

Control of dendritic development by the *Drosophila fragile X-related* gene involves the small GTPase Rac1

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Accepted 11 August 2003

Development 130, 5543-5552

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doi:10.1242/dev.00792

Summary

Fragile X syndrome is caused by loss-of-function mutations in the *fragile X mental retardation 1* gene. How these mutations affect neuronal development and function remains largely elusive. We generated specific point mutations or small deletions in the *Drosophila fragile X-related (Fmr1)* gene and examined the roles of *Fmr1* in dendritic development of dendritic arborization (DA) neurons in *Drosophila* larvae. We found that *Fmr1* could be detected in the cell bodies and proximal dendrites of DA neurons and that *Fmr1* loss-of-function mutations increased the number of higher-order dendritic branches. Conversely, overexpression of *Fmr1* in DA neurons dramatically decreased dendritic branching. In dissecting the mechanisms underlying *Fmr1* function in dendrite

development, we found that the mRNA encoding small GTPase Rac1 was present in the *Fmr1*-messenger ribonucleoprotein complexes *in vivo*. Mosaic analysis with a repressor cell marker (MARCM) and overexpression studies revealed that Rac1 has a cell-autonomous function in promoting dendritic branching of DA neurons. Furthermore, *Fmr1* and *Rac1* genetically interact with each other in controlling the formation of fine dendritic branches. These findings demonstrate that *Fmr1* affects dendritic development and that Rac1 is partially responsible for mediating this effect.

Key words: Fragile X syndrome, Dendrites, *Drosophila*, Rac1

Introduction

The normal development of dendritic structures of various neuronal subtypes is essential for the proper function of a nervous system. During the past few years, a number of studies in several model systems have demonstrated the essential roles of neuronal activity, extracellular cues and intrinsic factors in dendritic morphogenesis (for reviews, see McAllister, 2000; Scott and Luo, 2001; Cline, 2001; Jan and Jan, 2001; Whitford et al., 2002; Wong and Ghosh, 2002; Gao and Bogert, 2003). However, it remains largely unknown what limits the growth of a particular neuron, and in particular, the number and length of dendritic branches during development.

One family of proteins that may be important in controlling neuronal growth is that of mRNA-binding proteins. These proteins control gene expression at multiple steps of mRNA metabolism, such as splicing, transport, localization, translation and degradation (reviewed by Darnell, 2002; Dreyfuss et al., 2002). Some of these proteins are highly or solely expressed in neurons, and their functions are beginning to be revealed (reviewed by Musunuru and Darnell, 2001; Steward and Schuman, 2001). In this study, we focus on the role of the fly homolog of the *fragile X mental retardation 1 (FMR1)* gene in dendritic development.

The absence of the *FMR1* gene activity causes fragile X syndrome, the most common form of inherited mental retardation in humans, with an estimated incidence of 1 in 4000 males and 1 in 8000 females (reviewed by O'Donnell and Warren, 2002). *FMR1* encodes a putative RNA-binding protein

with two ribonucleoprotein K homology (KH) domains and an arginine- and glycine-rich domain (RGG box). The FMR1 protein preferentially binds to poly(G), poly(U) and a subset of brain mRNAs *in vitro* (Ashley et al., 1993; Siomi et al., 1993; Brown et al., 1998). In addition, FMR1 is associated with polyribosomes and a large number of mRNAs *in vivo*, some of which contain G quartet structures as FMR1-binding motifs (Feng et al., 1997a; Corbin et al., 1997; Sung et al., 2000; Darnell et al., 2001; Brown et al., 2001; Zalfa et al., 2003; Miyashiro et al., 2003). It remains to be determined which proteins encoded by mRNAs in FMR1-mRNP complexes are primarily responsible for the morphological and functional deficits caused by the absence of FMR1.

The exact molecular function of FMR1 *in vivo* remains largely unknown. Some studies suggest that FMR1 affects mRNA localization and translation (Darnell et al., 2001; Brown et al., 2001; Zalfa et al., 2003; Miyashiro et al., 2003), although the underlying mechanism is unclear (Antar and Bassell, 2003). The recent demonstration that *Drosophila* fragile X-related protein (*Fmr1*; previously dFXR) interacts with components of the RNA interference (RNAi) machinery raises the possibility that *Fmr1*/FMR1 may also function as part of a gene-silencing mechanism (Ishizuka et al., 2002; Caudy et al., 2002). FMR1 is highly expressed in neuronal perikaryon and dendrites and shuttles between the nucleus and the cytosol (Devys et al., 1993; Fridell et al., 1996; Feng et al., 1997b). Studies of individuals with fragile X syndrome, *Fmr1* knockout mice and cultured neurons, although not entirely consistent

with each other, raise the possibility that FMR1 is involved in the proper development of spines of central nervous system (CNS) neurons (Hinton et al., 1991; Comery et al., 1997; Braun and Segal, 2000; Nimchinsky et al., 2001).

We study the role of Fmr1 in the peripheral nervous system (PNS) of the *Drosophila* larva, which is relatively simple and consists of 44 sensory neurons in each abdominal hemisegment (Ghysen et al., 1986; Bodmer et al., 1989; Orgogozo et al., 2001). Dendritic arborization (DA) neurons, one subtype of PNS sensory neurons, elaborate extensive dendritic arbors just underneath the epidermis to receive sensory inputs (Bodmer and Jan, 1987; Gao et al., 1999). The ability to visualize the dendritic arbors in living *Drosophila* larvae allows us to quantitatively examine the effects of Fmr1 on dendritic development of identifiable neurons in vivo.

The *Fmr1* gene is the only fly homolog of the human *FMR1* gene that also has RNA-binding activity in vitro (Adams et al., 2000; Wan et al., 2000). It has been reported that *Fmr1* mutation impairs the synaptic function at the neuromuscular junction (Zhang et al., 2001). In addition, Fmr1 is required for normal circadian rhythm of adult flies (Dockendorff et al., 2002; Morales et al., 2002; Inoue et al., 2002). To study the role of *Fmr1* in dendritic growth, we isolated Fmr1 protein-deficient mutant fly lines in which specific point mutations or small deletions were introduced into the *Fmr1* gene. We report that Fmr1 is expressed in DA sensory neurons and limits dendritic branching during development. In addition, we show that the mRNA encoding the small GTPase Rac1 is present in Fmr1-mRNP complexes and that the function of Fmr1 in dendrite development is partially mediated by Rac1.

