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The Steady-State Level of the Nervous-System-Specific MicroRNA-124a Is Regulated by dFMR1 in *Drosophila*

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Fragile X syndrome is the most common form of inherited mental retardation caused by loss of the fragile X mental retardation protein 1 (FMRP). The detailed molecular pathways underlying the pathogenesis of this disorder remain incompletely understood. Here, we show that miR-124a, a nervous-system-specific miRNA, is associated with the *Drosophila* homolog of FMRP (dFMR1) *in vivo*. Ectopic expression of wild-type but not mutant miR-124a precursors decreased dendritic branching of dendritic arborization sensory neurons, which was partially rescued by the loss of dFMR1 activity, suggesting that the biogenesis and/or function of miR-124a are partially dependent on dFMR1. Indeed, in contrast with the complete loss of mature miR-124a in *Dicer-1* mutants, steady-state levels of endogenous or ectopically expressed mature miR-124a were partially reduced in *dfmr1* mutants, whereas the level of pre-miR-124a increased. This effect could be explained in part by the reduced abundance of the Dicer-1-Ago1 complex in the absence of dFMR1. These findings suggest a modulatory role for dFMR1 to maintain proper levels of miRNAs during neuronal development.

Key words: **miR-124a; processing; *Drosophila*; fragile X syndrome; dendrites; RNA**

Introduction

The loss of fragile X mental retardation protein 1 (FMRP) activity causes fragile X syndrome, the most common form of inherited mental retardation in humans (Verkerk et al., 1991). FMRP is an evolutionarily conserved RNA-binding protein with two ribonucleoprotein (RNP) K homology domains and an arginine- and glycine-rich domain (RGG box). It has been shown that FMRP preferentially binds to tertiary RNA structures named the "kissing complex" and the "G quartet" (Darnell et al., 2001, 2005). Although hundreds of mRNAs associate preferentially with FMRP-containing complexes (Brown et al., 2001; Miyashiro et al., 2003), the detailed mechanisms to account for FMRP action remain to be illustrated.

Drosophila has been used successfully as a model system to dissect the genetic pathways implicated in fragile X syndrome. The *Drosophila* fragile X mental retardation protein 1 (dFMR1) is involved in multiple aspects of neuronal development, including synapse formation, axonal growth and dendritic branching (Zhang et al., 2001; Dockendorff et al., 2002; Morales et al., 2002; A. Lee et al., 2003; Michel et al., 2004). dFMR1 is also implicated in the microRNA (miRNA) pathway (Caudy et al., 2002; Ishizuka et al., 2002), although the exact *in vivo* function of dFMR1 in this pathway is largely unknown.

During animal development, multiple genes must be expressed coordinately at precise levels both spatially and temporally. miRNAs are an important class of regulatory molecules that ensure the accuracy of gene expression. Primary miRNAs (pri-miRNAs) are usually transcribed by RNA polymerase II in the nucleus and processed mostly by the RNase III Drosha to generate 70–80 nt hairpin structures called precursor miRNAs (pre-miRNAs) (Y. Lee et al., 2003). Pre-miRNAs are transported to the cytoplasm by exportin-5 (Yi et al., 2003) and processed by Dicer into ~22 nt mature miRNAs (Bernstein et al., 2001; Grishok et al., 2001; Hutvágner et al., 2001). These small, noncoding RNAs destabilize target mRNAs or suppress their translation by binding to complementary sequences in the 3′ untranslated regions (3′UTRs) (Filipowicz et al., 2008).

Hundreds of miRNAs have been identified in worms, flies, and humans, and many are evolutionarily conserved at the nucleotide level and regulate various aspects of animal development (Bushati and Cohen, 2007; Hobert, 2008). Some miRNAs are specifically expressed in developing and mature nervous systems (Lagos-Quintana et al., 2002; Aboobaker et al., 2005; Wienholds et al., 2005), and their roles in neuronal development and function have begun to be unraveled in different model systems (Gao, 2008). One of them is miR-124a, an evolutionarily conserved and nervous-system-specific miRNA (Lagos-Quintana et al., 2002). Transcriptional activation of miR-124a expression during neuronal specification seems to require derepression by the RE1 silencing transcription factor (Conaco et al., 2006). Although its function has not been analyzed using genetic null mutations in any model system, miR-124a may be involved in downregulating target gene expression during neuronal specification (Cao et al., 2007; Makeyev et al., 2007; Visvanathan et al., 2007). In this

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study, we used the *Drosophila* dendritic arborization (DA) sensory neurons as an assay system and examined the interactions between miR-124a and dFMR1, providing novel mechanistic insights into the exact role of dFMR1 in the miRNA pathway and neuronal development.

Materials and Methods

Fly strains and genetics. All the flies were raised at 25°C on standard food medium. *Gal4*^{221} was used to visualize ddaE and ddaF neurons, and *Gal4*^{477} to visualize ddaC neurons and drive ectopic expression of pre-miR-124a in these cells. The UAS-pre-*miR-9a* fly line was generated in a previous study (Li et al., 2006). The *dfmr1*^{3} mutant fly line was obtained from Dr. T. Jongens (University of Pennsylvania, Philadelphia, PA) and the *dicer-1* mutants were from Dr. R. Carthrew (Northwestern University, Chicago, IL). The UAS-pre-*miR-124a* fly lines were generated by cloning a 92 bp genomic DNA fragment containing the 77 nt wild-type pre-*miR-124a* into the UAST vector between *Eco*RI and *Xba*I restriction enzyme sites. For UAS-mutant pre-*miR-124a*, a 6 nt long deletion was introduced into pre-*miR-124a*. UAS-pre-*miR-124a*, UAS-mutant pre-*miR-124a*, and UAS-pre-*miR-9a* were expressed either in the wild type background or in the *dfmr1*^{3}/*dfmr1*^{4} mutant background. *dcr-1* mutant alleles *dcr-1*^{d102} or *dcr-1*^{Q1147X} were recombined onto the same chromosome as FRT^{82B} and used for MARCM analysis as described previously (Li et al., 2004). The quantitative analysis of dendritic ends and dendritic fields was done also as described previously (Li et al., 2004).

