**Drosophila** FMRP controls miR-276-mediated regulation of *nejire* mRNA for space-filling dendrite development

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Abstract

MicroRNAs are enriched in neurons and play important roles in dendritic spine development and synaptic plasticity. MicroRNA activity is controlled by a wide range of RNA-binding proteins. FMRP, a highly conserved RNA-binding protein, has been linked to microRNA-mediated gene regulation in axonal development and dendritic spine formation. FMRP also participates in dendritic arbor morphogenesis, but whether and how microRNAs contribute to its function in this process remains to be elucidated. Here, using *Drosophila* larval sensory neurons, we show that a FMRP-associated microRNA, miR-276, functions in FMRP-mediated space-filling dendrite morphogenesis. Using EGF microRNA sensors, we demonstrate that FMRP likely acts by regulating miR-276a RNA targeting rather than by modulating microRNA levels. Supporting this conclusion, miR-276a communoprecipitated with FMRP and this association was dependent on the FMRP KH domains. By testing putative targets of the FMRP-miR-276a regulatory axis, we identified *nejire* as a FMRP-associated mRNA and, using EGF reporters, showed that the *nejire* 3′ untranslated region is a target of miR-276a in vivo. Genetic analysis places *nejire* downstream of the FMRP-miR-276a pathway in regulating dendrite patterning. Together, our findings support a model in which FMRP facilitates miR-276a-mediated control of *nejire* for proper dendrite space-filling morphology and shed light on microRNA-dependent dendrite developmental pathology of fragile X syndrome.

Keywords: Fragile X syndrome; FMRP; *nejire*; dendritic arborization; *Drosophila*; neuron

Introduction

MicroRNAs (miRNAs) are ~22-nt small noncoding RNAs that posttranscriptionally regulate gene expression ([Iwakawa and Tomari 2015]). miRNA biogenesis typically starts with synthesis of primary miRNAs (pri-miRNAs) which are processed by Drosha/DGCR8 to produce short hairpin precursor miRNAs (pre-miRNAs). Pre-miRNAs are exported from the nucleus to the cytoplasm by exportin 5, followed by cleavage by Dicer. One strand from the remaining duplex is subsequently loaded onto Argonaute (Ago), forming a miRNA-induced silencing complex (miRISC), which in turn promotes translational repression and/or degradation of target mRNAs by base pairing with complementary sequences in 3′ untranslated regions (3′ UTRs) ([Lai 2002; Ha and Kim 2014]). miRNAs are highly expressed in the nervous system where they play important roles in neuronal development and function ([Kosik 2006; Fineberg et al. 2009; Schratt 2009; McNeill and Van Vactor 2012; Rajman and Schratt 2017]). They are distributed to dendrites ([Sambandan et al. 2017]) and contribute to spine development and synaptic plasticity by locally regulating protein synthesis ([Schratt et al. 2006; Siegel et al. 2009]). Dysfunction of the miRNA pathway has been linked to many neurodevelopmental disorders, such as autism spectrum disorder, Rett syndrome, fragile X syndrome (FXS), and Tourette’s syndrome ([Kosik 2006; Fineberg et al. 2009; McNeill and Van Vactor 2012; Rajman and Schratt 2017]). However, the regulatory roles of miRNAs in the development of complex dendritic arbors are still poorly understood. miRNA biogenesis and function are highly regulated by RNA-binding proteins (RBPs) ([Ha and Kim 2014; Connett et al. 2015]). For example, TDP-43 interacts with Drosha and pri-miRNAs to facilitate pre-miRNA production ([Kawahara and Mieda-Sato 2012]). Xrn1, an exonuclease, regulates the turnover of mature miRNAs ([Bail et al. 2010]). In addition, recognition of RNA targets by miRNAs is controlled by a wide variety of RBPs ([Kim et al. 2021]). Pumilio ([Kedde et al. 2010]), IMP2 ([Degrauw et al. 2016]), FUS ([Zhang et al. 2018]), and Dnd1 ([Kedde et al. 2007]) have been reported to bind to and/or change 3′ UTR RNA structures to promote or suppress miRNA targeting for translational repression.

