence of specific functional domains influences FMRP's role in regulating axonal complexity. To

further investigate this question, we performed a focused structure-function analysis of isoform 7. These studies revealed that the FMRP-induced reduction in axonal complexity was independent of the KH and RGG RNA binding domains. Instead, this reduction required an intact NES and the ability to bidirectionally regulate the phosphorylation of serine 500 (S500). These studies therefore reveal that differential splicing of FMRP may regulate axonal complexity through the selective retention or exclusion of specific functional domains.

METHODS

Constructs

Constructs encoding mouse *Fmr1* splice forms 1, 3, 7, 8, and 9 including the 3' UTRs were provided by Dr. David Morris of the University of Washington. PCR primers (forward: 5'-GAGCTCAAGAGGAGCTGGTGGTGAAGTGC-3'; reverse: 5'-CCCGGGTTAGGGTACTCCATTCACCAGC-3') were used to subclone the coding sequence starting at amino acid two with 5' extensions to introduce *XmaI* and *SacI* restriction sites as well as adenines to place the *Fmr1* coding sequence in the appropriate frame. An N-terminal GFP was then placed upstream of these coding sequences. This cloning strategy is based on one previously used for cloning of human *FMR1* constructs (Darnell et al., 2005b). The resulting fusion constructs were then cloned into a pCAG-based vector. Sequences encoding mouse *Fmr1* splice forms 2, 4, 5, 6, 10, 11, and 12 were obtained as fragments from GeneArt Strings services (Life Technologies) with 5' extensions to introduce *XmaI* and *SacI* restriction sites as well as two adenines to place them in frame with an N-terminal EGFP. A sequence encoding an N-terminal GFP was placed upstream of these coding sequences as above, and the resulting fusion constructs were cloned into a pCAG-based vector. The accuracy of all mouse *Fmr1* splice forms was verified by sequencing. pCAG-based vectors expressing tdTomato or the various mutant forms of human FMRP splice form 7 were gifts from Dr. Jennifer Darnell of Rockefeller University and Dr. Justin Fallon of Brown University.

GFP::FMRP construct expression in HEK293T cells

HEK293T cells were grown to ~80% confluency in 6-well plates and then transfected using 200 ng of DNA and FuGENE transfection reagent (Promega) at a ratio of 3 μ l FuGENE to 1 μ g DNA according to the manufacturer's instructions. Cells were harvested approximately 24-hours post-transfection, washed with chilled 1x PBS, and lysed in RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS). Lysates were flash-frozen and stored at -80°C until use. Aliquots of these lysates were used to quantify total protein concentration using the Pierce BCA Protein Assay Kit (Thermo Fisher, 23225). 1 μ g of total protein from lysates from HEK cells expressing each GFP::FMRP construct or EGFP alone was separated on an 8% SDS-PAGE resolving gel. These blots were probed with primary antibodies against FMRP (1:1000; 2F5-1; Christie et al., 2009) and GFP (1:2000; Thermo Fisher, A11122), washed, then probed with secondary antibodies conjugated to IRDye 680 or IRDye 800 (1:10000; LI-COR Biosciences). After a final wash, these blots were imaged on a LI-COR Odyssey 9120. Each construct gave rise to a single band recognized by both antibodies (Supplemental Figure S1).

Neuronal Cell Cultures

Cover glasses for cortical neuron cultures were cleaned with nitric acid and sterilized with ethanol. They were prepared for cell culture by coating with 50 µg/mL PDL for 48 hours followed by 20 µg/mL laminin for 18 hours. E18 rat cortical neurons were obtained from Neurons-R-Us at the University of Pennsylvania and plated on cover glasses at a density of 400,000 neurons per well in a 24-well plate. Neurons were maintained in Neurobasal media supplemented with GlutaMax, penicillin-streptomycin, and B27 according to the manufacturer's instructions (Thermo Fisher). pCAGES-tdTomato and each FMRP construct were cotransfected at 3 days in vitro. Transfections were performed by magnetofection using Neuromag (Oz Biosciences) at a ratio of 1 µl Neuromag to 1 µg DNA (0.5ug TdTomato; 0.5ug EGFP::FMRP construct per well) according to the manufacturer instructions. For assessment of neuronal morphology in neurons expressing only endogenous FMRP, we transfected cells with 1 µg of the tdTomato plasmid alone. Neurons were fixed 7 days later after a total of 10 days in vitro using warmed 4% PFA/4% sucrose in 1x PBS for 15 minutes. Cultures were then washed 3x in PBS and stained with DAPI (1:10,000). The coverslips were mounted using NPG mounting medium (4% n-propylgallate (Sigma P3130), 85% glycerol, 50 mM phosphate pH 7.4) and stored at 4°C protected from light until imaging.

Imaging, Analysis, and Statistics

Cells selected for imaging exhibited a clear multipolar neuronal morphology. Axons were identified as the single process emerging from the soma that was long, thin, and untapered. We included neurons only if the axons either did not contact labeled processes from other cells or if they crossed these processes in a way that allowed unique identification (e.g., Figure 5). Neurons were imaged with a Leica SPE II confocal microscope using tile-scan and z-stacks to capture the entire length and depth of the axonal arbor using a 40X oil immersion objective (NA=1.15). For the wild type splice forms, the number of neurons examined were: endogenous FMRP, n=9; splice form 1, n=27; splice form 2, n=25; splice form 3, n=26; splice form 4, n=24; splice form 5, n=28; splice form 6, n=26; splice form 7, n=30; splice form 8, n=26; splice form 9, n=25; splice form 10, n=28; splice form 11, n=26; and splice form 12, n=26. For the mutant forms, the number of neurons examined were: endogenous FMRP, n=10; wild type, n=32; I241N, n=30; I304N, n=49; NES34A, n=44; S500A, n=55; S500D, n=48; RGG, n=41; and R290A, n=40. Image stacks were collapsed in the FIJI build of ImageJ. Axons were traced and puncta counted using Neurolucida (MBF Bioscience). Puncta had to be at least 3 pixels in diameter to be included. Traces were analyzed using "Branched Structure Analyses" in Neurolucida Explorer. These analyses provided the number of puncta, distance of each punctum from the soma, the length of the longest axon, the total size of the axonal arbor, complexity of the axonal arbor, highest branch order, the total number of nodes (branch points), and the number of terminal branches. Complexity was defined as $(\text{Sum of terminal orders} + \text{Number of terminals}) * (\text{Total axonal length})$. Branch order for each terminal branch was defined as the number of axonal segments (regions of the axon between nodes) needed to travel from the soma to that terminal branch. Most statistical comparisons were performed using Prism 6.0g for Mac (GraphPad Software). Multiple comparisons were performed using an ANOVA followed by post-hoc t-tests as indicated in results, except in situations where the data substantially violated ANOVA requirements (Bartlett's test: $p < 0.01$). In these cases, the data were instead

analyzed using the nonparametric Kruskal-Wallis test followed by post-hoc t-tests as indicated in the results. For analysis of distribution of individual puncta within the axonal arbor, one-way ANOVA followed by Dunnett t-test controlled for multiple observations of puncta within individual neurons was performed in R 3.1.2 (Pumpkin Helmet). All plots were made in Prism. All graphs represent mean $\pm$ SEM.