In situ *hybridization. In situ* hybridization was performed as described previously (Li et al., 2006). Briefly, *Drosophila* embryos at different developmental stages were collected and fixed. *Drosophila miR-124a* locked nucleic acid (LNA) probe was purchased from Exiqon, end-labeled with a DIG oligonucleotide 3′-end labeling kit (Roche), and purified on a G-25 Microspin column (Amersham Biosciences). The labeling with the LNA probe was detected by AP-conjugated anti-Dig antibody (Li et al., 2006).

miR-124a-Gal4 fly line. Primers 5′-GGGCGGCCGCGGACCTAGCTTTGTGCGTG-3′ and 5′-GGAGATCTCGTGCCTTATGGTGGAAATAC-3′ were used to obtain 3.2 kb PCR fragments corresponding to the *miR-124a* promoter region, which was subsequently cloned into the pPTGAL vector (Sharma et al., 2002) between the *Not*I and *Bgl*II restriction enzyme sites. The resulting vector was used to generate transgenic flies.

Quantification of dendritic ends. The number of dendritic ends of different DA neurons was quantified as previously described (Li et al., 2004). Briefly, dendritic ends of DA neuron images were identified visually and highlighted with dots. The number of dots was counted using the Illustrator software.

Immunoprecipitation and Western blot analysis. The brains and ventral nerve cords from hundreds of third instar larvae were dissected out in PBS on ice and homogenized in cold RNase-free lyses buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 0.5% Nonidet P-40, 1 mM dithiothreitol, and protease inhibitors). In some case, EDTA was used at a final concentration of 10 mM. The protein extracts was prepared by centrifugation for 15 min at 4°C. The supernatant was incubated with anti-hemagglutinin (HA) antibody (Roche) and protein-G-agarose beads at 4°C for 4 h. The precipitated complex was washed with PBS three times. The associated RNA was isolated with Trizol (Invitrogen).

Western blot analysis was performed according to the standard protocol provided by Bio-Rad. Briefly, protein extracts was prepared from three instar larvae as above. Protein (25 μg), as measured by the Bio-Rad reagent, was separated on 8% SDS-PAGE followed by transferring onto PVDF membranes and immunoblotting with appropriate antibodies. The primary antibodies, anti-dFMR1 (5A11) and anti-tubulin (E7) were from the Developmental Studies Hybridoma Bank at the University of Iowa. Anti-Ago1 antibody (1B8) is a gift from Dr. H. Siomi (Keio University, Tokyo, Japan). Anti-Dcr-1 antibody was purchased from the Abcam. The HRP-conjugated anti-mouse IgG antibody was used as secondary antibody (Jackson Laboratory).

Northern blot. Northern blot was performed as described previously (Li et al., 2006) with some minor modifications. Total RNA (30 μg) extracted from third instar larvae with Trizol were resolved on 12.5% polyacrylamide gels (Sequagel; National Diagnostics) and transferred to Nytran Super Charge Signal membrane (Whatman Schleicher and Schuell) or Zeta-Probe GT membrane (Bio-Rad). Antisense oligonucleotides complementary to mature microRNA sequences were 5′-labeled with ^{32}P (GE Healthcare) and purified with Quick Spin columns (Sigma). The following oligonucleotides were used to detect mature microRNAs: 5′-CTTGGCATTCACCGCGTGCCTTA-3′ for miR-124a, 5′-CTCCATACTTCTTTACATTCCA-3′ for miR-1, and 5′-ACAACAAAATCACTAGTCTTCCA-3′ for miR-7. In some cases, the membrane was stripped by boiling in 0.1% SDS for 3 min and then re-probed with different ^{32}P-labeled oligonucleotides. The intensity of miRNA bands on Northern blot was quantified on a scanning densitometer and averaged from multiple independent experiments.

Quantitative real-time PCR analysis. Total RNA was extracted from third instar larvae as described above, treated with DNase I and purified with RNeasy mini kit (Qiagen). The purified RNA was used in reverse transcription reaction using Taqman reverse transcription reagent (Applied Biosystems). The first-strand cDNA was used as template for quantitative real-time PCR (qRT-PCR) in a final volume of 25 μl containing primers and SYBR Green PCR master mix (Applied Biosystems). The reaction was done using the ABI7700 sequence detection system. For mature microRNAs, expression levels were measured by qRT-PCR analysis with TaqMan miRNA reverse transcription kit and TaqMan microRNA assays containing specific primers for mature miR-124a (Applied Biosystems). A standard curve was run in each PCR. Individual values were normalized with the value of the gene encoding the ribosomal protein RP-49. For qRT-PCR analysis of the primary and precursor transcripts of miR-124a, the following primers were used: forward primer for pri-miR-124a: 5′-CACTTTCGGTGACCTCA-3′; reverse primer for pri-miR-124a: 5′-CCAATGGCGAGAATATCCTTG-3′; forward primer for pre-miR-124a: 5′-ACGTTTTTCTCCTGGTATCCACTG-3′; reverse primer for pre-miR-124a: 5′-CACCGCGTGCCTTATGG-3′; forward primer for rp-49: 5′-AGATCGTGAAGAAGCGCACCAAG-3′; and reverse primer for rp-49: 5′-CACCAGGAACTTCTTGAATCCGG-3′. All reactions were done three times in triplicate, and relative expression of RNAs was calculated by using the standard curve method and the Delta-Delta Ct method.