Materials and methods

Fly lines and genetic crosses

All the flies were raised at 25°C and fed standard food. The P-element insertion line EP(3)3517 was from the Szeged Stock Center (Hungary). Genomic rescue analysis confirmed that the P-element was inserted into the intron between the first and second exon of the *Fmr1* gene. The ATG start codon is located in the second exon of the gene. To separate the P-element insertion from the second-site semi-lethal mutation, we crossed the EP(3)3517 line with isogenized *w¹¹¹⁸* flies and established multiple recombinant lines that contained the P-element insertion. We selected one of the recombinant lines in which flies homozygous for the P-element could develop into adulthood at the expected Mendelian ratios for further experiments. To isolate mutations in the *Fmr1* gene, we fed male flies from this recombinant line with a 1% sucrose solution containing ethyl methanesulfonate (EMS) and mated them en masse with *tubulin-Gal4* virgin females. The EP line contains an upstream activation sequence (UAS) in the P-element (Rørth, 1996); therefore, the *tubulin-Gal4* could drive overexpression of the endogenous wild-type *Fmr1* gene in all cell types, which led to a lethal phenotype. Individual surviving flies from this cross might contain a mutated *Fmr1* gene on the chromosome that contained the P-element insertion; therefore, the presence of the Gal4 in the same flies would not cause the death of the flies. These surviving flies were crossed with flies containing balancer chromosomes to establish stable lines. The absence of Fmr1 in these lines was confirmed by western blot and sequence analysis on the cloned genomic DNA fragments (see below).

Other flies lines used in this study were *UAS-mCD8::GFP* (Bloomington Stock Center), *UAS-Rac1* (Bloomington Stock Center), *UAS-Fmr1* (Wan et al., 2000), *Fmr1³/TM6* (Dockendorff et al., 2002), the MD neuron-specific Gal4 line 109(2)80 (Gao et al., 1999),

Rac1¹¹¹, *FRT^{2A}/Cyo* (Ng et al., 2002) and *UAS-Fmr1-GFP* (this study). A fly line that contains a 14 kb fragment that spans the *Fmr1* transcriptional unit (Dockendorff et al., 2002) was also used in this study. To study Fmr1 overexpression phenotypes, we selected third instar larvae with the genotype of *Gal4 109(2)80, UAS-mCD8-GFP/+; UAS-Fmr1/+* and visualized mCD8::GFP-labeled DA neurons in which Fmr1 was overexpressed. Similar genetic crosses were made to overexpress Rac1 in DA neurons.

Western blot analysis

The expression of Fmr1 in *Drosophila* larvae was analyzed by western blot according to the standard protocol provided by BioRad. Wild-type or *Fmr1* mutants at the third instar larval stage were used to prepare protein extracts. Anti-Fmr1 monoclonal antibody (Wan et al., 2000) was used as the primary antibody (1:1000 dilution). Horseradish peroxidase-conjugated donkey anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories) was used as the secondary antibody (1:200).

In situ analysis and immunohistochemistry

The expression of *Fmr1* mRNA was analyzed according to a standard in situ protocol. For antibody immunostaining of DA neurons in dissected third instar larvae, monoclonal antibody against Fmr1 (1:100) was used as the primary antibody. Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, 1:100) was used as the secondary antibody. The dissected larvae were mounted in 90% glycerol in PBS, and confocal images were obtained with a confocal microscope (BioRad Radiance 2000). For this experiment, *Fmr1* mutant larvae were used as the negative control.

Quantification of dendritic processes

To measure the total number of terminal dendrites of ventral DA neurons in segments 5 and 6, images of mCD8::GFP-labeled dendrites were taken with a confocal microscope and were converted with Photoshop 6.0 into grayscale with a bright background. The dendritic processes were counted in a specific area between two contralateral ventral clusters of DA neurons from zoom-in images and presented as the number of processes per 1000 μm^2 .

Immunoprecipitation and RT-PCR

Cell lysates were made from *Drosophila* larvae of the desired genotypes and used in immunoprecipitation experiments in which a monoclonal antibody raised against Fmr1 (Wan et al., 2000) was used to pull down Fmr1-mRNP complexes. Total RNA was extracted from the precipitated complexes and used to generate cDNAs in reverse transcription (RT) reactions, which served as the template for polymerase chain reactions (PCR). PCR was performed with oligonucleotide primers specific for Rac1 or other control mRNAs, and the resulting DNA fragments were analyzed by electrophoresis in 2% agarose gels.

MARCM analysis of Rac1 function in DA neuron dendrite development

Single-cell analysis of *Rac1* function in DA neuron dendrite development was performed as described (Sweeney et al., 2002). Briefly, *Rac1¹¹¹*, *FRT^{2A}/TM6B* male flies were crossed with *GAL4^{C155}*, *UAS-mCD8::GFP*, *hs-FLP1/FM7* virgin female flies. Then, *GAL4^{C155}*, *UAS-mCD8::GFP*, *hs-FLP1/+*; *Rac1¹¹¹*, *FRT^{2A}/+* male flies were crossed with *GAL4^{C155}*, *UAS-mCD8::GFP*, *hs-FLP1/+*; *tubP-GAL80*, *FRT^{2A}* virgin female flies. Embryos from this cross were collected and incubated at 25°C for 3 hours. At 3-6 hours after egg laying (AEL), embryos were heat-shocked in a 37°C water bath for 40 minutes to induce mitotic recombination. Vials were then kept at 25°C for 3-4 days. Third instar larvae were collected and examined for the presence of a single mCD8::GFP-labeled dorsal cluster PNS neuron, and images of dendritic morphology were obtained as described above.

Results

Generation of *Fmr1* mutant flies

To generate *Fmr1* mutant flies, we obtained a semi-lethal fly line, EP(3)3517, from the Szeged Stock Center in which a P-element insertion was found in the first intron of the *Fmr1* gene, which is located on the third chromosome (Fig. 1A). As *Fmr1/FMR1* loss-of-function mutations in mouse and human are not lethal (reviewed by O'Donnell and Warren, 2002), we reasoned that the semi-lethal phenotype in the original EP(3)3517 stock was due to an unidentified second-site mutation. Therefore, we carried out genetic recombination experiments and obtained a viable, isogenic fly line that contained the P-element insertion in the *Fmr1* gene but did not have the second-site mutation that caused the semi-lethal phenotype (see Materials and methods).