RESULTS

Wild type splice forms

FMRP plays roles in a variety of axonal and presynaptic functions. Here, we investigated its role in the regulation of axon growth and branching. In order to investigate potential differential roles for FMRP splice forms in the regulation of axonal arbors, we transfected individual GFP-tagged FMRP splice forms into cultured wild type rat cortical neurons that had been grown in vitro for three days. We co-transfected these neurons with tdTomato in order to visualize the entire neuronal arbor. We examined 12 splice forms of mouse FMRP, each containing specific functional domains (Figure 1). GFP::FMRP and tdTomato expression in these neurons was then visualized one week later after a total of ten days in vitro. This approach allowed us to quantify the morphology of the axonal arbors that were overexpressing FMRP during a period of robust axonal outgrowth (Dotti et al., 1988). Further, we could assess the distribution of GFP::FMRP within that axonal arbor.

We began by asking whether overexpressing individual FMRP splice forms resulted in a decrease in axonal complexity. Complexity was measured using a standardized formula that reflects the total length of the axonal arbor as well as the number of branches (see Methods). Indeed, axonal complexity was altered in FMRP-overexpressing neurons (Figure 2D; Kruskal-Wallis, $p < 0.0001$). Curiously, not all splice forms exhibited the ability to alter axonal complexity. Dunn's pairwise comparisons between neurons overexpressing individual splice forms and control neurons indicated that splice forms 1 ($p = 0.0345$), 7 ($p = 0.0034$), and 9 ($p = 0.0042$) had significantly reduced axonal complexities while the other splice forms did not ($p > 0.05$). Elevated expression of specific FMRP splice forms thus resulted in a decrease in axonal complexity.

A reduction in complexity could reflect a decrease in the size of the axonal arbor and/or the number of branches in that arbor. We addressed the first possibility by assessing axon growth via two related but distinct measures. In the first measure, we examined the total length of all axonal branches in neurons expressing GFP::FMRP constructs. Overexpression of the FMRP splice forms resulted in a reduction in the summed length of all axonal branches (Figure 2E), as compared to control cells expressing tdTomato alone (ANOVA, $p = 0.0006$), suggesting that FMRP regulates axon growth. Of the individual splice forms, overexpression of splice form 1 (Dunnett's, $p = 0.0493$) and splice form 9 (Dunnett's, $p = 0.0422$) resulted in significant reductions in total length. Specific splice forms of FMRP therefore regulate the total size of the axonal arbor.

A reduction in total size could reflect either decreased outgrowth by individual branches or a reduction in the number of branches. To address the first possibility, we examined whether overexpression of FMRP altered the length of the longest single axon branch. We observed

no detectable effect on the length of the longest axon branch in cells expressing any of the splice forms (Supplemental Figure S2A; ANOVA, $p=0.1473$). FMRP therefore does not appear to regulate axon arbor size by altering the outgrowth of individual portions of that arbor.

A decrease in the total size of the axonal arbor with no change in the outgrowth of the longest branch suggests that FMRP regulates axonal arborization via limiting the number of branches. We therefore asked whether complexity differences reflected alterations in the branching patterns of the axonal arbor. We assessed axonal branching in each neuron via three related measures: the total number of nodes (branch points) across the entire arbor, the total number of terminal branches, and the highest branch order (see Methods).

Overexpression of FMRP splice forms affected each of these measures (Kruskal-Wallis, $p<0.0001$ for all). Post-hoc pairwise comparisons revealed that overexpressing splice form 1, 7, or 9 (Dunn's; $p=0.0268$, $p=0.0019$, $p=0.0074$, respectively) resulted in a lower branch order (Figure 2F). Interestingly, despite affecting branch order, overexpression of splice form 1 did not detectably alter either the number of nodes or the total number of terminal branches (although both findings trended towards significance; Dunn's, $p=0.0864$ and $p=0.0877$, respectively). These observations suggest that splice form 1 results in a rearrangement of the axonal arbor such that nodes occur more often in the proximal than the distal axon. In contrast, overexpressing either splice form 7 (Dunn's, $p=0.0024$) or splice form 9 (Dunn's, $p=0.0064$) resulted in a decrease in the total number of nodes across the entire arbor (Supplemental Figure S3A). Similarly, the total number of terminal branches (Supplemental Figure S3B) was also diminished in neurons overexpressing splice form 7 (Dunn's, $p=0.0025$) or splice form 9 (Dunn's, $p=0.0059$). No other splice form detectably altered axonal arbor complexity (Dunn's, $p>0.05$). FMRP splice forms 1, 7, and 9 therefore regulate axonal arbor morphology by affecting branching.

Differential regulation of the axonal arbor by the various FMRP splice forms could, in principle, reflect differences in protein abundance, localization, or function. To address the first possibility, we examined splice form abundance by measuring GFP intensity in individual neurons expressing each of the splice forms. There were no differences in the expression levels between splice form constructs (ANOVA, $p = 0.6694$; Supplemental Figure S4A). The observed differences in axonal complexity among the isoforms therefore do not reflect variation in expression levels of the isoforms.

We next asked whether differences in the axonal complexity could be explained by differential axonal localization of the isoforms. We could readily detect axonal GFP::FMRP expression for each of the splice forms (Figure 3), indicating that transport into axons is not sufficient for FMRP to regulate axon morphology. We therefore asked whether splice forms that modulate axonal complexity exhibit differential efficiency in axonal localization. For this assay, we counted the total number of GFP puncta in each axonal arbor and normalized it to the total length of that axonal arbor. This analysis revealed an overall difference in puncta density between the splice forms (Figure 3B; Kruskal-Wallis, $p=0.0125$). Pairwise comparisons among the splice forms indicated that splice form 3 showed less axonal localization than splice forms 9 and 12 (Figure 3B; $p=0.0315$ and $p=0.0309$, respectively, by Dunn's post-hoc t-test). No other pairwise comparisons reached significance (Dunn's,

$p>0.05$). Notably, no differences were observed between the splice forms that regulate axonal complexity and the other splice forms. The number of FMRP-containing puncta in the axon therefore does not correlate with the ability of that FMRP to reduce axonal complexity.

In addition to the total number of axonal puncta, we asked whether these puncta were distributed differently within the axonal arbor. To address this question, we measured the distance that each individual GFP punctum was found along an axon from the cell soma. Comparing the overall axonal distribution of these puncta across the splice forms revealed that each of the splice forms, when controlling for the total number of puncta, was transported equivalent distances into the axonal arbor (Figure 3C; ANOVA, $p=0.4203$). All splice forms therefore localize to axons with only slight differences between the constructs in the number of FMRP puncta formed. Differential axonal localization therefore cannot account for differences in axonal complexity among the isoforms.