Fragile X mental retardation protein (FMRP) is a highly conserved RBP that has been implicated in miRNA-mediated gene regulation. FMRP was found to be associated with key components of the miRNA biogenesis pathway, including Ago1 and Dicer ([Jin et al. 2004]), and to bind to several miRNAs, such as bantam, let-7, mir-125b, mir-132, and mir-181d ([Yang et al. 2009; Edbauer et al. 2010; Wang et al. 2015]), suggesting that miRNA dysfunction may contribute to FXS. In neuronal development, FMRP was previously reported to regulate translation of synaptic miRNAs by interacting with individual miRNAs and promoting the formation of miRISC for proper synaptic structure and function ([Edbauer et al. 2010; Muddashetty et al. 2011]), to mediate axonal transport of mir-181d and local regulation of map1b and map2b. However, the regulatory roles of miRNAs in the Axon guidance and synapse formation is a complex process controlled by a wide range of RNA-binding proteins. FMRP, a highly conserved RNA-binding protein, has been linked to microRNA-mediated gene regulation in axonal development and dendritic spine formation. FMRP also participates in dendritic arbor morphogenesis, but whether and how microRNAs contribute to its function in this process remains to be elucidated. Here, using *Drosophila* larval sensory neurons, we show that a FMRP-associated microRNA, miR-276, functions in FMRP-mediated space-filling dendrite morphogenesis. Using EGF microRNA sensors, we demonstrate that FMRP likely acts by regulating miR-276a RNA targeting rather than by modulating microRNA levels. Supporting this conclusion, miR-276a communoprecipitated with FMRP and this association was dependent on the FMRP KH domains. By testing putative targets of the FMRP-miR-276a regulatory axis, we identified *nejire* as a FMRP-associated mRNA and, using EGF reporters, showed that the *nejire* 3′ untranslated region is a target of miR-276a in vivo. Genetic analysis places *nejire* downstream of the FMRP-miR-276a pathway in regulating dendrite patterning. Together, our findings support a model in which FMRP facilitates miR-276a-mediated control of *nejire* for proper dendrite space-filling morphology and shed light on microRNA-dependent dendrite developmental pathology of fragile X syndrome.

**Keywords:** Fragile X syndrome; FMRP; *nejire*; dendritic arborization; *Drosophila*; neuron
Materials and methods

Fly strains

The following transgenic stocks were used: ppk-GAL4, UAS-CD4:tdGFP (Bhogal et al. 2016); UAS-mCherry scramble.sponge (Bloomington Stock 61501); ppk-GAL4 (Bloomington Stock 32079); UAS-mCherry.miri-276a.sponge (Bloomington Stock 61406); UAS-mCherry.miri-276b.sponge (Bloomington Stock 61407); UAS-mCherry.miri-9c.sponge (Bloomington Stock 61376); UAS-mCherry.miri-125.sponge (Bloomington Stock 61393); UAS-Fmr1.LZ (Bloomington Stock 6931); UAS-fmr1-RNAi (Bloomington Stock 34944); TRIP HMSO0248; UAS-NF1-RNAi (Bloomington Stock 53322; TRIP HMC03551) (validated by Moscato et al. 2020); UAS-nej-RNAi (Bloomington Stock 37489; TRIP HMS01507) (validated by Jia et al. 2015); UAS-inaj-RNAi (Bloomington Stock 64885; TRIP HMC0575); UAS-fmr1-2RNAi (Bloomington Stock 61853; TRIP HJ23347); UAS-Ric-RNAi (Bloomington Stock 82973; TRIP HMC06651); UAS-Mkp3-RNAi (Bloomington Stock 57030; TRIP HMS04475); and UAS-Axin-RNAi (Bloomington Stock 62434; TRIP HMJ23888) (validated by Nye et al. 2020). ppk-GAL4 was used to drive expression of UAS transgenes specifically in C4da neurons. To enhance GAL4/UAS efficiency, the experiments UAS-pre-miri-276a transgenes