The UAS was engineered into the P-element (Fig. 1A); therefore, the downstream gene could be expressed in the presence of the yeast transcription activator Gal4 (Rørth, 1996). When the P-element insertion line was crossed with the tubulin-Gal4 line, which drives target-gene expression ubiquitously in developing embryos as well as in later stages, the overexpression of the endogenous *Fmr1* gene led to a lethal phenotype (data not shown). We reasoned that if *Fmr1* were mutated with EMS, flies containing chromosomes harboring both tubulin-Gal4 and the P-element insertion would survive. Using this strategy, we isolated three *Fmr1* mutant fly lines. Larvae that were homozygous for the mutated chromosome were analyzed by western blot with an anti-Fmr1 monoclonal antibody (Wan et al., 2000). None of the three *Fmr1* mutant lines, *Fmr1*¹, *Fmr1*² and *Fmr1*⁴, expressed the Fmr1 protein (Fig. 1B). We cloned and sequenced the genomic DNA at the *Fmr1* locus and identified an 11-nucleotide deletion in the *Fmr1*¹ allele that, owing to the frame shift, resulted in a stop codon after amino acid 126 (Fig. 1C). A single nucleotide mutation (C to T) at amino acid 289 in the *Fmr1*⁴ allele changed the codon to a stop codon (Fig. 1C). We did not find mutations in the *Fmr1*-coding region in the *Fmr1*² allele, indicating that the loss-of-function mutations are probably in the promoter or introns.

Mutant flies with the genotypes of *Fmr1*⁴/*Fmr1*⁴, *Fmr1*¹/*Fmr1*⁴ or *Fmr1*²/*Fmr1*⁴ are viable and develop into adulthood at the predicted Mendelian ratios, consistent with some of the previous reports that loss-of-function mutations in *Fmr1* are not lethal events (reviewed by Gao, 2002). No obvious morphological defects were found in adult *Fmr1* mutant flies. In this study, we mainly analyzed developmental abnormalities of *Fmr1* mutant larvae.

Fmr1 is expressed in dendritic arborization (DA) neurons

We have been studying dendritic growth in the *Drosophila* embryonic and larval PNS, in which each abdominal hemisegment contains 44 sensory neurons that can be grouped into dorsal, lateral and ventral clusters. In the dorsal cluster, eight MD neurons, but not the four external sensory (ES) neurons, can be labeled by green fluorescent protein (GFP) driven by Gal4 109(2)80 (Gao et al., 1999) (Fig. 2A). DA neurons, a subclass of MD neurons, develop complex dendritic branching patterns (Bodmer and Jan, 1987; Gao et al., 1999; Gao et al., 2000).

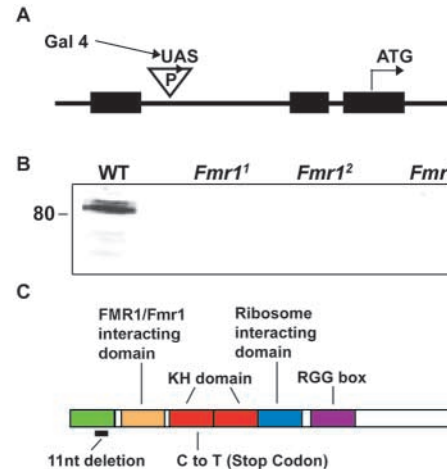


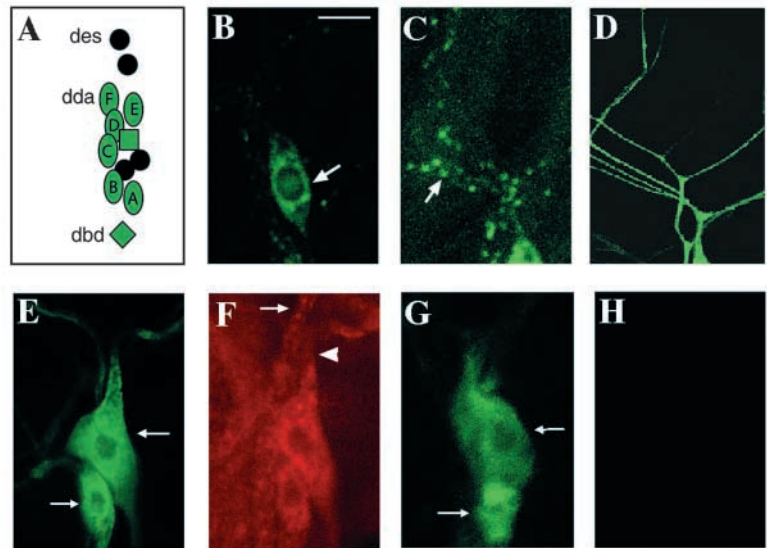
Fig. 1. Generation of *Fmr1* mutant flies. (A) A P-element inserted in the first intron of the *Fmr1* gene can be overexpressed because of the Gal4-binding sites in the P-element. (B) Western blot analysis indicated that Fmr1, an 80 kDa protein with multiple isoforms, is not expressed in mutant lines *Fmr1*¹, *Fmr1*² and *Fmr1*⁴. (C) Fmr1 contains several highly conserved domains, including the FMR1/FXR-interacting domain, KH domains and the ribosomal-association domain. EMS-induced mutations have been identified. For example, a point mutation in the *Fmr1*⁴ allele changes amino acid 289 to a stop codon, and a small deletion in the *Fmr1*¹ allele causes a frame-shift in the N terminus after the amino acid 126.

To test whether *Fmr1* affects the dendritic growth of DA neurons, we first confirmed the expression of Fmr1 in these neurons. Consistent with a previous report (Wan et al., 2000), *Fmr1* mRNA was expressed at high levels in the embryonic nervous system and in body wall muscles (data not shown). To examine the subcellular localization of Fmr1 in DA neurons of live larvae, we generated a *UAS-Fmr1-GFP* transgenic fly line. When Fmr1-GFP was expressed in DA neurons driven by Gal4 109(2)80, we observed Fmr1 expression in the cytoplasm of DA neurons (Fig. 2B) and in particle-like structures in dendrites (Fig. 2C). To further confirm that the endogenous Fmr1 is expressed in DA neurons, we performed immunostaining analysis on dissected larvae using a monoclonal antibody raised against Fmr1 (Wan et al., 2000). We found that Fmr1 was present in DA neurons and was expressed predominantly in the cytoplasm (Fig. 2F,H). The expression of Fmr1 in the proximal dendrites of DA neurons and in body wall muscle fibers was also detectable (Fig. 2F). Owing to the high level of Fmr1 expression in muscles, the localization of endogenous Fmr1 in distal dendrites was barely visible with confocal microscopy (not shown). The subcellular localization of Fmr1 in DA neurons is consistent with the subcellular localization of FMR1 in mammalian neurons (Devys et al., 1993). As a negative control, Fmr1 signal was not detected in either DA neurons or muscle fibers in *Fmr1* mutant larvae (Fig. 2H).