Mutant isoforms of splice form 7

Differences among the splice forms in regulation of the axonal arbor appear to reflect the selective retention or exclusion of specific functional domains. We therefore asked whether mutating functional domains in a particular splice form would similarly modulate axonal arbor complexity. Splice form 7 is the most highly expressed isoform in the developing mouse brain (Brackett et al., 2013), and our results show that selective overexpression of this splice form reduces axonal arbor complexity. Human splice form 7 constructs have been extensively tested in mouse and rat neurons, where the wild type construct associates with polyribosomes, localizes normally to dendrites, and rescues morphological and electrophysiological phenotypes of FMRP null neurons (Antar et al., 2004, 2006; Darnell et al., 2005b; Pfeiffer and Huber, 2007). We therefore used wild type and mutant forms of human splice form 7 (hSF7) to examine how functional domains in FMRP contribute to the regulation of axonal morphology. The mutations examined included mutations that impact RNA binding domains, regulation of translation by a conserved serine, and the nuclear export sequence. These mutations collectively allow us to interrogate the effect of mutations that affect RNA binding as well as other distinct FMRP functions.

As above, in these studies we cotransfected neurons with tdTomato and GFP-tagged FMRP constructs and assessed both the morphology of the axonal arbor and the distribution of FMRP within that arbor. Across this set of experiments, a small number of neurons (30/338; 8.88%) did not have detectable GFP puncta in their axons. Unfortunately, their small number prevented a meaningful comparison to neurons that did exhibit axonal localization of the same construct. Therefore neurons lacking axonal GFP-tagged FMRP were excluded from further analyses.

We first examined the effects of overexpressing the wild type construct on axonal arbor complexity. Neurons overexpressing this wild type FMRP exhibited significantly reduced axonal arbor complexity as compared to neurons expressing only endogenous FMRP (Supplemental Figure S5A; two-tailed t-test, $p<0.0001$). We then asked whether the various mutant forms (discussed below) of hSF7 FMRP differed in their effects on axonal complexity. There was an overall difference in axonal complexity among the hSF7 isoforms

(Figure 4A; Kruskal Wallis, $p=0.0038$), indicating that at least some of these functional domains impact the regulation of axonal complexity by FMRP.

We began by analyzing whether mutations that impact RNA binding had an effect on the regulation of axonal complexity. FMRP has three well-characterized RNA-binding domains, two KH domains (KH1 and KH2) and an RGG box. Here we examined three functionally similar mutations in the KH domains (I241N, R290A, and I304N). The KH domains bind to a particular RNA structure termed a “kissing complex” (Darnell et al., 2005a). An I304N missense mutation in KH2, identified in a patient with a particularly severe form of Fragile X, greatly diminishes FMRP association with kissing complex RNAs and polyribosomes and results in the full Fragile X phenotype in mouse models (De Boulle et al., 1993; Feng et al., 1997a; Lagerbauer et al., 2001; Darnell et al., 2005a; Zang et al., 2009). The RGG box of FMRP binds a different RNA structure, the G-quadruplex, that is found in a subset of FMRP target mRNAs (Darnell et al., 2001; Ramos et al., 2003; Vasilyev et al., 2015). Deletion of the RGG box therefore inhibits association of FMRP with G-quadruplex-containing mRNAs. Interestingly, pairwise comparisons between neurons overexpressing the mutant forms and the wild type forms revealed that mutations affecting RNA binding (I241N, R290A, I304N, and RGG) did not alter the regulation of axonal complexity by FMRP (Figure 4A; Dunn's, $p>0.05$). This regulation therefore appears to be independent of RNA binding.

We next asked whether mutations in the conserved serine 500 (S500) impacted FMRP regulation of axonal complexity. The ability of FMRP to inhibit translation of its target mRNAs is impacted by the phosphorylation state of serine 500 (S500). Phosphorylated FMRP inhibits translation without affecting FMRP binding to mRNA (Ceman et al., 2003). The S500A phospho-null mutant therefore mimics a state in which FMRP is permissive to ongoing translation while the S500D phosphomimetic mutant mimics constitutive repression of translation. Curiously, both the S500A (Dunn's, $p=0.0045$) and S500D (Dunn's, $p=0.0172$) mutants diminished the effect of overexpressed FMRP on axonal morphology (Figure 4A). These findings therefore implicate the phosphorylation of FMRP S500 in the regulation of the axonal arbor.

Finally, we examined mutations in the NES, which facilitates mRNA-bound FMRP export from the nucleus (Eberhart et al., 1996; Sittler et al., 1996). This domain is not absolutely required for nuclear export, as FMRP lacking this domain can be exported from the nucleus in a complex with other NES-containing proteins (Kim et al., 2008; Levenga et al., 2009). FMRP's regulation of axonal complexity was affected in the NES34A mutant (Figure 4A; Dunn's, $p=0.0015$). As discussed in subsequent paragraphs, this mutant was not retained in the nucleus and was quite efficiently transported into axons. Therefore an intact NES is required for FMRP regulation of axonal morphology, independent of any role this domain might play in export from the nucleus.

As with the splice forms, we examined whether this alteration in complexity reflected changes in axon elongation and/or in the number of branches. In contrast to neurons overexpressing the mouse splice form 7 discussed above, neurons overexpressing wild type hSF7 exhibited a reduction in axonal arbor size (Supplemental Figure S5B; two-tailed t-test,

$p=0.0024$). We next asked if any of the mutants modified this effect on the axonal arbor. However, pairwise comparisons revealed no differences between these mutants and the wild type in their modulation of arbor size (Figure 4B, Dunn's; $p>0.05$ for all). As with the splice forms, the various mutant forms had no effect on the size of the longest individual axon branch (ANOVA, $p=0.0785$; Supplemental Figure S2B). These findings therefore suggest that differences in complexity among the mutant forms reflected differential effects on the number of branches.