Plasmid construction

The UAS-pre-miri-276a transgenes were generated using the same strategy as previously described (Xu et al. 2008). A 112-bp fragment containing 98 nt of the pre-miri-276a sequence was amplified from genomic DNA with the following pairs of primers: Fwd_pUAS_pre-miri-276a (5`-GATCCTGAATTTTCCTTTTACTCGGTTTTT-3`) and Rev_pUAS_pre-miri-276a (5`-GGCTATCTAGAAGCGACCATATGAAGTCTATTAACGTTAACGT-3`). The PCR products and pUAST-UAS vector were digested with EcoRI and Xhol and ligated together to generate pUAST-pre-miri-276a.

pTub-nuc-EGFP-2x-miri-276a transgenes

A fragment containing 2 copies of perfectly complementary sequence to mir-276a was generated by overlap extension PCR with the following pairs of primers: Fwd_Xbal_2x-miri-276a (5`-GCTATCTAGAAGCGACCATATGAAGTCTATTAACGTTAACGT-3`) and Rev_Xhol_2x-miri-276a (5`-GGCGGCTACCTCCTGAG-3`). The PCR products and pCaSpeR4_Tub-nuc-EGFP (by courtesy of E. Lai) were digested with Xbal and Xhol and then ligated together to produce pCaSpeR4_Tub-nuc-EGFP_2x-miri-276a. Tub-nuc-EGFP and Tub-nuc-EGFP_2x-miri-276a fragments were amplified from pCaSpeR4_Tub-nuc-EGFP and pCaSpeR4_Tub-nuc-EGFP_2x-miri-276a, respectively, using Fwd_EcoRI_Tub-nuc-EGFP (5`-GATCCTCTCATTACGCTAG-3`) and Rev_BglII_Tub-nuc-EGFP (5`-GACAGTAGTACTCTGAGCATAGATCTG-3`) primers. The PCR products were digested with EcoRI and BglII and ligated individually into the pattB vector digested with EcoRI and BglII to produce pTub-nuc-EGFP and pTub-nuc-EGFP_2x-miri-276a.

pTub-nuc-EGFP-nej-3`UTR transgenes

To generate the intact reporter, a 493-bp fragment of the nej 3` UTR was amplified from genomic DNA using Fwd_Xba1_nej-3`UTR (5`-GGCTATCTAGAAGCGACCATATGAAGTCTATTAACGTTAACGT-3`) and Rev_Xhol_nej-3`UTR (5`-GATCCTCTCATTACGCTAG-3`) and Rev_Esp3i_nej-3`UTR_frag1 (5`-ATTACGTCGTACGCGAGCAAGGA-3`) and Rev_Esp3i_nej-3`UTR_frag2 (5`-CGCGCCGTACGCTAG-3`) primers and digested with Xbal and Xhol. The fragment was then ligated into pCaSpeR4_Tub-nuc-EGFP digested with Xbal and Xhol to produce pCaSpeR4_Tub-nuc-EGFP_nej-3`UTR. To generate the mutant reporter, the mir-276a seed sequence was deleted from the nej 3` UTR fragment by amplifying 2 regions of the nej 3` UTR fragment using Fwd_Xba1_nej-3`UTR (5`-GGCTATCTAGAAGCGACCATATGAAGTCTATTAACGTTAACGT-3`) and Rev_Esp3i_nej-3`UTR_frag1 (5`-ATTACGTCGTACGCGAGCAAGGA-3`) and Rev_Esp3i_nej-3`UTR_frag2 (5`-CGCGCCGTACGCTAG-3`) primers. Two PCR products were digested with indicated enzymes and then ligated into pCaSpeR4_Tub-nuc-EGFP digested with Xbal and Xhol to produce pCaSpeR4_Tub-nuc-EGFP_nej-3`UTR-mut. Tub-nuc-EGFP_nej-3`UTR and Tub-nuc-EGFP_nej-3`UTR-mut were amplified from pCaSpeR4 plasmids using Fwd_EcoRI_Tub-nuc-EGFP (5`-GACAGTAGTACTCTTGAGCATAGATCTG-3`) and Rev_BglII_Tub-nuc-EGFP (5`-GACAGTAGTACTCTGAGCATAGATCTG-3`) primers and digested with EcoRI and BglII for insertion into the pattB vector to produce pTub-nuc-EGFP_nej-3`UTR and pTub-nuc-EGFP_nej-3`UTR-mut.