The development of DA neuron terminal dendrites is affected by *Fmr1* mutations

Because *Fmr1* mutants were viable, we were able to directly examine the effects of *Fmr1* mutations on dendritic

Fig. 2. Subcellular localization of *Fmr1* in DA neurons in *Drosophila* larvae. (A) Schematic representation of all the neurons in the dorsal cluster of an abdominal hemisegment. des, dorsal external sensory neurons; dda, dorsal dendritic arborization neuron; dbd, dorsal bipolar dendritic neuron. Only the dda and dbd neurons are labeled by GFP using Gal4 109(2)80. (B) Cytoplasmic localization of *Fmr1*-GFP fusion protein in a ddaF neuron is indicated by the arrow. The expression of *Fmr1*-GFP was driven by Gal4 109(2)80. Scale bar: 10 μm . (C) The *Fmr1*-GFP signal in dendrites (arrow) is relatively weak compared with that in the cell body. This image was enhanced using Photoshop to demonstrate the localization of *Fmr1*-GFP in dotted structures in dendrites of dorsal cluster DA neurons. (D) UAS-GFP driven by Gal4 109(2)80 in these DA neurons indicates the dendritic branching patterns. (E) GFP-labeled DA neurons in a wild-type third instar larva are indicated by arrows. (F) Antibody staining of the same DA neurons as in E demonstrates the cytoplasmic localization of endogenous *Fmr1*. The arrowhead indicates the proximal segments of dendrites. The arrow indicates a muscle fiber that was also labeled by *Fmr1*-specific monoclonal antibody. (G) UAS-GFP-labeled DA neurons in a *Fmr1* mutant third instar larva are indicated by arrows. (H) Antibody staining of the same DA neurons in Panel G demonstrates the absence of *Fmr1* positive signals in *Fmr1* mutants.



development of specific neurons in a large number of live flies. To label all dendritic processes, we expressed UAS-mCD8::GFP, which targets to the cell membrane, in all DA neurons. We selected third instar larvae 4-5 days after egg laying (AEL) and recorded the images of dendrites of ventral DA neurons from segments 5 and 6 in live animals. We found that the *Fmr1* mutant larvae exhibited more dendritic processes than wild-type larvae (Fig. 3A,B). To quantify the difference, we counted the number of ends of all dendritic terminal processes. To reduce variation between larvae of the same genotype, we calculated the number of ends per 1000 μm^2 between two ventral cluster DA neurons, to reflect the density of dendritic processes in a particular area. On average, *Fmr1* mutations increased the number of terminal dendritic processes of ventral DA neurons by 25% ($n=30$, $P<0.001$) (Fig. 3C). To demonstrate that the increased number of terminal dendritic processes in *Fmr1* mutants was indeed due to the absence of *Fmr1* activity, we introduced one copy of the wild-type *Fmr1* gene into the *Fmr1*⁴ mutant background and found that the transgene could rescue the dendritic defects in *Fmr1* mutants (Fig. 3C). This *Fmr1* transgene construct was previously shown to be able to rescue

the circadian defects in *Fmr1* mutants (Dockendorff et al., 2002). As shown in Fig. 3D, a large number of segments in wild-type and *Fmr1* mutant larvae exhibit a similar number of terminal dendritic processes, indicating that there is a large variation among individual larvae of a given genotype and that *Fmr1* mutations cause subtle changes in neuronal morphology.

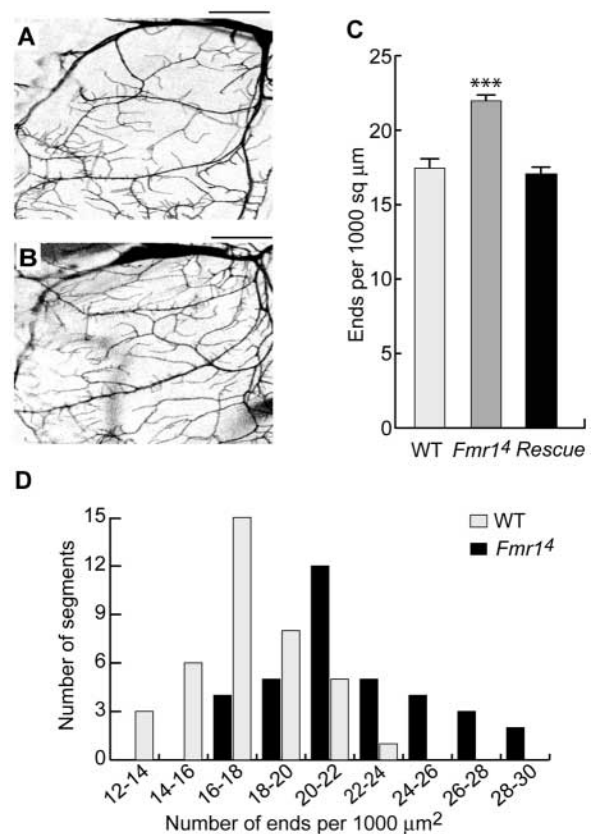


Fig. 3. Morphological alterations in DA neurons caused by *Fmr1* mutations. (A,B) Ventral DA neurons in the A5 segment of a wild-type larva (*w*¹¹¹⁸) (A) and a *Fmr1*⁴ mutant larva (B) were labeled by UAS-mCD8::GFP. The images were taken from live animals, and all the dendritic processes could be visualized. (C) All ends in a specific area between the two ventral clusters of DA neurons in the A5 segment were counted to determine the number of dendritic processes per 1000 μm^2 ($n=38$ for wild-type, $n=35$ for mutants, *** $P<0.001$). All the values are mean \pm s.e.m. To rescue the dendritic phenotype in *Fmr1* mutants, a chromosome containing a 14 kb fragment that spans the *Fmr1* transcriptional unit (Dockendorff et al., 2002) was introduced into the *Fmr1*⁴ mutant background. (D) The distribution of individual larvae with different numbers of dendritic ends illustrates the differences among individual animals of the same genotype and between wild-type and mutant larvae. Scale bars: 40 μm .