As above, we investigated the possibility that alterations in complexity arose from perturbations of the axonal branching patterns. Overexpression of the wild type hSF7 resulted in a decrease in the highest branch order, total number of nodes, and terminal branch number (Supplemental Figure S5C-E, two-tailed t-test; $p<0.0001$ for each). We next asked which domains of FMRP are required for this regulation of branching by comparing each mutant hSF7 to the wild type. Comparisons among neurons overexpressing the various hSF7 FMRP constructs indicated that the mutations affected highest branch order, total number of nodes, and terminal branch number (Figure 4C-D, Supplemental Figure S3C; Kruskal-Wallis, $p<0.0001$ for each). As with complexity, RNA binding domains were not required for the effect on branching as the I241N, R290A, I304N, and RGG mutant forms had no effect on any of these measures (Figure 4C-D, Supplemental Figure S3C; Dunn's, $p>0.05$ for each construct/measure). Relative to neurons expressing the wild type FMRP, branch order was altered in neurons expressing NES34A (Dunn's, $p<0.0001$), S500A (Dunn's, $p=0.0014$), or S500D (Dunn's, $p=0.0008$) mutants. Similarly, the total number of nodes was altered by NES34A (Dunn's, $p=0.0003$), S500A (Dunn's, $p=0.0085$), and S500D (Dunn's, $p=0.0025$) mutations. Finally, the number of terminal branches was also altered by these same mutations (Dunn's: NES34A, $p=0.0003$; S500A, $p=0.0106$; S500D, $p=0.0033$). Regulation of axonal branching by FMRP therefore requires S500 and an intact NES, but is independent of RNA binding.

To determine whether the differences among the mutant constructs reflected differential expression levels, we measured the somatic GFP fluorescence in neurons expressing each of the mutant constructs. Overall, the GFP fluorescence varied among the various constructs (Supplemental Figure S4C; ANOVA, $p=0.0152$). However, as with the splice forms, the expression of each of the EGFP::FMRP mutant forms was not detectably different from the expression of the wild type form, with only I304N approaching significance (Supplemental Figure S4C, $p=0.0681$). The effects observed above on axonal complexity and branching therefore do not reflect differences in expression levels among the various mutants.

We next asked whether differing effects on the axonal arbor complexity could be accounted for by differences in axonal distribution of these mutant constructs. As above, only neurons in which GFP::FMRP localized to the axon were included in this analysis. As an assay of the efficiency of axonal localization, we measured the number of puncta in each axonal arbor normalized to the total length of that arbor. As a group, mutant forms exhibited differential localization (Figure 4E; Kruskal-Wallis, $p<0.0001$). S500A, S500D, and RGG mutant isoforms exhibited significantly fewer puncta localized within the axon, compared to the wild type construct (Dunn's: $p<0.0001$, $p<0.0001$, and $p=0.0408$, respectively; Figure 4E). The other constructs were indistinguishable from the wild type construct in terms of the

number of axonal puncta per length of the axon (Dunn's, $p > 0.05$). Mutations affecting the RGG box or phosphorylation at S500 therefore impact the frequency of axonal GFP::FMRP puncta. The limited number of axonal puncta in S500 mutants may contribute to the reduced regulation of axonal complexity by these constructs.

We next asked whether the various mutant forms exhibited differential localization within the axonal arbor, independent of the total number of puncta exhibiting axonal localization. The distance of individual puncta from the soma was measured. As a group, the localization was significantly different among the mutant forms (Figure 4F; ANOVA, $p = 0.003908$). Surprisingly, the only mutant form to exhibit differential efficiency of transport relative to the wild type construct was the NES34A mutant, which exhibited increased localization to the distal axonal arbor (Figure 4F; Dunnett, $p = 0.00428$). As differences in localization do not correlate with differences in complexity, differential distribution of GFP::FMRP puncta within the axonal arbor is unlikely to account for the effect of FMRP on complexity.

Elongated fibril-like structures

An interesting but uncommon feature of some wild type splice forms was the formation of elongated structures. In contrast to puncta, which had an apparent size of $\sim 1 \mu\text{m}$, the elongated fibril-like structures were up to $15 \mu\text{m}$ in length (Figure 5A). These elongated structures were most commonly seen in neurons expressing splice form 11, in which $\sim 20\%$ of neurons contained such structures (Table 1). These elongated FMRP structures were not observed in neurons expressing splice form 3, splice form 7, or splice form 8. Several of the mutant FMRP forms exhibited particularly elongated structures (Figure 5B) that differed markedly from the more commonly observed puncta. Further, in contrast to the fibril-like structures seen with the normal splice forms, which never exceeded $15 \mu\text{m}$ in length, several mutant forms (I241N, I304N, and NES34A) exhibited structures that were observed extending as long as $175 \mu\text{m}$ without break. Particular forms of FMRP thus appear prone to the formation of fibril-like structures.

DISCUSSION

Summary

FMRP exists as a combination of twelve different splice forms, whose relative abundance varies with both brain region and age (Verkerk et al., 1993; Denman and Sung, 2002; Dolzhanskaya et al., 2006; Xie et al., 2009; Brackett et al., 2013). Here we investigated how these splice forms regulate the morphology of the axonal arbor by overexpressing each splice form individually in cultured wild type cortical neurons. Splice forms 1, 7, and 9 altered the morphology of the axons, resulting in a reduction in the complexity of the axonal arbor. These findings were not consistent with a role for FMRP in the regulation of axonal outgrowth. Instead, FMRP appears to regulate axonal complexity largely by limiting branching. To gain further insight into this regulation, we further examined the domains required for complexity reduction by splice form 7, the most highly expressed splice form in the brain. These studies do not support a requirement for intact RNA binding by the KH domains in the regulation of axonal complexity. Instead, our findings implicate both the NES

and phosphorylation of S500 in splice form-dependent regulation of axonal arbor complexity by FMRP.

Axonal arbor complexity

Our findings provide evidence that FMRP-dependent regulation of axonal arbor complexity is carried out primarily by three FMRP splice forms. These three splice forms are expressed at low levels during most of early development, with a peak in late embryonic development (Brackett et al., 2013). During postnatal development, there is an increase with age in the expression of all three, in particular splice forms 7 and 9 (Brackett et al., 2013). These findings suggest a model in which the balance of FMRP splice forms favors axon elongation and branching during early development, with a shift towards restricting these processes in the adult nervous system. It will be of interest to determine whether paradigms that induce axonal structural plasticity in adult animals, such as seizure and injury models that lead to axonal sprouting, affect the relative levels of FMRP splice forms.

Our results are consistent with experiments in *Drosophila* in which overexpression of dFMR led to a simplified axonal arbor as well as with experiments from both rodents and flies in which loss of FMRP/dFMR resulted in overelaborate axonal arbors (Dockendorff et al., 2002; Ivanco and Greenough, 2002; Morales et al., 2002; Michel et al., 2004). The differential effects on axonal complexity seen among the various constructs did not obviously correspond to expression levels of the constructs or assembly into fibrils. As all of the FMRP isoforms tested exhibited axonal localization, we could not directly test whether FMRP regulates axonal structure primarily locally in the axon or in the soma. However, FMRP isoforms with a mutated S500 had reduced axonal localization as well as impaired regulation of the axonal arbor, raising the possibility that at least a portion of the structural regulation occurs via local control within the axonal arbor. Future studies will be required to disentangle the contribution of S500 to axonal localization from its regulation of axonal complexity. Our findings thus provide insights into the mechanisms underlying regulation of the axonal arbor and the domains of FMRP critical for this regulation.