Results

miR-124a is associated with dFMR1 *in vivo*

To examine the role of miR-124a in neuronal development and its biogenesis in *Drosophila*, we first confirmed its expression pattern during development. miR-124a is specifically expressed in the developing nervous system, mostly in the brain and the ventral nerve cord, as shown by *in situ* hybridization (Fig. 1*A*). Other laboratories reported similar findings during the course of our work (Aboobaker et al., 2005; Stark et al., 2005). To further examine this expression pattern at a high resolution, we generated transgenic flies in which Gal4 expression from the pPTGAL vector (Sharma et al., 2002) was directly under the control of a minimal promoter and a 3.2-kb genomic DNA fragment upstream of the pre-*miR-124a* sequence. When the *miR-124a*-Gal4 line was crossed with the UAS-GFP line, GFP was specifically expressed in the developing embryonic nervous system (Fig. 1*B*), and this expression pattern persisted to adulthood (data not shown). In *Drosophila* embryos and larvae, GFP expression driven by *miR-124a* promoter is high in the brain, ventral nerve cord and motor neurons but lower in DA neurons in the peripheral nervous system.

The exact *in vivo* function of dFMR1 in the miRNA pathway is largely unknown. We set out to determine whether dFMR1 and miR-124a are present in the same RNP complex. We generated UAS transgenic flies that express dFMR1 with C-terminally tagged HA epitope (dFMR1-HA), expressed dFMR1-HA in all neurons with Elav-Gal4, and dissected out the brain and ventral nerve cord from a large number of third instar larvae. Dissected

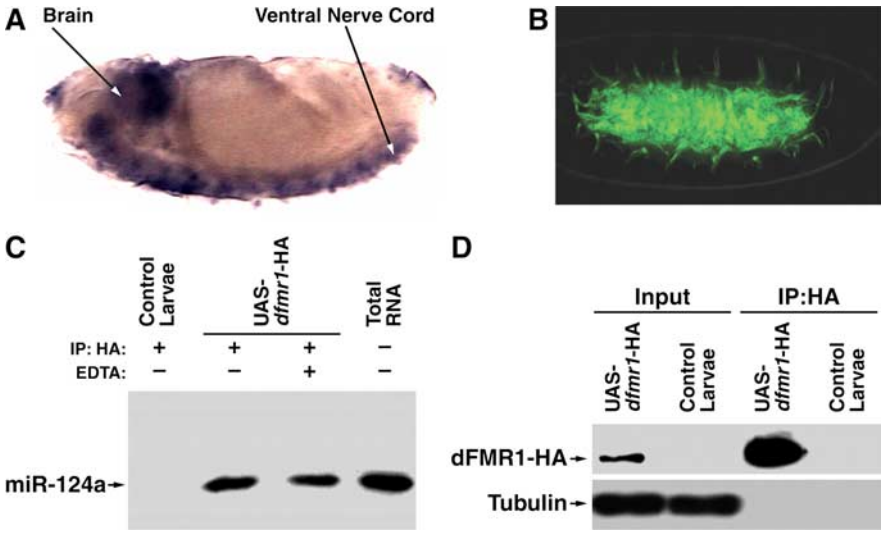

Figure 1. miR-124a and dFMR1 are present in the same RNP complex in the *Drosophila* nervous system. ***A***, *In situ* analysis shows prominent *miR-124a* expression in the brain and ventral nerve cord in a stage 13 *Drosophila* embryo. ***B***, *miR-124a*-Gal4 drives GFP expression specifically in the embryonic nervous system, and the expression persists to adulthood. The axon bundles from motor neurons are visible in this image. ***C***, Immunoprecipitation (IP) experiments demonstrate that miR-124a and dFMR1 are present in the same RNP complex independent of polyribosomes. dFMR1-containing RNP complexes were pulled down with HA antibody, and miR-124a in the immunoisolates was detected with a ^{32}P-labeled ribonucleotide probe. A portion of total RNA from control larvae before IP was loaded as a positive control. ***D***, Western blot analysis of immunoisolates with HA antibody. HA-tagged dFMR1 was readily detectable in lysates isolated from larval brains or in immunoisolates after IP. The absence of tubulin in immunoisolates indicates the specificity of the IP experiment with HA antibody. In ***C*** and ***D***, control larvae express *elav*-Gal4 alone, and other larvae express dFMR1 under the control of *elav*-Gal4.