Overexpression of *Fmr1* in DA neurons reduces dendritic branches

To further understand the function of *Fmr1* in regulating dendritic growth, we overexpressed *Fmr1* in all DA neurons of wild-type wandering larvae. To do so, we crossed *UAS-Fmr1* flies with *Gal4 109(2)80* flies and examined the third-instar larvae 4 days AEL. We found that the numbers of terminal dendritic processes were dramatically reduced ($n=10$, $P<0.001$) in both ventral (Fig. 4A-C) and dorsal DA neurons (Fig. 4D,E) when *Fmr1* was overexpressed. The length of remaining terminal processes was also greatly reduced (Fig. 4B,E). This phenotype caused by *Fmr1* overexpression is 100% penetrant.

Drosophila larvae increase their body surface over 50-fold from the first to the third instar larval stages. Correspondingly, the dendritic fields of DA neurons increase substantially during this period of development. In larvae overexpressing *Fmr1*, the major dendritic branches were still capable of extending more than fivefold during larval development. However, most terminal processes failed to form or fully extend even at the first instar stage (data not shown). This demonstrates that

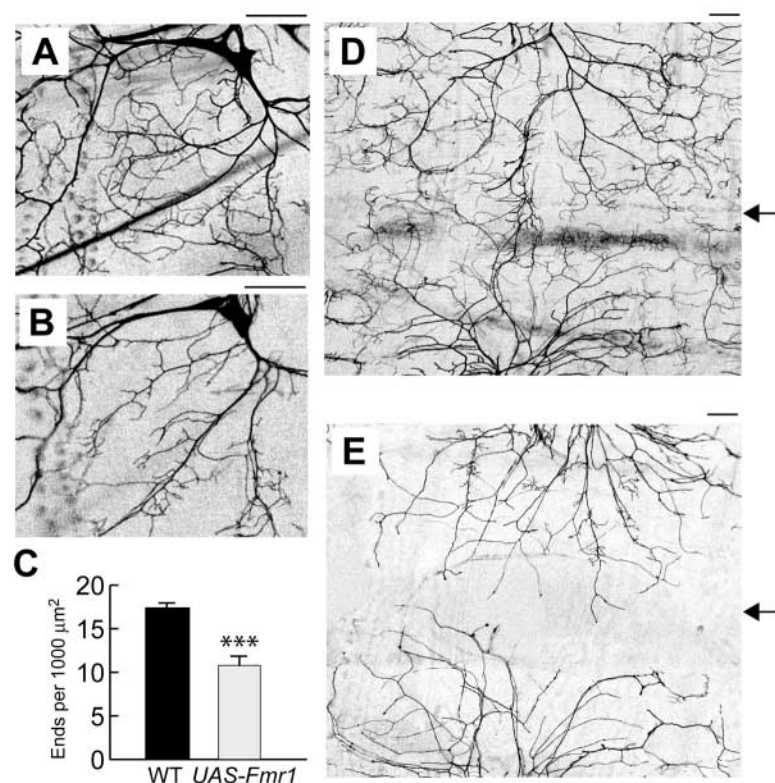


Fig. 4. Dendritic defects caused by overexpression of *Fmr1* in DA neurons of wild-type larvae. (A) Ventral DA neurons in each hemisegment elaborate their dendrites underneath the ventral epidermis. The dendritic field near the cell bodies of ventral cluster DA neurons in a wild-type larva is shown. (B) Overexpression of *Fmr1* in ventral DA neurons reduced the number of dendritic processes. (C) All dendritic processes in a specific area in the A5 segment were counted, and the average numbers for wild-type and *Fmr1* mutant larvae are presented ($n=25$, $***P<0.001$). (D) Dorsal cluster DA neurons in the A5 segment of a wild-type larva elaborated higher-order dendritic processes near the dorsal midline. (E) *Fmr1* overexpression reduced the number of terminal dendritic processes near the dorsal midline. Scale bars: 40 μm. The arrows in D,E indicate the dorsal midline.

overexpression of *Fmr1* blocks the formation of higher-order dendritic branches and reduces the complexity of DA neuron dendrites during development.

Rac1 mRNA is associated with *Fmr1*-mRNP complex in vivo

The KH domains of *Fmr1* share more than 70% identity with the mammalian FMR1 proteins. Indeed, *Fmr1* and human FMR1 have similar RNA-binding properties in vitro (Wan et al., 2000). A number of recent studies have identified a large number of mRNAs that are associated with FMR1 in mammalian systems (Darnell et al., 2001; Brown et al., 2001; Miyashiro et al., 2003). However, systematic identification of *Fmr1*-binding targets in flies has not been carried out. To gain mechanistic insights into *Fmr1* function in controlling dendritic growth in flies, we carried out co-immunoprecipitation experiments to identify mRNAs that are associated with the *Fmr1*-mRNP complex in vivo. In this study, using primers specific for genes encoding small GTPase *Rac1*, α -tubulin, and the voltage-gated K^+ channel molecule Hyperkinetic, we performed RT-PCR analyses on either total

RNAs or the RNAs that were immunoprecipitated by an anti-*Fmr1* monoclonal antibody, from lysates derived from third instar larvae. All three mRNAs could be readily detected from total RNAs, while only the *Rac1* mRNA was associated with *Fmr1* in lysates derived from wild-type larvae as shown by co-immunoprecipitation experiments (Fig. 5). The lysate derived from *Fmr1* mutant larvae was used as a negative control, in which no *Rac1* mRNA was detected from identical co-immunoprecipitation experiments (Fig. 5). These studies demonstrate that *Rac1* mRNA is associated with *Fmr1*-mRNP complexes in vivo.

Rac1 is required for the development of higher-order dendritic branches of DA neurons in *Drosophila* larvae

Based on our finding that *Rac1* mRNA is present in *Fmr1*-mRNP complexes in vivo, we hypothesized that the effect of *Fmr1* on dendritic development in DA neurons may be partially mediated by *Rac1*. *Rac1* is expressed in many cell types in *Drosophila* (Luo et al., 1994). In addition, there is no specific antibody to recognize *Rac1* in flies nor to distinguish it from other highly homologous small GTPases, *Rac2*, and Mig2-like (*Mtl*). Therefore, we were not able to test our hypothesis biochemically.