Binding of the axon guidance molecule Semaphorin-3A to receptors on the axon leads to growth cone collapse in a local protein synthesis-dependent manner (Campbell and Holt, 2001). The ability of Semaphorin-3A to cause growth cone collapse is severely attenuated in the absence of FMRP, contributing to a hypercomplex axonal arbor in FMRP null neurons (Li et al., 2009). This FMRP-dependent response includes local synthesis of the microtubule associated protein MAP1b (Li et al., 2009), but may also involve other FMRP targets such as calmodulin (Wang et al., 2015). Whether these effects arise solely from altered axonal translation or also include perturbations in FMRP-dependent axonal RNA transport remains unclear (Wang et al., 2015; Zhang et al., 2015). Together with our findings that overexpression of FMRP leads to a decrease in axon complexity, these observations suggest a model in which axonal responses to axonal guidance cues are mediated and/or enhanced by local regulation of translation by axonal FMRP.

The phosphorylation state of S500 modulates how FMRP regulates translation, with phospho-FMRP repressing translation. The presence of this serine is not required to regulate axonal complexity, as splice form 9, which lacks S500, suppressed axonal complexity. Given

this finding, it was surprising that mutations in S500 abrogate the ability of splice form 7 to reduce axonal complexity. Curiously, this effect is seen in both the phosphomimetic and phosphonull mutations, suggesting that bidirectional regulation of phosphorylation of this residue is required. Alternately, the presence of S500A or S500D within an FMRP granule may alter the function and/or presence of other granule components. It should also be noted that splice form 9 also does not have a functional RGG box (Dolzhanskaya et al., 2006). In contrast, splice form 8, which lacks S500 but contains a functional RGG box, did not detectably impact axonal morphology (although the complexity measure approached significance, $p=0.0882$). Therefore, S500 may only modulate FMRP's effect on the axonal arbor when FMRP can interact with RNA via its RGG box. Directly testing this possibility would require analyzing the effects of double mutants in which S500 mutants are combined with deletion of the RGG box.

In addition to regulating translation, FMRP also regulates the function and distribution of axonal and presynaptic ion channels (Deng et al., 2013; Ferron et al., 2014). For example, FMRP directly binds to and gates ion channels independent of the RNA binding domains as N-terminal fragments of FMRP are sufficient to carry out these effects (Brown et al., 2010; Deng et al., 2013). Our findings are consistent with FMRP regulating axonal complexity in a manner that is independent of RNA binding and translational control. Two of the splice forms that regulate axonal complexity, splice forms 1 and 7, differ only in that splice form 7 has a shorter KH2 RNA binding domain than does splice form 1. The inclusion of exon 12 in splice form 1 reduces affinity of FMRP for both kissing complex RNAs and polyribosomes (Darnell et al., 2009). These observations suggest that either the effect on axon arbors is independent of RNA binding or that the specific binding activity is not affected by the exclusion of exon 12 in splice form 7. Further consistent with the suggestion that RNA binding is not required for axonal arbor modification, mutations in the KH and RGG RNA binding domains of splice form 7 do not modify the effect of this splice variant on axon arborization. Exogenous FMRP can therefore exert its influence on axonal morphology in an RNA binding-independent manner. This control may be exerted either by altering the composition of other FMRP-containing RNA granules and/or through interactions with other proteins such as ion channels.

Axonal localization

FMRP localizes to axonal granules in both the brain and in cultured neurons (Feng et al., 1997b; Antar et al., 2006; Price et al., 2006; Christie et al., 2009; Akins et al., 2012). Our results indicate that, in principle, all FMRP splice variants may be components of these axonal FMRP granules. Surprisingly, the hSF7 mutant lacking the NES exhibited the most efficient localization to the distal axon. NES-lacking FMRP forms are retained in the nucleus when expressed in an *Fmr1* null background (Sittler et al., 1996). However, coexpression of NES-lacking FMRP constructs in the presence of an NES-containing isoform leads to nuclear export of both proteins in a single complex (Levenga et al., 2009). Since NES-lacking FMRP is expressed along with NES-containing splice forms in the wild type brain (Brackett et al., 2013), this suggests that endogenous splice forms lacking the nuclear export signal are at least partially resident in the cytoplasm normally. Whether the NES influences the composition of the FMRP-containing granules that are exported from

the nucleus is unknown. However, the increased ability of the NES mutant to be transported to the distal axon suggests that an intact NES facilitates FMRP interaction with proteins and/or complexes that are preferentially retained in the cell body or proximal axon.

The ability of all splice forms to localize to axons suggests that the sequences required for this transport are common to all these forms. As all FMRP splice forms are identical for the first eleven exons, these findings suggest that interactions mediated by the N-terminus are required for axonal transport. FMRP interacts with many proteins through its N-terminus, including the related proteins FXR1P and FXR2P as well as 82-FIP, NUFIP, and CYFIP (Zhang et al., 1995; Bardoni et al., 1999, 2003; Schenck et al., 2001; Ramos et al., 2006). FMRP likely associates with a subset of these proteins to form granules that are transported into axons. The only differences observed among splice forms in axonal localization were fewer axonal puncta containing splice form 3 as compared to splice forms 9 and 12. Of these isoforms, only splice form 3 contains exon 12, which interferes with FMRP binding of RNA (Darnell et al., 2009). Why this difference is not consistent among splice forms that contain or lack exon 12 is not currently clear. However, this observation suggests that RNA binding may facilitate, but is not required for, axonal localization depending on other motifs present in the molecule. Determining the FMRP domains that influence axonal localization requires a detailed structure-function analysis of other splice forms analogous to the studies that we conducted here on splice form 7.

As our experiments were conducted in the context of wild type FMRP expression, the GFP::FMRP is expected to assemble into granules along with the endogenous FMRP. At this point, we are unable to determine the number of FMRP molecules in an individual granule or whether the endogenous granules contain multiple splice forms. However, as neurons normally contain a mix of FMRP splice forms, axonal FMRP granules also most likely contain a combination of splice forms. All twelve splice forms are expressed in neurons in a complex pattern that reflects both cell type and developmental stage (Brackett et al., 2013). As the splice forms exhibit differences in both functional and regulatory motifs, variation in the abundance of FMRP splice forms could lead to a diverse population of axonal FMRP granules whose function varies among brain regions as well as throughout development.