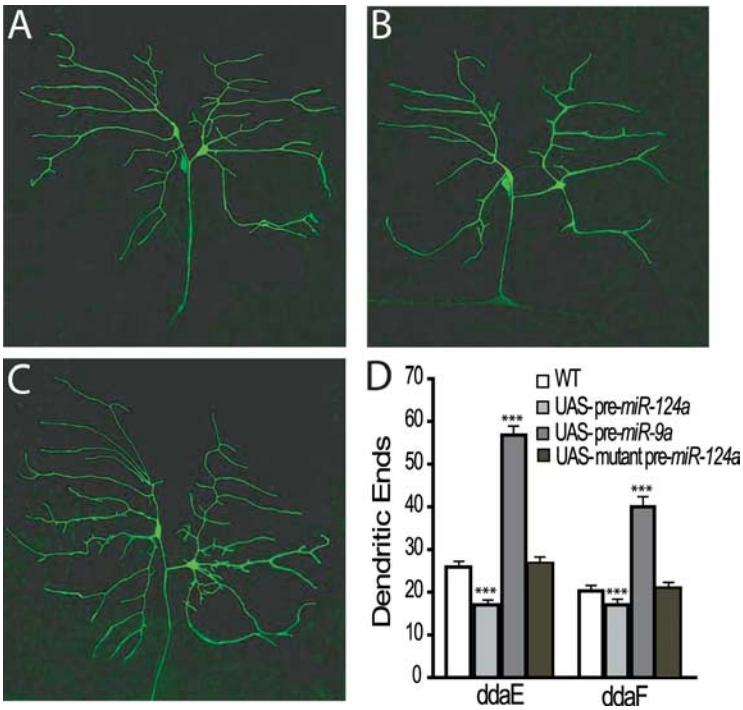

Figure 2. miR-124a suppresses dendritic branching of DA neurons in *Drosophila*. ***A***, Wild-type ddaE and ddaF neurons in the dorsal cluster were labeled with mCD8-GFP driven by Gal4^{221}. ***B***, Ectopic expression of pre-*miR-124a* reduced dendritic branching of DA neurons. ***C***, Ectopic expression of pre-*miR-9a* increased dendritic branching. ***D***, Quantification of dendritic ends in wild-type (WT) ddaE or ddaF neurons and in neurons expressing pre-*miR-124a*, pre-*miR-9a*, and mutant pre-*miR-124a*. The values are mean ± SEM. ***$p < 0.001$ versus wild type.

brain tissues provide "cleaner" lysates for immunoprecipitation experiments than whole larvae, in which contents from guts are problematic. dFMR1-containing RNP complexes were pulled down with the HA antibody, and RNA was extracted from immunoisolates for analysis by Northern blot.

miR-124a was found in immunoisolates from flies expressing both *Elav*-Gal4 and dFMR1-HA but not in those from *Elav*-Gal4 control flies (Fig. 1*C*). The specific isolation of dFMR1-containing RNP complexes was confirmed by Western blot analysis, which revealed dFMR1 but not tubulin in the immunoisolates (Fig. 1*D*). To confirm that the presence of both miR-124a and dFMR1 in the same RNP complex did not depend on polyribosomes, we also treated larval brain lysates with 10 mM EDTA before immunoprecipitation and obtained the same result (Fig. 1*C*). Thus, it is unlikely that miR-124a and dFMR1 are associated with polyribosomes through separate RNP complexes, instead, they are present in the same RNP complex. This result raises the possibility that dFMR1 affects the biogenesis and/or function of miR-124a *in vivo*.

miR-124a suppresses dendritic branching

Before we examine the biological significance of the association of miR-124a and dFMR1 in the same RNP complex, we first set out to examine the effects of miR-124a on dendritic morphology and take this as the readout for our following studies on the functional interactions between the two genes. To this end, we ectopically expressed pre-*miR-124a* in *Drosophila* DA sensory neurons, a model system we have used extensively to investigate mechanisms of dendritic morphogenesis. The pre-*miR-124a* sequence was cloned into the UAST vector, and transgenic flies were generated. In ddaE and ddaF neurons in the dorsal cluster (Sweeney et al., 2002), expression of pre-*miR-124a* with *Gal4*^{221} resulted in significantly fewer dendritic ends than in wild-type neurons [ddaE: 17.0 ± 0.6 ($n = 24$) vs 25.9 ± 1.0 ($n = 20$), $p < 0.001$; ddaF: 17.0 ± 0.7 ($n = 24$) vs 20.3 ± 0.6 ($n = 20$), $p < 0.001$, quantified as we did before in other studies (Li et al., 2004)] (Fig. 2*B*,*D*). A similar phenotype was observed in some other DA neurons (supplemental Fig. S1, available at www.jneurosci.org as supplemental material).

To exclude the possibility that ectopic expression of any small RNA was responsible for this effect, we generated a mutant pre-*miR-124a* molecule with a 6 nt deletion (supplemental Fig. S2, available at www.jneurosci.org as supplemental material). Expression of this mutant construct did not affect the dendritic morphology of DA neurons (Fig. 2*D*). Moreover, ectopic expression of pre-*miR-9a* significantly increased the number of dendritic ends of both ddaE neurons [56.8 ± 1.9 ($n = 18$) vs 25.9 ± 1.0 ($n = 20$), $p < 0.001$) and ddaF neurons (40.0 ± 3.1 ($n = 10$) vs 20.3 ± 0.6 ($n = 10$), $p < 0.001$]