We were, however, able to test the hypothesis genetically. We first examined the function of *Rac1* in dendritic growth and branching of DA neurons in *Drosophila* embryos. We used *Rac1^{J11}*, a null allele previously characterized based on biochemical and genetic criteria (Ng et al., 2002; Hakeda-Suzuki et al., 2002). We used *Gal4 109(2)80* (Gao et al., 1999) to drive the expression of GFP in DA neurons in *Rac1^{J11}* mutant embryos and did not observe gross defects in dendritic branching patterns in later embryogenesis stages (data not shown). DA neuron dendrites develop in discrete phases from the embryonic to larval stages (Gao et al., 1999; Gao et

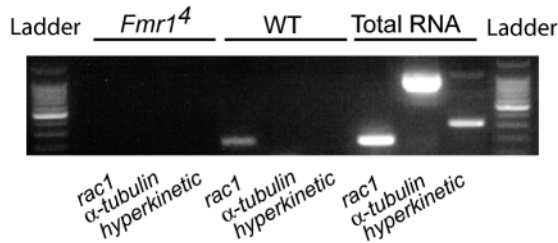


Fig. 5. Rac1 mRNA is present in Fmr1-mRNP complexes in vivo. DNA fragments obtained from PCR were analyzed by electrophoresis in a 2% agarose gel. DNA ladders were run on both sides of the gel. Total RNA: DNA fragments derived from RT-PCR reactions with total RNA isolated from third instar larvae. Primers specific for the voltage-gated K⁺ channel molecule Hyperkinetic gave rise to DNA fragments of 0.3 kb. The band for α -tubulin is 1.0 kb, and the band for Rac1 is 0.2 kb. WT: DNA fragments derived from RT-PCR reactions with RNAs isolated from the Fmr1-mRNP complexes immunoprecipitated from wild-type third instar larvae. *Fmr1*⁴: DNA fragments derived from RT-PCR reactions with RNAs isolated from the Fmr1-mRNP complexes immunoprecipitated from *Fmr1* mutant third instar larvae.

al., 2000). In embryos, dorsal dendrites of DA neurons extend from cell bodies first, and stop elongation 16-17 hours AEL, falling short of the dorsal midline. The lateral dendrites start to extend toward adjacent segment boundaries and cover the hemisegment before hatching (22-23 hours AEL). Our findings in *Rac1*^{J11} mutant embryos suggest that Rac1 is not required for the initial growth of dorsal dendrites during embryogenesis.

During larval stages, the dendritic fields of DA neurons

expand many-fold in accordance with the increase of larval body size. Higher-order dendritic branches further develop to cover the whole epidermal surface of each hemisegment (Gao et al., 2000; Sugimura et al., 2002). Here, we used the MARCM technique (Lee and Luo, 1999) to examine the role of endogenous Rac1 in dendritic growth in the third instar larval stage. We generated single GFP-labeled wild-type or *Rac1* mutant DA neurons in abdominal segments and counted the number of terminal dendritic branches (Fig. 6). We found that *Rac1* mutant ddaC neurons developed fewer dendritic branches than wild-type neurons (Fig. 6A-F), a phenotype similar to that caused by *Fmr1* overexpression. Different *Rac1*^{J11} mutant ddaC neurons exhibited varying severities of dendritic defects. On average, there was a 23% reduction in the number of dendritic branches due to the *Rac1* mutation (Fig. 6F). Similar dendritic defects were also found in other DA neurons (data not shown). These findings demonstrate that Rac1 is required for normal dendritic branching of DA neurons in vivo, consistent with several previous studies that rely on the ectopic expression of dominant mutant forms of Rac1 (Threadgill et al., 1997; Gao et al., 1999; Ruchhoeft et al., 1999; Li et al., 2000).

Overexpression of Rac1 in DA neurons causes increased dendritic branching

To support the notion further that Rac1 is partially responsible for the effect of *Fmr1* on dendritic development, we overexpressed Rac1 in DA neurons in third instar larvae with the UAS-Gal4 system (Brand and Perrimon, 1993). Consistent with our finding that Rac1 loss-of-function resulted in a decreased number of terminal dendritic branches,

overexpression of Rac1 promoted dendritic branching of DA neurons with 100% penetrance (Fig. 7B,D). This result is also in line with previous studies that ectopic expression of the constitutively active form of Rac1 promotes dendritic branching (Luo et al., 1996; Threadgill et al., 1997; Ruchhoeft et al., 1999; Li et al., 2000). The enhanced dendritic branching caused by Rac1 overexpression is much more dramatic than that caused by *Fmr1* loss-of-function, and this is presumably due to the high level of ectopic expression of Rac1.

Because *Fmr1* (or its mammalian homologue FMR1) can function as a translation inhibitor (Li et al., 2001; Zhang et al., 2001) (reviewed by O'Donnell and Warren, 2002), we wondered whether the elevated Rac1 expression using the UAS-Gal4 system would partially rescue the

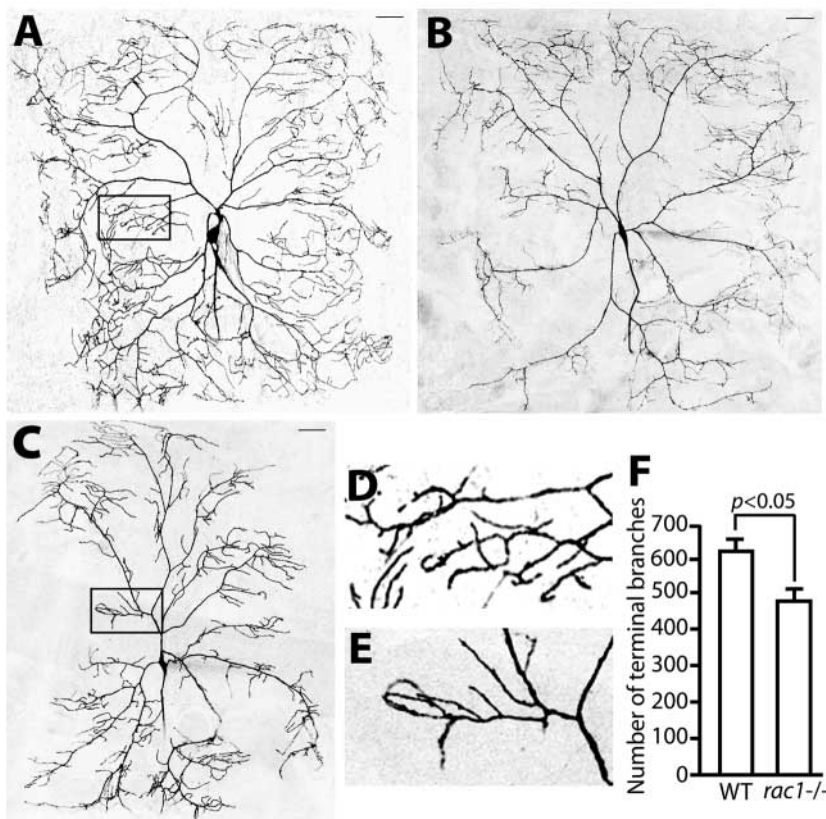


Fig. 6. Rac1 is required for dendritic branching of DA neurons in *Drosophila* larvae. (A) A wild-type ddaC neuron that elaborates extensive dendritic arbors. (B,C) *Rac1* mutant ddaC neurons with fewer dendritic branches. (D) An enlarged image of the area indicated by a square in A. (E) An enlarged image of the area indicated by a square in C. (F) Statistical analysis of the numbers of terminal dendritic branches for wild-type ($n=15$) and *Rac1* mutant ddaC neurons ($n=7$) ($P<0.01$). Scale bars: 50 μ m.