Fibril formation

In a subset of neurons, overexpressed FMRP was found in both granules and elongated fibril-like structures. RNA binding proteins can assemble in vitro into fibrils upon RNA binding, a process that is thought to resemble the assembly into granules in the normal cellular environment (Buchan, 2014; Calabretta and Richard, 2015; Xiang et al., 2015). This assembly into fibrils is aided by the binding of RNA and the consequent changes in protein conformation. The normal splice variants occasionally assembled into small fibril-like structures, a phenomenon that may reflect an exaggerated version of normal FMRP oligomerization due to increased concentration of these proteins in transfected neurons. Further, we cannot rule out the possibility that the formation of these structures may be initiated by the GFP tag itself rather than the FMRP. However, the differences among the various isoforms in the size and frequency of these structures suggest that the FMRP sequence influences the assembly of these structures. In particular, several mutant forms of

splice form 7 (I241N, I304N, and NES34A) exhibited especially elongated structures that were found in approximately half of the transfected neurons. Whether these represent an even more exaggerated form of fibril formation seen with wild type FMRP or an entirely novel structure present only in the mutants is unclear. These findings thus raise the possibility that the KH and NES domains normally modulate FMRP oligomerization and that mutations in these domains dysregulate this process.

Conclusion

Taken together, our findings suggest that the relative abundance of the FMRP splice forms expressed by a cell impact the axonal morphology of that cell by regulating axonal branching. The FMRP-induced regulation of axonal complexity was independent of the KH and RGG RNA binding domains. Instead, this reduction required an intact NES and the ability to bidirectionally regulate the phosphorylation of serine 500 (S500). These studies therefore reveal that FMRP exhibits domain-specific functions in the regulation of axon morphology in a splice form-dependent manner. Natural variation in the relative abundance of FMRP splice forms among neuronal cell types may contribute to the diversity of axonal morphology seen throughout the mammalian nervous system. Further, loss of all FMRP isoforms in Fragile X syndrome may lead to circuit-dependent changes in axonal morphology, and therefore function, that reflect differential FMRP splice form expression across brain regions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Grant support: MH090237 to MRA.

We thank K. Shepard for technical assistance. We thank D. Morris, J. Darnell, and J. Fallon for generously supplying plasmids for use in these studies.

References

- Akins MR, LeBlanc HF, Stackpole EE, Chyung E, Fallon JR. Systematic mapping of fragile X granules in the mouse brain reveals a potential role for presynaptic FMRP in sensorimotor functions. *J Comp Neurol.* 2012; 520:3687–3706. [PubMed: 22522693]
- Antar LN, Afroz R, Dictenberg JB, Carroll RC, Bassell GJ. Metabotropic Glutamate Receptor Activation Regulates Fragile X Mental Retardation Protein and Fmr1 mRNA Localization Differentially in Dendrites and at Synapses. *J Neurosci.* 2004; 24:2648–2655. [PubMed: 15028757]
- Antar LN, Li C, Zhang H, Carroll RC, Bassell GJ. Local functions for FMRP in axon growth cone motility and activity-dependent regulation of filopodia and spine synapses. *Mol Cell Neurosci.* 2006; 32:37–48. [PubMed: 16631377]
- Bardoni B, Castets M, Huot M-E, Schenck A, Adinolfi S, Corbin F, Pastore A, Khandjian EW, Mandel J-L. 82-FIP, a novel FMRP (Fragile X Mental Retardation Protein) interacting protein, shows a cell cycle-dependent intracellular localization. *Hum Mol Genet.* 2003; 12:1689–1698. [PubMed: 12837692]
- Bardoni B, Schenck A, Mandel J. A novel RNA-binding nuclear protein that interacts with the fragile X mental retardation (FMR1) protein. *Hum Mol Genet.* 1999; 8:2557–2566. [PubMed: 10556305]

- Bassell GJ, Warren ST. Fragile X Syndrome: Loss of Local mRNA Regulation Alters Synaptic Development and Function. *Neuron*. 2008; 60:201–214. [PubMed: 18957214]
- Bhakar AL, Dölen G, Bear MF. The Pathophysiology of Fragile X (and What It Teaches Us about Synapses). *Annu Rev Neurosci*. 2012; 35:417–443. [PubMed: 22483044]
- Brackett DM, Qing F, Amieux PS, Sellers DL, Horner PJ, Morris DR. Fmr1 Transcript Isoforms: Association with Polyribosomes; Regional and Developmental Expression in Mouse Brain. *PLoS ONE*. 2013; 8:e58296. [PubMed: 23505481]
- Brown MR, Kronengold J, Gazula V-R, Chen Y, Strumbos JG, Sigworth FJ, Navaratnam D, Kaczmarek LK. Fragile X mental retardation protein controls gating of the sodium-activated potassium channel Slack. *Nat Neurosci*. 2010; 13:819–821. [PubMed: 20512134]
- Buchan JR. mRNP granules. *RNA Biol*. 2014; 11:1019–1030. [PubMed: 25531407]
- Calabretta S, Richard S. Emerging Roles of Disordered Sequences in RNA-Binding Proteins. *Trends Biochem Sci*. 2015; 40:662–672. [PubMed: 26481498]
- Campbell DS, Holt CE. Chemotropic Responses of Retinal Growth Cones Mediated by Rapid Local Protein Synthesis and Degradation. *Neuron*. 2001; 32:1013–1026. [PubMed: 11754834]
- Ceman S, O'Donnell WT, Reed M, Patton S, Pohl J, Warren ST. Phosphorylation influences the translation state of FMRP-associated polyribosomes. *Hum Mol Genet*. 2003; 12:3295–3305. [PubMed: 14570712]
- Christie SB, Akins MR, Schwob JE, Fallon JR. The FXG: A Presynaptic Fragile X Granule Expressed in a Subset of Developing Brain Circuits. *J Neurosci*. 2009; 29:1514–1524. [PubMed: 19193898]
- Darnell JC, Fraser CE, Mostovetsky O, Darnell RB. Discrimination of common and unique RNA-binding activities among Fragile X mental retardation protein paralogs. *Hum Mol Genet*. 2009; 18:3164–3177. [PubMed: 19487368]
- Darnell JC, Fraser CE, Mostovetsky O, Stefani G, Jones TA, Eddy SR, Darnell RB. Kissing complex RNAs mediate interaction between the Fragile-X mental retardation protein KH2 domain and brain polyribosomes. *Genes Dev*. 2005a; 19:903–918. [PubMed: 15805463]
- Darnell JC, Jensen KB, Jin P, Brown V, Warren ST, Darnell RB. Fragile X Mental Retardation Protein Targets G Quartet mRNAs Important for Neuronal Function. *Cell*. 2001; 107:489–499. [PubMed: 11719189]
- Darnell JC, Mostovetsky O, Darnell RB. FMRP RNA targets: identification and validation. *Genes Brain Behav*. 2005b; 4:341–349. [PubMed: 16098133]
- De Boule K, Verkerk AJMH, Reyniers E, Vits L, Hendrickx J, Van Roy B, Van Den Bos F, de Graaff E, Oostra BA, Willems PJ. A point mutation in the FMR-1 gene associated with fragile X mental retardation. *Nat Genet*. 1993; 3:31–35. [PubMed: 8490650]
- Deng P-Y, Rotman Z, Blundon JA, Cho Y, Cui J, Cavalli V, Zakharenko SS, Klyachko VA. FMRP Regulates Neurotransmitter Release and Synaptic Information Transmission by Modulating Action Potential Duration via BK Channels. *Neuron*. 2013; 77:696–711. [PubMed: 23439122]
- Denman RB, Sung YJ. Species-Specific and Isoform-Specific RNA Binding of Human and Mouse Fragile X Mental Retardation Proteins. *Biochem Biophys Res Commun*. 2002; 292:1063–1069. [PubMed: 11944923]
- Dockendorff TC, Su HS, McBride SMJ, Yang Z, Choi CH, Siwicki KK, Sehgal A, Jongens TA. *Drosophila* Lacking dfmr1 Activity Show Defects in Circadian Output and Fail to Maintain Courtship Interest. *Neuron*. 2002; 34:973–984. [PubMed: 12086644]
- Dolzanskaya N, Merz G, Denman RB. Alternative splicing modulates protein arginine methyltransferase-dependent methylation of fragile X syndrome mental retardation protein. *Biochemistry (Mosc)*. 2006; 45:10385–10393.
- Dotti CG, Sullivan CA, Banker GA. The establishment of polarity by hippocampal neurons in culture. *J Neurosci*. 1988; 8:1454–1468. [PubMed: 3282038]
- Eberhart DE, Malter HE, Feng Y, Warren ST. The Fragile X Mental Retardation Protein is a Ribonucleoprotein Containing Both Nuclear Localization and Nuclear Export Signals. *Hum Mol Genet*. 1996; 5:1083–1091. [PubMed: 8842725]
- Feng Y, Absher D, Eberhart DE, Brown V, Malter HE, Warren ST. FMRP Associates with Polyribosomes as an mRNP, and the I304N Mutation of Severe Fragile X Syndrome Abolishes This Association. *Mol Cell*. 1997a; 1:109–118. [PubMed: 9659908]