RNA immunoprecipitation and RT-qPCR

Drosophila S2 cell culture, transfection, and RNA immunoprecipitation were conducted as previously described (Li and Gavis 2022). FMRP variants were detected by immunoblotting with 1:2,000 DYKDDDK DKO monoclonal antibody (Invitrogen, Cat # MA-191878) and 1:2,000 HRP sheep anti-mouse antibody (VWR, Cat # 95017-332). Ten nanograms of total RNA was reverse transcribed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Cat # 4366596). Real-time PCR was then performed with TaqMan Fast Advanced Master Mix (Applied Biosystems, Cat # 4444556) and dme-miR-276a TaqMan miRNA Assays (Applied Biosystems, Cat # 4440868). Poly(A) mRNA was reverse transcribed using SuperScript III First-Strand Synthesis System (Invitrogen, Cat # 18080051) and real-time PCR analysis was performed with SYBR Green PCR Master Mix (Thermo Fisher, 2016).
immunofluorescence

Late 3rd instar larva preparation and staining were performed as described (Bhogal et al. 2016). For better immunostaining efficiency, the larval body wall muscles were removed as described (Tenenbaum and Gavis 2016). FMRP expression was detected with anti-FMRP monoclonal antibody (1:100, Abcam, ab10299) and AlexaFluor 568 goat anti-mouse (1:500, Life technologies, A-11004) secondary antibody. Neuronal membranes were visualized using Alexa 568-conjugated anti-HRP (1:200, Jackson, 123-605-021) and Alexa 647-conjugated anti-HRP (1:200, Jackson, 123-605-021). All antibodies were incubated in blocking buffer containing PBS/0.3% TritonX-100 with 5% normal goat serum either overnight at 4°C (primary antibodies) or for 1 h at room temperature (secondary antibodies). Larval filies were mounted between a coverslip and slide with VECTASHIELD Antifade Mounting Medium (Vector Laboratory, H-1000-10) and were imaged using a Leica SP5 laser scanning confocal microscope with a 63×/1.4 NA oil objective and sequential scanning. All images are confocal z series projections. Relative nuclear EGF expression levels were measured by ROI with IntDen function in ImageJ software (https://image.nih.gov/ij/) and background was subtracted.

Analysis of dendrite morphology

C4da (ddaC) neurons from live larvae were mounted individually in 80% glycerol between a slide and a coverslip and imaged by a Leica SP5 laser scanning confocal microscope (40×/1.25 NA oil objective). All images are confocal z series projections. For consistency, class IV ddaC neurons from abdominal segments A3–A5 were imaged. At least 10 neurons from 5 or more larvae were imaged and analyzed for each genotype. Quantitative analysis of dendrite morphology was performed with ImageJ software. The dendritic arbor field coverage was quantified by overlaying a grid of 200 × 20 squares on the image of interest and counting the number of empty squares. The dendritic field coverage ratio = # empty squares/400.

Statistical analysis

All data were analyzed and plotted using GraphPad Prism 9 (https://www.graphpad.com/). Comparisons between 2 groups were performed with the unpaired Student’s t-test. For 3 or more groups, 1-way ANOVA with Dunnett’s or Tukey’s multiple comparisons test was used. Values are mean ± SD; ns, not significant; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Results