dendritic phenotype caused by *Fmr1* overexpression. To test this hypothesis, we expressed *Fmr1* and *Rac1* simultaneously in DA neurons driven by Gal4 109(2)80. Overexpression of *Fmr1* decreased the number of higher-order dendritic branches (Fig. 4 and Fig. 7E), but could be partially rescued by co-expression of *Rac1* (Fig. 7F). In addition, the number of terminal dendritic branches in *Fmr1*⁴ mutants with a reduced *rac1* dosage (18.9 ± 0.5 , $n=20$) was significantly lower than that in *Fmr1*⁴ mutants (21.7 ± 0.6 , $n=20$, $P < 0.001$). These findings support the notion that *Rac1* is one of the downstream components of *Fmr1* function in controlling dendritic development.

Discussion

FMR1 is an RNA-binding protein, the activity of which is absent in individuals with fragile X mental retardation, one of the most common developmental neurological disorders in humans (reviewed by O'Donnell and Warren, 2002). How FMR1 affects the normal development and function of the mammalian nervous system remains largely unknown. We study the role of *Fmr1*, the FMR1 homolog in the fruit fly *Drosophila*, in the development of dendritic arbors of larval sensory neurons. We provide the first evidence that *Fmr1* is required to limit dendritic branching in flies and that this effect is partially mediated by the actions of the small GTPase *Rac1*.

Dendritic defects caused by the loss of *Fmr1* activity

During the past couple of years, several labs have independently generated *Fmr1* mutant fly lines (Zhang et al., 2001; Dockendorff et al., 2002; Inoue et al., 2002) (this study). All of these groups used fly lines provided by fly stock centers that contain a P-element inserted in the first intron of the *Fmr1* gene. Some labs used the 'imprecise hop-out' approach to excise the P-element and generated deletions in the range of several thousand base pairs in the *Fmr1* locus (Zhang et al., 2001; Dockendorff et al., 2002; Inoue et al., 2002). In this study, we took advantage of the presence of Gal4-binding sites in the P-element (Rørth, 1996) and designed a different approach to create *Fmr1* loss-of-function alleles (Fig. 1). Consistent with some of the previous reports, our *Fmr1* mutant flies, which contain either point mutations or small deletions, are fully viable and develop into adulthood at the expected Mendelian ratios without gross morphological defects.

The viability of *Fmr1* mutant flies allowed us to directly examine the role of *Fmr1* in dendritic development in live larvae. We focused on DA neurons because these neurons elaborate their dendrites in a two-dimensional manner just

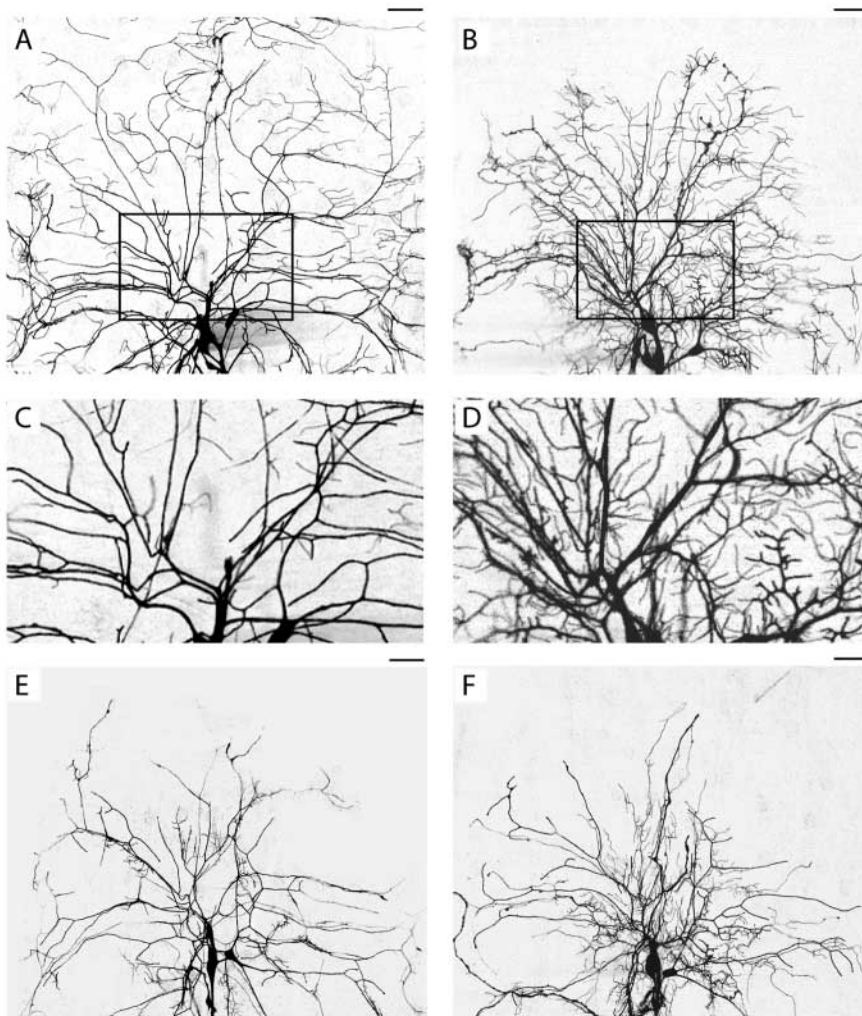


Fig. 7. Expression of *Rac1* increases dendritic branching of DA neurons in *Drosophila* larvae. (A) Dendritic branching pattern of dorsal cluster DA neurons in wild-type third instar larvae. (B) Increased dendritic branching when *Rac1* is overexpressed in DA neurons. (C,D) Enlarged images of dendrites in A,B, respectively. (E) Reduced dendritic branching when *Fmr1* is overexpressed in dorsal cluster DA neurons. (F) Reduced dendritic branching caused by *Fmr1* overexpression (E) can be partially reversed by expression of *Rac1*. Scale bars: 40 μm .