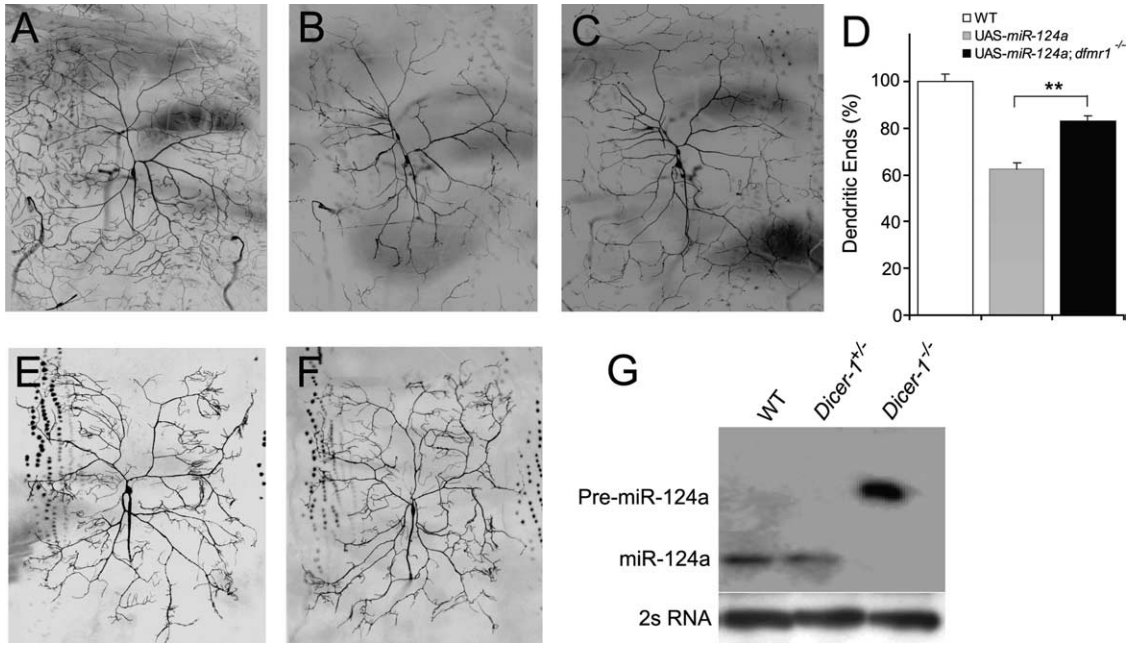

Figure 3. dFMR1 is required for pre-miR-124a to exert its effect on dendritic morphogenesis of DA neurons. ***A***, A wild-type (WT) ddaC neuron is labeled with mCD8-GFP whose expression is driven by *Gal4*^{477}. ***B***, Reduced dendritic branching and dendritic field size in a ddaC neuron expressing pre-*miR-124a*. ***C***, Expression of pre-*miR-124a* in ddaC neurons in *dfmr1* mutant background (*dfmr1*^{4}/*dfmr1*^{4} or *dfmr1*^{3}/*dfmr1*^{4}) resulted in a dendritic field of nearly normal size. ***D***, Quantification of dendritic ends of wild-type (WT) ddaC neurons and neurons expressing pre-*miR-124a* in normal or *dfmr1* mutant backgrounds. The values are mean ± SEM. $^{**}p < 0.01$. ***E***, A MARCM-generated *dcr-1* mutant ddaC neuron. ***F***, A *dcr-1* mutant ddaC neuron expressing pre-*miR-124a*. ***G***, Northern blot analysis of miR-124a processing in WT or *dcr-1* mutant larvae demonstrates that Dcr-1 is absolutely required for the production of mature miR-124a *in vivo*.

(Fig. 2*C*,*D*). Ectopic expression of pre-*miR-124a* and pre-*miR-9a* also had opposite effects on dendritic branching in vpda sensory neurons (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). These findings indicate that miR-124a specifically affects dendritic branching and that different miRNAs may exert opposite effects on neuronal morphology, probably by modulating the expression of different sets of downstream target mRNAs.

Genetic interactions between dfmr1 and pre-miR-124a

Based on our finding that dFMR1 and miR-124a are present in the same RNP complex (Fig. 1*C*,*D*), we performed genetic interaction experiments to determine if miR-124a and *dfmr1* indeed interact to control the dendritic branching of DA neurons. We did not perform similar experiments for miR-9a because unlike miR-124a, miR-9a is not detectable in postmitotic neurons. For this experiment, we used ddaC neurons that elaborate extensive dendritic arbors to innervate a large area of the epidermis in the hemisegment (Sweeney et al., 2002). Ectopic expression of pre-*miR-124a* in ddaC neurons decreased the number of dendritic ends compared with wild-type neurons [342.5 ± 21.2 ($n = 18$) vs 548.2 ± 13.1 ($n = 18$), $p < 0.001$] and the size of the dendritic field [0.27 ± 0.01 mm^2 ($n = 10$) vs 0.42 ± 0.01 mm^2 ($n = 10$) vs $p < 0.001$] (Fig. 3*B*,*D*), consistent with dendritic phenotypes exerted by miR-124a in other DA neurons (Fig. 2*B*; supplemental Fig. S1, available at www.jneurosci.org as supplemental material). To examine whether dFMR1 mediates the effects of pre-*miR-124a*, we expressed pre-*miR-124a* in DA neurons in mutant flies containing two independently generated *dfmr1* loss-of-function alleles: *dfmr1*^{3} (Dockendorff et al., 2002) and *dfmr1*^{4} (A. Lee et al., 2003). The effects of pre-*miR-124a* expression in ddaC neurons were significantly attenuated by loss of *dfmr1* activity (Fig. 3*C*,*D*): the number of dendritic ends was partially rescued [437.2 ± 21.0 ($n = 18$) vs 342.5 ± 21.2 ($n = 18$), $p < 0.01$], although not to the wild type level [437.2 ± 21.0 ($n = 18$) vs 548.2 ± 13.1 ($n = 20$), $p < 0.001$] (Fig. 3*D*). Interestingly, the dendritic fields of ddaC neurons were similar in size to those of wild-type neurons [0.40 ± 0.02 mm^2 ($n = 10$) vs 0.42 ± 0.01 mm^2 ($n = 10$), $p > 0.4$]. These results indicate that dFMR1 is required *in vivo*, at least in part, for pre-*miR-124a* to exert its effects on dendritic morphology. Our earlier published work indicated that loss of *dfmr1* led to increased dendritic branching of DA neurons (A. Lee et al., 2003), raising the possibility that attenuated effects of endogenous miRNAs on dendritic branching could be a potential underlying mechanism.