- Feng Y, Gutekunst C-A, Eberhart DE, Yi H, Warren ST, Hersch SM. Fragile X Mental Retardation Protein: Nucleocytoplasmic Shuttling and Association with Somatodendritic Ribosomes. *J Neurosci*. 1997b; 17:1539–1547. [PubMed: 9030614]
- Ferron L, Nieto-Rostro M, Cassidy JS, Dolphin AC. Fragile X mental retardation protein controls synaptic vesicle exocytosis by modulating N-type calcium channel density. *Nat Commun*. 2014; 5:3628. [PubMed: 24709664]
- Ivanco TL, Greenough WT. Altered mossy fiber distributions in adult Fmr1 (FVB) knockout mice. *Hippocampus*. 2002; 12:47–54. [PubMed: 11918288]
- Kim M, Bellini M, Ceman S. Fragile X mental retardation protein FMRP binds mRNAs in the nucleus. *Mol Cell Biol*. 2008 MCB.01377–08.
- Laggerbauer B, Ostareck D, Keidel E-M, Ostareck-Lederer A, Fischer U. Evidence that fragile X mental retardation protein is a negative regulator of translation. *Hum Mol Genet*. 2001; 10:329–338. [PubMed: 11157796]
- Levenga J, Buijsen RAM, Rifé M, Moine H, Nelson DL, Oostra BA, Willemsen R, de Vrij FMS. Ultrastructural analysis of the functional domains in FMRP using primary hippocampal mouse neurons. *Neurobiol Dis*. 2009; 35:241–250. [PubMed: 19464371]
- Li C, Bassell GJ, Sasaki Y. Fragile X mental retardation protein is involved in protein synthesis-dependent collapse of growth cones induced by Semaphorin-3A. *Front Neural Circuits*. 2009; 3:11. [PubMed: 19826618]
- Michel CI, Kraft R, Restifo LL. Defective Neuronal Development in the Mushroom Bodies of *Drosophila* Fragile X Mental Retardation 1 Mutants. *J Neurosci*. 2004; 24:5798–5809. [PubMed: 15215302]
- Morales J, Hiesinger PR, Schroeder AJ, Kume K, Verstreken P, Jackson FR, Nelson DL, Hassan BA. *Drosophila* Fragile X Protein, DFXR, Regulates Neuronal Morphology and Function in the Brain. *Neuron*. 2002; 34:961–972. [PubMed: 12086643]
- Pan L, Zhang YQ, Woodruff E, Broadie K. The *Drosophila* Fragile X Gene Negatively Regulates Neuronal Elaboration and Synaptic Differentiation. *Curr Biol*. 2004; 14:1863–1870. [PubMed: 15498496]
- Penagarikano O, Mulle JG, Warren ST. The Pathophysiology of Fragile X Syndrome. *Annu Rev Genomics Hum Genet*. 2007; 8:109–129. [PubMed: 17477822]
- Pfeiffer BE, Huber KM. Fragile X Mental Retardation Protein Induces Synapse Loss through Acute Postsynaptic Translational Regulation. *J Neurosci*. 2007; 27:3120–3130. [PubMed: 17376973]
- Price TJ, Flores CM, Cervero F, Hargreaves KM. The RNA binding and transport proteins stau and fragile X mental retardation protein are expressed by rat primary afferent neurons and localize to peripheral and central axons. *Neuroscience*. 2006; 141:2107–2116. [PubMed: 16809002]
- Ramos A, Hollingworth D, Adinolfi S, Castets M, Kelly G, Frenkiel TA, Bardoni B, Pastore A. The Structure of the N-Terminal Domain of the Fragile X Mental Retardation Protein: A Platform for Protein-Protein Interaction. *Structure*. 2006; 14:21–31. [PubMed: 16407062]
- Ramos A, Hollingworth D, Pastore A. G-quartet-dependent recognition between the FMRP RGG box and RNA. *RNA*. 2003; 9:1198–1207. [PubMed: 13130134]
- Schenck A, Bardoni B, Moro A, Bagni C, Mandel J-L. A highly conserved protein family interacting with the fragile X mental retardation protein (FMRP) and displaying selective interactions with FMRP-related proteins FXR1P and FXR2P. *Proc Natl Acad Sci*. 2001; 98:8844–8849. [PubMed: 11438699]
- Sittler A, Devys D, Weber C, Mandel J. Alternative splicing of exon 14 determines nuclear or cytoplasmic localisation of fmr1 protein isoforms. *Hum Mol Genet*. 1996; 5:95–102. [PubMed: 8789445]
- Vasilyev N, Polonskaia A, Darnell JC, Darnell RB, Patel DJ, Serganov A. Crystal structure reveals specific recognition of a G-quadruplex RNA by a β -turn in the RGG motif of FMRP. *Proc Natl Acad Sci*. 2015; 112:E5391–E5400. [PubMed: 26374839]
- Verkerk AJ, de Graaff E, De Boulle K, Eichler EE, Konecki DS, Reyniers E, Manca A, Poustka A, Willems PJ, Nelson DL. Alternative splicing in the fragile X gene FMR1. *Hum Mol Genet*. 1993; 2:399–404. [PubMed: 8504300]