miR-276 is required for proper C4da dendritic field coverage

Several miRNAs identified in FMRP immunoprecipitates from wild-type Drosophila ovaries, including miR-9c, miR-125, and miR-276a (Yang et al. 2009), have been implicated in the regulation of mammalian dendritic growth and spine formation (Edbauer et al. 2010; Giusti et al. 2014) and Drosophila olfactory memory formation (Li et al. 2013). To determine if these potential FMRP-associated miRNAs play a role in dendrite morphogenesis in Drosophila, we disrupted their activity in C4da neurons using miRNA sponges, which act as competitive inhibitors by sequestering miRNAs (Fulga et al. 2015). Each miRNA sponge was expressed selectively in C4da neurons using ppk-GAL4 (Fig. 1, a–e) and measured in regulation of olfactory memory formation in mushroom bodies (Lee et al. 2003; Li and Gavis 2022) and to associate with miR-276a in the Drosophila ovary (Yang et al. 2009). miR-276a and miR-276b, which differ by a single nucleotide at position 10, are members of the Drosophila dme-miR-276 family (Supplementary Fig. 1, a and b). We therefore asked if miR-276a and/or miR-276b sponges are involved in FMRP-mediated dendritic regulation. Overexpression of fmr1, which encodes FMRP, in C4da neurons (fmr1OE) results in a sparse dendritic arbor and dramatically reduced field coverage (Li and Gavis 2022). Expression of either the miR-276a or miR-276b sponge, but not the scrambled sponge, in fmr1OE neurons partially rescued this dendritic field coverage defect (Fig. 1, f–h and i). Together, these results support the idea that FMRP function in space-filling dendrite morphogenesis is mediated in part by its interaction with miR-276.

The single nucleotide difference between miR-276a and miR-276b falls within the bulge that forms between each miRNA sponge and the bound miRNA (position 9–11, Supplementary Fig. 3c). Thus, miR-276a and miR-276b should each be sequestered by both the miR-276a and miR-276b sponges. miR-276a has functions in regulation of olfactory memory formation in mushroom body neurons (Li et al. 2013) and circadian rhythms in the central nervous system (Chen and Rosbash 2016). Because of its known activity in the nervous system, we focused on miR-276a in subsequent experiments, although we cannot rule out the possibility that miR-276a and miR-276b function redundantly in C4da dendrite regulation.

miR-276a levels are not altered by loss or gain of FMRP in C4da neurons

We next sought to determine how FMRP and miR-276a are mechanistically linked in regulating dendritic patterning. One possibility is that FMRP functions in miRNA maturation and/or stability to regulate steady-state levels of miR-276a (Fig. 2a). Alternatively, FMRP might function in miR-276a RNA targeting (Fig. 2b). To test the first possibility, we generated transgenic flies ubiquitously expressing nuclear EGFP sensors to monitor relative miR-276a levels in vivo (Fig. 2, c and d). EGFP sensors fused to the SV40 3’ UTR, with or without 2 copies of perfectly complementary sequences to miR-276a, were expressed in wild-type larvae or in larvae with C4da neuron-specific fmr1 RNAi or overexpression. If miR-276a levels are regulated by FMRP, EGFP expression should depend on the level of FMRP. With the control sensor lacking
Fig. 1. miR-276 genetically interacts with FMRP to regulate C4da dendritic field coverage. Representative images of C4da neurons expressing the mCherry-scramble-sponge (SP) (a–a'), mCherry-miR-276a-sponge (b–b'), mCherry-miR-276b-sponge (c–c'), mCherry-miR-9c-sponge (d–d'), and mCherry-miR-125-sponge (e–e') driven by ppk-GAL4. Representative images of C4da neurons overexpressing fmr1 (fmr1\textsuperscript{OE}) together with the mCherry-scramble-sponge (f–f'), mCherry-miR-276a-sponge (g–g'), or mCherry-miR-276b-sponge (h–h'). Representative images of C4da neurons expressing nej RNAi together with mCherry-scramble-sponge (i–i'), mCherry-miR-276a-sponge (j–j'), or mCherry-miR-276b-sponge (k–k'). C4da neuronal membranes were labeled with CD4-tdGFP. Expression of the mCherry-miRNA-sponges was confirmed by direct mCherry fluorescence in (a–k). i) Quantification of the dendritic field coverage ratio in C4da neurons with the indicated genotypes. All images are confocal z series projections. Scale bar, 50 μm. Each data point represents a value for one neuron. Values are mean ± SD; ns, not significant; **P < 0.01, ****P < 0.0001, one-way ANOVA with Tukey’s multiple comparisons test.