As a positive control for dFMR1, we performed a similar analysis with *dicer-1* mutants. Genetic analysis of zebrafish suggested that Dicer is essential for proper brain morphogenesis (Giraldez et al., 2005). In *Drosophila*, Dicer-1 (Dcr-1), but not Dicer-2 (Dcr-2), is required for the processing of pre-miRNAs into mature miRNAs (Lee et al., 2004). Because *dcr-1*^{Q1147X} and *dcr-1*^{d102} mutants die at the first or second instar larval stage, the dendritic morphology of DA neurons cannot be analyzed in detail in mutant animals. To examine the cell-autonomous effects of Dcr-1 and miR-124a on dendritic morphology of DA neurons in third instar larvae, we used either allele to generate GFP-labeled single *dcr-1* mutant ddaC neurons in an otherwise wild-type animal with mosaic analysis with a repressible cell marker (MARCM) (Lee and Luo, 1999).

Expression of pre-*miR-124a* in *dcr-1* mutant neurons did not affect the number of dendritic ends [613.0 ± 14.3 ($n = 7$) vs 586 ± 44.6 ($n = 9$), $p > 0.5$] (Fig. 3*E*,*F*), indicating that Dcr-1 is absolutely essential for pre-*miR-124a* to exert its biological function in neurons, as one would expect. Indeed, Northern blot analysis demonstrates the absence of endogenous mature miR-124a in *dcr-1*^{Q1147X} or *dcr-1*^{d102} mutant first instar larvae and a corresponding accumulation of endogenous pre-miR-124a (Fig. 3*G*). It is interesting to note that loss of Dcr-1 activity by itself in

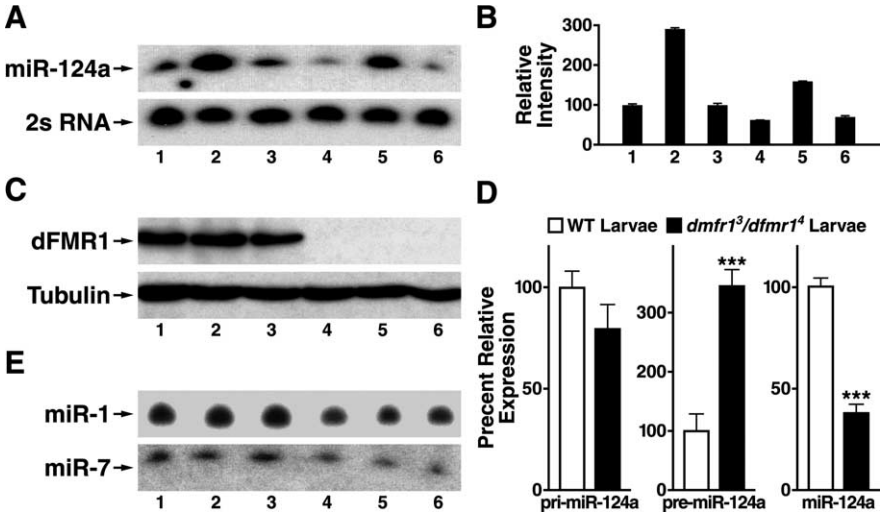

Figure 4. dFMR1 is required to ensure normal steady-state levels of mature miR-124a in the *Drosophila* nervous system. ***A***, Northern blot analysis of miR-124a levels in *Drosophila* third instar larvae. Lane 1: *Gal4 109(2)80*, *UAS-mCD8-GFP/*± larvae (controls). Lane 2: *Gal4 109(2)80*, *UAS-mCD8-GFP/*+; *UAS-pre-miR-124a/*+ larvae that express miR-124a in all MD neurons and a small number of CNS neurons. Lane 3: *Gal4 109(2)80*, *UAS-mCD8-GFP/*+; *UAS-mutant pre-miR-124a/*+ larvae. Lane 4: *Gal4 109(2)80*, *UAS-mCD8-GFP/*+; $dfmr1^3/dfmr1^4$ larvae. Lane 5: *Gal4 109(2)80*, *UAS-mCD8-GFP/UAS-pre-miR-124a;* $dfmr1^3/dfmr1^4$ larvae. Lane 6: *Gal4 109(2)80*, *UAS-mCD8-GFP/UAS-mutant pre-miR-124a;* $dfmr1^3/dfmr1^4$ larvae. Equal amounts of total RNAs were loaded for each genotype, and 2 s RNA was used as the internal control. ***B***, Relative expression levels of miR-124a in larvae with different genetic backgrounds and transgene expression. The data (mean ± SEM) were derived from three independent experiments and normalized against the levels of 2s RNA. ***C***, Western blot analysis verified the absence of dFMR1 in *dfmr1* mutant larvae as described in ***A***. ***D***, Quantitative RT-PCR analysis of relative expression levels of pri-miR-124a, pre-miR-124a, and mature miR-124a in wild-type (WT) and *dfmr1* mutant larvae. ***E***, The Northern blot membrane used in ***A*** was stripped and reprobed for miR-1 and miR-7 which showed that the *in vivo* expression levels of miR-1 and miR-7 were also reduced in *dfmr1* mutant larvae. The values are mean ± SEM. ***$p < 0.001$ versus wild type.