- Wang B, Pan L, Wei M, Wang Q, Liu W-W, Wang N, Jiang X-Y, Zhang X, Bao L. FMRP-Mediated Axonal Delivery of miR-181d Regulates Axon Elongation by Locally Targeting Map1b and Calm1. *Cell Rep.* 2015; 13:2794–2807. [PubMed: 26711345]
- Xiang S, Kato M, Wu LC, Lin Y, Ding M, Zhang Y, Yu Y, McKnight SL. The LC Domain of hnRNP A2 Adopts Similar Conformations in Hydrogel Polymers, Liquid-like Droplets, and Nuclei. *Cell.* 2015; 163:829–839. [PubMed: 26544936]
- Xie W, Dolzhanskaya N, Lafauci G, Dobkin C, Denman RB. Tissue and developmental regulation of fragile X mental retardation 1 exon 12 and 15 isoforms. *Neurobiol Dis.* 2009; 35:52–62. [PubMed: 19362146]
- Zang JB, Nosyreva ED, Spencer CM, Volk LJ, Musunuru K, Zhong R, Stone EF, Yuva-Paylor LA, Huber KM, Paylor R, Darnell JC, Darnell RB. A Mouse Model of the Human Fragile X Syndrome I304N Mutation. *PLoS Genet.* 2009; 5:e1000758. [PubMed: 20011099]
- Zhang P, Abdelmohsen K, Liu Y, Tominaga-Yamanaka K, Yoon J-H, Ioannis G, Martindale JL, Zhang Y, Becker KG, Yang IH, Gorospe M, Mattson MP. Novel RNA- and FMRP-binding protein TRF2-S regulates axonal mRNA transport and presynaptic plasticity. *Nat Commun.* 2015; 6:8888. [PubMed: 26586091]
- Zhang Y, O'Connor JP, Siomi MC, Srinivasan S, Dutra A, Nussbaum RL, Dreyfuss G. The fragile X mental retardation syndrome protein interacts with novel homologs FXR1 and FXR2. *EMBO J.* 1995:14.

Figure 1. Splice forms of mouse *Fmr1*

FMRP is encoded by twelve related splice variants. Splice form 1 represents the full-length transcript, which includes all 17 exons. Isoforms 2 and 3 are produced by the use of alternate splice acceptor sites in exon 15. Isoform 2 lacks serine 499 (S499), which is phosphorylated to modulate translational regulation by FMRP (Ceman et al., 2003). Isoform 3 lacks S499 as well as a methylation signal, which is required for RNA binding by the RGG box (Dolzhanskaya et al., 2006). Isoforms 7–9 are identical to isoforms 1–3, except that they lack exon 12, which encodes an amino acid loop within the second KH domain that alters binding to kissing complex RNA (Darnell et al., 2009). Isoforms 4–6 and 10–12 are similar to isoforms 1–3 and 7–9, respectively, but lack exon 14. The exclusion of exon 14 produces a frame shift resulting in entirely different C-terminal sequences than those found in isoforms 1–3 and 7–9. These alternate C-termini lack the nuclear export sequence (NES), S499, the methylation signal, and the RGG box.

Figure 2. Axonal arbor complexity is selectively regulated by specific splice forms of FMRP (A–B) Neuron (A) co-transfected with tdTomato (red) and GFP::FMRP splice form (SF) 1 (green). The corresponding tracing is shown in (B). (C) Tracing of a neuron transfected with tdTomato only. Note the increased length and complexity of the axon, compared to the neuron shown in A–B. Scale bar, 25μm. (D) Axonal complexity of neurons transfected with FMRP splice forms as compared to neurons expressing only endogenous FMRP (endog). Neurons overexpressing SF1, 7, and 9 are significantly less complex than neurons transfected with tdTomato only. (E) The cumulative length of all axonal branches. Neurons transfected with SF1 and SF9 are significantly different from neurons transfected only with tdTomato. (F) The highest branch order of individual cells, averaged over all cells analyzed. Neurons transfected with SF1, SF7, and SF9 show significantly lower branch orders compared with neurons transfected with tdTomato alone. (* $p < 0.05$, ** $p < 0.005$).

Figure 3. Axonal localization of FMRP is not dependent on specific splice forms

(A) Neuron cotransfected with tdTomato (red) and GFP::FMRP SF9 (green). Arrows indicate individual GFP-expressing puncta. Scale bar, 25µm, inset, 5µm. (B) The average density of GFP-expressing puncta for all neurons transfected with a given individual splice form. Neurons transfected with SF9 and SF12 exhibited significantly greater puncta density compared to neurons transfected with SF3 (* $p < 0.05$). (C) The average distance of individual GFP-expressing puncta from the soma (averaged for each neuron) did not differ among the splice forms.

Figure 4. FMRP-mediated regulation of axonal morphology does not require RNA binding domains

(A) Morphological complexity of neurons transfected with wild type human splice form 7 (hSF7) or various mutant constructs of hSF7. (B) The cumulative length of axons transfected with hSF7 or various mutant constructs of hSF7. (C–D) The highest branch order (C) and number of nodes (D) of individual cells, averaged over all cells analyzed. Neurons expressing the mutant splice forms NES34A, S500A, and S500D show significantly increased branch order or number of nodes, compared to neurons transfected with wild type hSF7. (E–F) The density of GFP-expressing puncta (E) and the average distance of each punctum from the soma (F) for neurons transfected with wild type hSF7 or mutant isoforms. *p<0.05; **p<0.005; ***p<0.0005; #p<0.0001.

Figure 5. Differential assembly into larger complexes among FMRP splice and mutant forms
(A) A neuron co-transfected with tdTomato (red) and GFP::FMRP SF4 (green), showing individual puncta along the axon. Such elongated puncta were observed in a population of neurons expressing a subset of the splice forms. **(B)** A neuron co-transfected with tdTomato (red) and GFP::FMRP hSF7-I304N (green) shows particularly elongated FMRP structures. Scale bar, 5 μ m.

Table 1
Formation of FMRP fibril-like structures in axons

Indicated is the percentage of neurons containing elongated FMRP structures in their axons for each FMRP construct.

FMRP Isoform	Neurons Containing Elongated GFP::FMRP Structures in Axons (%)
<i>Mouse Splice Forms</i>	
Splice Form 1	7.69
Splice Form 2	4.00
Splice Form 3	0
Splice Form 4	12.50
Splice Form 5	3.57
Splice Form 6	3.84
Splice Form 7	0
Splice Form 8	0
Splice Form 9	12.00
Splice Form 10	7.14
Splice Form 11	19.23
Splice Form 12	3.84
<i>Mutant Forms (Human Splice Form 7)</i>	
Wild Type	0
I241N	58.62
R290A	5.41
I304N	55.81
RGG	2.63
NES34A	42.11
S500A	0
S500D	0