Fig. 2. miR-276a levels in C4da neurons are not altered by FMRP. a and b) Two hypotheses for FMRP-mediated dendrite patterning through miR-276a. Schematic illustration of the pTub-nuc:EGFP sensor without (c) or with (d) 2 copies of miR-276a perfect complementary sequences in the SV40 3' UTR. Nuclear EGFP signals detected in wild-type larvae (e and f), larvae with C4da-specific fmr1 RNAi (g), or overexpression (OE) (h). FMRP (e–h') was detected by anti-FMRP immunofluorescence. Neuronal membranes were visualized by anti-HRP immunofluorescence. e–h') Merged images. Dashed circles denote the somas of C4da neurons. All images are confocal z series projections. Scale bar, 20 μm. Panels (a–d) were created with BioRender.com.
miR-276a complementary sequences, EGFP was detected in the nuclei of epidermal cells and all 4 classes of da neurons (Fig. 2e). By contrast, EGFP expression was dramatically reduced throughout larvae expressing the miR-276a sensor (Fig. 2f), indicating ubiquitous expression of miR-276a at the late 3rd instar larval stage. Expression of the miR-276a sponge, but not the scrambled sponge, in C4da neurons together with the miR-276a EGFP sensor. More importantly, decreasing or increasing FMRP levels, as confirmed by anti-FMRP immunofluorescence, did not affect EGFP expression in C4da neurons (Fig. 2, g–h), indicating that FMRP does not regulate levels of miR-276a.

FMRP is associated with miR-276a through KH domains

Since FMRP does not control miR-276a levels, we asked whether it might instead function in miR-276a target RNA interaction. We first tested whether FMRP interacts with miR-276a by RNA communoprecipitation. Flag-tagged FMRP (FMRP-3xFlag; Fig. 3a) was expressed in Drosophila S2 cells and immunoprecipitated with anti-DYKDDDDK antibody (Fig. 3b). RT-qPCR analysis of RNA extracted from the immunoprecipitates showed that amount of mature miR-276a was similar in S2 cells with or without induction of FMRP-3xFlag expression (Fig. 3c), which is consistent with results from the EGFP sensor experiments showing that miR-276a levels were unaffected by overexpression of FMRP in C4da neurons (Fig. 2, f and h). Mature miR-276a was enriched 2-fold in the FMRP-3xFlag immunoprecipitate compared to the control (Fig. 3d), indicating that FMRP associates with miR-276a in vivo, either directly or indirectly (see Discussion).

FMRP has 3 RNA-binding domains (RBDs)—KH1, KH2, and RGG. The KH domains were previously shown to facilitate miRNA: mRNA complex formation in vitro (Plante et al. 2006). To assess the involvement of FMRP’s RBDs in its association with miR-276a, we generated a set of constructs to express Flag-tagged FMRP variants with individual RBD deleted in S2 cells (Fig. 3a). Following immunoprecipitation of the FMRP variants (Fig. 3b), the amount of communoprecipitated miR-276a was quantified by RT-qPCR. Deletion of either the KH1 or the KH2 domain resulted in the loss of miR-276a enrichment in FMRP immunoprecipitates (Fig. 3d), indicating that both KH domains are indispensable for FMRP to bind to miR-276a.

Predicted FMRP–miR-276a regulatory targets nej, Ric, and Mkp3 are required for C4da dendrite arborization

To identify potential targets of the FMRP–miR-276a regulatory axis, we focused on the overlap between previously identified FMRP targets (Darnell et al. 2011; Ascano et al. 2012; Maurin et al. 2018) and predicted miR-276 targets (TargetScan 7.2), which includes nej, Neurofibromin 1 (Nf1), Mitogen-activated protein kinase phosphatase 3 (Mkp3), Ras-related protein interacting with calmodulin (Ric), inactivation no afterpotential E (inaE), Histone gene-specific Epigenetic Repressor in late S phase (Hers), and Axin (Axn). To determine if these putative targets function in C4da dendrite arborization, we specifically knocked them down in C4da neurons using RNAi driven by ppk-GAL4. miRNAs typically downregulate their targets by promoting mRNA degradation and/or translational repression (Iwakawa and Tomari 2015). However, because miRNAs often have only modest regulatory effects, we expected that deleting targets of FMRP–miR-276a regulatory pathway would at least partially mimic fmr1 overexpression in C4da neurons. Knockdown of Nf1, inaE, Hers, and Axn had no obvious phenotypic consequences (Fig. 4, a, d–g, and n). Ric and Mkp3 RNAi each resulted in increased dendritic field coverage, which resembles fmr1 knockdown rather than overexpression (Fig. 4, a–c, i, and n).