MARCM clones did not significantly reduce the number of dendritic ends of ddaC neurons; however, terminal branches fail to elaborate properly, suggesting an essential role for Dcr-1 and the miRNA pathway in some aspects of dendritic morphogenesis.

dFMR1 is required to maintain the steady-state level of miR-124a in *Drosophila*

The similarity between the absolute requirement for Dcr-1 (Fig. 3*E*,*F*) and the partial requirement for dFMR1 (Fig. 3*A*–*D*) in the effect of pre-miR-124a on dendritic branching prompted us to examine the exact role of dFMR1 in miR-124a biogenesis and the biochemical basis for the genetic interaction we observed between *miR-124a* and *dfmr1*. Because Dcr-1 is absolutely required for pre-*miR-124a* processing (Fig. 3*G*), we tested the hypothesis that dFMR1 might affect the steady-state levels of mature miR-124a *in vivo*. Ectopic expression of pre-miR-124a in a subset of PNS sensory neurons with *109(2)80*-Gal4 led to increased level of mature miR-124a as shown by Northern blot (Fig. 4*A*, lane 2 vs lane 1 as the control). As expected, ectopic expression of mutant pre-*miR-124a* did not increase steady-state level of mature miR-124a *in vivo* (Fig. 4*A*, lane 3), consistent with the finding that mutant pre-*miR-124a* did not cause any dendritic phenotype (Fig. 2*D*). However, in $dfmr1^3/dfmr1^4$ mutant larvae, endogenous mature miR-124a was decreased by 42% (Fig. 4*A*,*B*, lane 4), and mature miR-124a processed from ectopically expressed pre-miR-124a was also decreased similarly by 47% (Fig. 4*A*,*B*, lane 5). The absence of dFMR1 in mutant larvae was confirmed by Western blot analysis (Fig. 4*C*, lanes 4–6). These results suggest that the steady-state level of miR-124a in the *Drosophila* nervous system is modulated by dFMR1. Interestingly, overexpression of dFMR1 did not lead to increased steady-state level of mature miR-124a *in vivo* (supplemental Fig. S3, available at www.jneurosci.org as supplemental material), suggesting that dFMR1 is not a rate-limiting factor in miRNA biogenesis.

To further support the notion that dFMR1 modulates miR-124a biogenesis, we analyzed the steady-state levels of miR-124a and its precursors in wild-type and *dfmr1* mutant larvae by qRT-PCR. Consistent with the Northern blot analysis, $dfmr1^3/dfmr1^4$ mutant larvae had a lower level of mature miR-124a than wild-type larvae and a correspondingly higher steady-state level of pre-miR-124a (Fig. 4*D*). Thus, it appears that dFMR1 modulates the processing efficiency and the steady-state levels of miR-124a *in vivo*.

To determine if dFMR1 is required for the proper biogenesis of other miRNAs, we examined by Northern blot analysis muscle-specific miR-1 and miR-7 that is highly expressed in the nervous system. The steady-state levels of both miRNAs were reduced by ~40–45% (Fig. 4*E*, lanes 4–6). Moreover, the levels of pre- and mature but not pri-miR-7 and miR-1 as measured by qRT-PCR were also decreased (supplemental Fig. S4, available at www.jneurosci.org as supplemental material), suggesting that loss of dFMR1 affects the levels of multiple miRNAs in *Drosophila*. Because each miRNA may target hundreds of mRNAs, these results suggest that the expression of a very large number of genes may be affected to some extent by the loss of dFMR1.

The Dcr-1–Ago1 complex is less abundant in the absence of dFMR1

In *Drosophila*, Dcr-1 is primarily responsible for converting pre-miRNAs to mature miRNAs, whereas Dcr-2 is required for processing siRNA precursors (Lee et al., 2004). Although different miRNAs can associate predominantly with either Ago1- or Ago2-containing RNA-induced silencing complex (RISC) (Förstemann et al., 2007), it seems that Ago1 is more essential for miRNA biogenesis (Okamura et al., 2004). To provide further mechanistic insight for the action of dFMR1 in the miRNA pathway, we performed co-immunoprecipitation experiments to examine the abundance of the Dcr-1-Ago1 complex in the presence or absence of dFMR1. We prepared lysates from a large number of dissected larval brains and used Ago1 antibody to pull down the complex. We found that Ago1 was associated with Dcr-1 and dFMR1 *in vivo* (Fig. 5*A*). Interestingly, based on three independent experiments, there was an ~44% decrease in the abundance of the Dcr-1-Ago1 complex attributable to the absence of dFMR1 (Fig. 5*A*,*B*). This novel finding suggests a functional role for dFMR1 in facilitating the formation of the Dcr-1-Ago1 complex. This difference is not attributable to reduced Dcr-1 and Ago1 expression levels in *dfmr1* mutants (Fig. 5*C*,*D*). These findings provide a molecular explanation for the observed lower levels of miR-124a in the developing *Drosophila* nervous system.