![Fig. 3.](image-url)
On the contrary, nej RNAi led to a dramatic decrease in the dendritic coverage ratio, to an extent comparable to fmr1OE neurons (Fig. 4, h, i, l, and n). nej encodes *Drosophila* CREB-binding protein, which acts as a transcriptional coactivator and acetylates histones to regulate gene expression (Akimaru et al. 1997; Das et al. 2009). Overexpression of precursor miR-276a (pre-miR-276a) in C4da neurons resulted in similar dendritic coverage defects (Fig. 4, k–m and o), suggesting that nej might act in C4da dendritic morphogenesis through the FMRP-miR-276a regulatory pathway.

**nej interacts with FMRP in regulating C4da dendrite patterning**

We further tested if nej is controlled by FMRP using genetic and biochemical analyses. Double RNAi of fmr1 and nej rescued both the increased dendritic field coverage caused by fmr1 RNAi and the decreased dendritic field coverage caused by nej RNAi (Fig. 4, h–j and n), suggesting that nej genetically interacts with fmr1 in regulating C4da space-filling dendrite arborization. To determine if nej physically interacts with FMRP, we performed RT-PCR for RNAs that coimmunoprecipitated with FMRP-3xFlag from S2 cells. nej, as well as other 2 putative RNA targets, ntf1 and 14-3-3ζ, coimmunoprecipitated with FMRP (Fig. 4p). Along with the phenotypic analysis above, our results suggest that nej mRNA is a target of FMRP in C4da dendrite patterning.

The **nej 3′ UTR is a target of miR-276a in C4da neurons**

nej has a predicted miR-276a recognition element in its 3′ UTR (positions 1,567–1,573; TargetScan7.2) (Fig. 5a). To confirm that nej 3′ UTR is a target of miR-276a in C4da neurons, we generated a ubiquitously expressed nuclear EGFP reporter with a 493-bp fragment from the nej 3′ UTR containing the predicted miR-276a recognition site (intact reporter) and a corresponding reporter with the recognition site deleted (mut reporter) (Fig. 5b) inserted in the SV40 3′ UTR. EGFP expression from the mut reporter was significantly increased relative to that from the intact reporter (Fig. 5, c–e), consistent with the idea that the reporter expression is regulated by miR-276a. However, EGFP expression from the mut reporter was much lower than that of the EGFP control sensor with only the SV40 3′ UTR (Fig. 2, a and c), indicating that other factors may target this 493-bp region to control nej expression.

As a further test that nej acts downstream of miR-276a, we knocked down nej by RNAi in C4da neurons expressing either of the miR-276 sponges. The increase in dendritic field coverage observed for the sponges was rescued by nej RNAi, consistent with a role for miR-276 in downregulating nej (Fig. 1, i–l). In sum, our results provide evidence that regulation of nej by binding of miR-276 to its 3′ UTR is necessary for proper C4da dendritic field coverage.