Discussion

The major novel findings in this study are that elevated miR-124a expression decreases dendritic branching and dFMR1, the fly ho-

molog of the human protein responsible for fragile X syndrome, modulates the biogenesis and function of miRNAs in the *Drosophila* nervous system. This conclusion is supported by the presence of dFMR1 and miR-124a in the same RNP complex, the genetic interaction between the two genes, and biochemical analysis of steady-state levels of miRNAs in the absence of dFMR1. Moreover, we found that the Dcr-1-Ago1 complex was less abundant in the nervous system of *dfmr1* mutants, providing a mechanistic explanation for the observed effect of loss of dFMR1 activity on miRNA biogenesis.

miR-124a is one of the most abundant miRNAs in the brain and whose nucleotide sequence is 100% conserved across species yet it functions in the brain remain incompletely understood (Lagos-Quintana et al., 2002; Gao, 2008). Our findings suggest that elevated pre-miR-124a level, such as in pathological conditions, can lead to decreased dendritic branching, which may be detrimental to neuronal function and connectivity. The effect of pre-*miR-124a* on dendritic branching *in vivo* seems to require dFMR1. Although dFMR1 is implicated in neuronal development through translational control, the exact mechanism remains unclear. Our findings shed new light on the precise roles of dFMR1 in the developing nervous system. dFMR1 is not an absolute essential factor for the biogenesis of mature miRNAs *in vivo*, consistent with earlier findings in cultured S2 cells (Caudy et al., 2002; Ishizuka et al., 2002). Nonetheless, the finding that dFMR1 modulates the steady-state levels of miR-124a and other miRNAs is of considerable significance, because multiple genes must be expressed coordinately at precise levels both spatially and temporally during brain development. Because miRNAs fine-tune protein synthesis of many genes (Baek et al., 2008; Selbach et al., 2008), the developmental and functional defects seen in *dfmr1* mutants are likely the consequence of changes in multiple proteins and pathways.

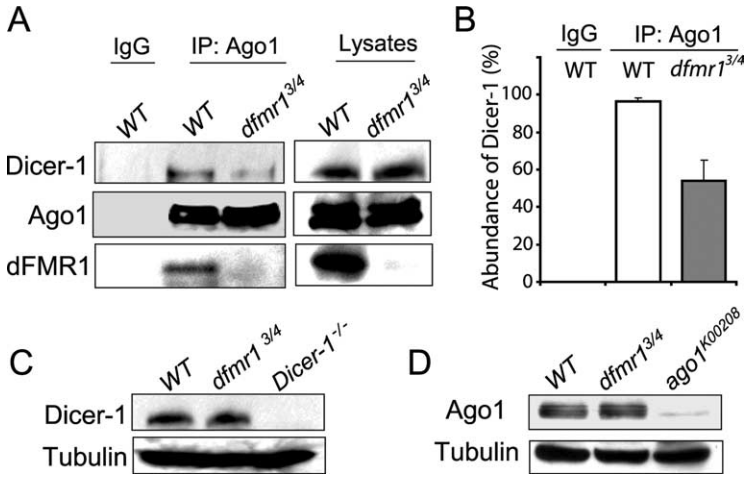

Figure 5. The Dcr-1-Ago1 complex is less abundant in the absence of dFMR1. ***A***, Control mouse IgG or Ago1 antibody were used to immunoprecipitate Ago1-containing complexes from lysates of dissected wild-type (WT) or *dfmr1* mutant larval brains. The presence or absence of Dcr-1 or dFMR1 was detected by Western blot. This experiment was repeated three times. ***B***, Quantification of the abundance of Dicer-1 in Ago1 immunoprecipitates based on three independent experiments. The values are mean ± SEM. $p < 0.01$ versus wild type. ***C***, The protein band recognized by the Dcr-1 antibody obtained from Abcam is absent in the *dcr-1* mutants. ***D***, An Ago1 antibody (Okamura et al., 2004) identifies Ago1 isoforms that are absent in *ago1* mutants. Note that the expression levels of Dcr-1 and Ago1 are the same in *dfmr1* mutants and wild-type larvae. IP, Immunoprecipitation.

As an RNA-binding protein, dFMR1 is present in multiple distinct RNP complexes that are likely involved in many aspects of RNA metabolism. For instance, dFMR1 forms a complex with PIWI in germ cells and play a role in the biogenesis and function of piRNAs (Megosh et al., 2006). dFMR1 is also associated with Ago2 and likely plays a role in the RNAi pathway (Caudy et al., 2002; Ishizuka et al., 2002). Here, we show that endogenous dFMR1 and Ago1 are associated with each other in the developing *Drosophila* nervous system, consistent with a recent finding in oocytes (Yang et al., 2007). More importantly, we found that the Dcr-1-Ago1 complex in the nervous system is less abundant in the absence of dFMR1, providing novel mechanistic insight into the exact role of dFMR1 in the miRNA pathway. Considering the essential roles of the Dcr-1-Ago1 complex in miRNA biogenesis (Okamura et al., 2004; Förstemann et al., 2007), impaired association between Dcr-1 and Ago1 attributable to the absence of dFMR1 seems to be responsible, at least in part, for the observed lower steady-state levels of the nervous-system-specific miR-124a and other miRNAs *in vivo*. Global regulation of miRNA levels by RNA-binding proteins was also reported in the case of Rbm3, a glycine-rich RNA-binding protein that regulates global protein synthesis under cold-stress conditions (Dresios et al., 2005).

In a human B-cell line, FMRP was associated with small 20 nt RNAs in a common RNP complex (Jin et al., 2004). This small RNA is unlikely to be miR-124a, which is nervous-system-specific and 23 nt long as many other miRNAs. dFMR1 has three mammalian homologs, FMRP, FXR1, and FXR2. Their exact functions in the miRNA pathway remain largely unknown, although one recent report suggested that FXR1 was involved in some aspects of miRNA function in HeLa cells (Vasudevan et al., 2007). It is possible that these three mammalian proteins may have redundant functions in the miRNA pathway. Alternatively, different paralogs may have evolved to carry out at least some distinct molecular functions in postmitotic neurons in mammals. It will be interesting to determine the extent to which dFMR1 and FMRP are functionally conserved and whether the miRNA pathway is also misregulated in mouse models of fragile X syndrome.

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