**Discussion**

miRNAs contribute to the regulation of synaptic structure and axon elongation by FMRP, a highly conserved RBP that functions in different aspects of neuronal development (Edbauer et al. 2010; Muddashetty et al. 2011; Wang et al. 2015). However, their roles in FMRP-mediated dendrite patterning remained unclear. Here, we uncover a role for a FMRP-associated miRNA, miR-276, in FMRP-dependent regulation of space-filling dendrite morphology. FMRP has been shown to regulate the steady-state levels of miRNAs including miR-124 in *Drosophila* larvae (Xu et al. 2008). However, our results are most consistent with a role for FMRP in regulating RNA targeting by miR-276 rather than mature miR-276a levels, indicating multiple regulatory roles of miR-276 in miRNA-mediated gene expression control of neuronal development. Given the wide range of RNAs identified as FMRP targets (Darnell et al. 2011; Ascano et al. 2012; Maurin et al. 2018), association with miR-276a might contribute to FMRP’s target selectivity and/or provide yet another point of control in addition to translation initiation (Napoli et al. 2008) and elongation (Darnell et al. 2011; Chen et al. 2014).
Our findings support a model in which FMRP facilitates miR-276a targeting of *nej* mRNA for posttranscriptional regulation. *nej*-associated FMRP may help recruit miRISC to the transcript for regulation of gene expression. Deletion of the KH domains significantly alleviated FMRP-mediated enhancement of miRNA:mRNA complex formation in vitro (Plante et al. 2006). Consistent with this, mature miR-276a coimmunoprecipitated with FMRP from Drosophila S2 cells and this interaction was dependent on both the KH1 and KH2 domains, suggesting that FMRP may interact directly with miR-276a or the miR-276a-*nej* complex through the KH domains. Alternatively, since FMRP was found to coimmunoprecipitate with Ago (Jin et al. 2004), it might function in miR-276a targeting via KH-dependent interactions with protein components of miRISC. Lastly, FMRP could indirectly facilitate miR-276a binding to target mRNAs. For example, FMRP might function together with other proteins, such as the RNA helicase MOV10, to help unwind RNA secondary structure and expose miRNA-recognition elements for miRISC targeting (Kenny et al. 2014). In addition to their synergistic effects, miR-276a and FMRP might independently contribute to the regulation of dendrite space-filling morphogenesis. A previous study demonstrated that phosphorylation of FMRP inhibits miR-125a-mediated translational regulation of PSD-95 mRNA (Muddashetty et al. 2011); therefore, as an added layer of complexity, posttranslational modifications could affect FMRP’s functions in miRNA-mediated gene regulation.

Since individual miRNAs often have modest regulatory effects on their targets (Baek et al. 2008; Selbach et al. 2008), and removal of the miR-276a-recognition element from the *nej* 3' UTR did not completely restore expression of the EGFP reporter, it is likely that other factors also contribute to regulation of *nej* expression. For example, the 493-bp *nej* 3' UTR fragment used in our analysis contains a predicted recognition element for dme-miR-2/5/6/11/13/308 (positions 1,651–1,657; TargetScan 7.2), indicating an involvement of these miRNAs in *nej* 3' UTR regulation. Moreover, given the critical roles of RBPs in RNA posttranscriptional regulation (Glisovic et al. 2008), they are also likely to contribute to 3' UTR-mediated regulation of *nej*.

Interestingly, knockdown of 2 FMRP target RNAs—Ric and Mkp3—that are also predicted to be miR-276a targets led to increased dendritic field coverage, similar to that of *fmr1*
knockdown neurons. This effect could be explained by an overlap in the binding sites for FMRP and miR-276a, resulting in competition between FMRP and miRISC for the target RNAs (Kim et al. 2021). Thus, FMRP might function to prevent Ric and Mkp3 mRNAs from being targeted by miR-276a-induced silencing complex, thereby upregulating their expression. Whether these RNAs are controlled by FMRP and/or miR-276a or act independently in dendrite morphogenesis warrants further study.

**Data availability**

Plasmids and transgenic flies are available upon request. The data underlying this article are available within the article and in the Supplementary material. Supplemental material is available at G3 online.

**Acknowledgments**

We thank the Bloomington Drosophila Stock Center for fly stocks and the Drosophila Genomic Resource Center and E. Lai for plasmids. We thank the Princeton Confocal Imaging Facility, a Nikon Center of Excellence in the Department of Molecular Biology, for assistance with confocal imaging and J. Yan for comments on the article.

**Funding**

This work was supported by a fellowship to H.L. from the China Scholarship Council (CSC), based on the April 2015 Memorandum of Understanding between the CSC and Princeton University.

**Conflicts of interest**

None declared.

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