beneath the epidermis (Gao et al., 1999; Gao et al., 2000); therefore, it was easy to visualize and quantify the number of GFP-labeled dendritic branches. Secondly, there are only six DA neurons in the dorsal cluster and four in the ventral cluster in each abdominal hemisegment (Ghysen et al., 1986; Bodmer et al., 1989; Orgogozo et al., 2001). We can examine the dendrites of these same DA neurons in an area free of other neurons in a large number of live animals. Our studies indicate that loss of *Fmr1* activity slightly increased the number of terminal dendritic processes, which can be rescued by expressing wild-type *Fmr1* in the mutant background (Fig. 3). In addition, specific overexpression of *Fmr1* solely in DA neurons in wild-type larvae dramatically decreased the number of terminal dendritic branches (Fig. 4), further supporting a role for *Fmr1* in controlling dendritic development.

The dendritic phenotype of DA neurons in *Fmr1* mutant larvae is relatively subtle compared with that of *Fmr1*

overexpression. The average number of terminal dendritic processes of ventral DA neurons in *Fmr1* mutant larvae showed a statistically significant difference from that of wild-type larvae; however, the numbers of dendrites in a large number of segments in *Fmr1* mutants show a similar number of terminal dendritic processes as that seen in wild-type larvae (Fig. 3D). This finding suggests that the loss of *Fmr1* activity has low expressivity in terms of the dendritic phenotype, consistent with the wide range of physical and mental abnormalities found in fragile X patients (Hagerman, 2002).

mRNA targets regulated by Fmr1

Recent studies suggest that a large number of mRNAs can be found in association with FMR1-mRNA complexes in mouse brain, and many of them contain an FMR1-binding motif, the G quartet structure (Darnell et al., 2001; Brown et al., 2001; Zalfa et al., 2003; Miyashiro et al., 2003). As *Fmr1* shares a high degree of amino acid sequence homology with human and mouse FMR1, and these proteins behave in several similar ways (Wan et al., 2000; Gao, 2002), it seems highly likely that *Fmr1* also regulates multiple mRNAs during neural development. Systematic identification of *Fmr1* binding targets in *Drosophila* has not been carried out. It was reported before that mRNA encoding the microtubule-binding protein Futsch could be found in *Fmr1*-mRNP complexes (Zhang et al., 2001). We found that *Rac1* mRNA is also present in *Fmr1*-mRNP complexes, as shown by co-immunoprecipitation and RT-PCR analyses (Fig. 5). Interestingly, part of the *Rac1*-coding region is highly conserved at the nucleotide level from *Drosophila* to humans. This region contains G-rich nucleotide sequences, which are not identical but are similar to the FMR1-binding sequences identified from the in vitro RNA selection experiments (Darnell et al., 2001).

Several pieces of evidence indicate that *Rac1* is partially responsible for the effects of *Fmr1* on dendritic development of DA neurons. First, *Rac1* mRNA is present in *Fmr1*-mRNP complexes in vivo (Fig. 5). Second, MARCM analysis demonstrates that *Rac1* is required for dendritic branching of DA neurons in a cell-autonomous manner, consistent with a previous report that three small GTPases together, *Rac1*, *Rac2* and *Mtl*, are required for dendritic branching in mushroom body neurons (Ng et al., 2002). Third, overexpression of *Rac1* promotes dendritic branching of DA neurons, a phenotype partially similar to that caused by *Fmr1* loss-of-function mutations. Fourth, decreased dendritic branching caused by ectopic expression of *Fmr1* can be partially rescued by co-expression of *Rac1*. It is worth noting that overexpression of *Rac1* increases dendritic branching more dramatically than *Fmr1* loss-of-function mutations. We suspect that this is because of the level of overexpression of *Rac1* in the UAS-Gal4 system being much higher than *Rac1* expression in *Fmr1* mutant DA neurons. Conversely, overexpression of *Fmr1* causes a more dramatic decrease in dendritic branching than *Rac1* mutations in MARCM-generated single neurons. This can potentially be accounted for in two ways. The first is that, in MARCM-generated single mutant neurons, wild-type *Rac1* protein and mRNA inherited from its precursor cell before the FRT-mediated recombination event may reduce the severity of the dendritic phenotype (Ng et al., 2002). Second, it is highly likely that the expression of more than one protein

encoded by mRNAs in *Fmr1*-mRNP complexes is affected by the overexpression of *Fmr1*. Indeed, our co-immunoprecipitation experiments indicate that other mRNAs are also associated with *Fmr1* in vivo (K.X. and F.-B.G., unpublished).

FMR1/*Fmr1* proteins are found in dendrites of mammalian or fly neurons (Devys et al., 1993; Feng et al., 1997b) (this study). Although difficult to prove in vivo, local regulation of *Rac1* expression by FMR1/*Fmr1* may play a role in controlling the branching process of terminal dendrites. The identification of *Rac1* mRNA as one of the targets of *Fmr1* may also provide a partial explanation for the reported subtle axon guidance defects found in *Fmr1* mutant flies (Morales et al., 2002; Dockendorff et al., 2002).

After the submission of this manuscript, Schenck et al. (Schenck et al., 2003) reported the biochemical association between *Rac1* and CYFIP, and between CYFIP and *Fmr1* in vitro and in transfected S2 cells. Based on biochemical and genetic analyses in different cell types, the authors proposed that *Rac1* regulates *Fmr1* activity through CYFIP (Schenck et al., 2003). Taken together, their studies and the findings reported here may suggest that there is feedback loop between *Rac1* and *Fmr1* functions in vivo.

Molecular functions of Fmr1

How does *Fmr1* function at the molecular level? Recent studies demonstrate that *Fmr1* is associated with some proteins that are known to function in the RNAi complex (Ishizuka et al., 2002; Caudy et al., 2002). Although *Fmr1* can affect the efficiency of the RNAi, *Fmr1* is not required for the process, and its exact role in the complex remains unclear (Ishizuka et al., 2002; Caudy et al., 2002). RNAi can act at the post-transcriptional level to influence the stability of mRNAs with sequences complementary to the silence trigger (Fire et al., 1998), or they can inhibit protein synthesis without causing message degradation (Olsen and Ambros, 1999). In our *Fmr1* mutants, we did not detect any significant change in quantity of *Rac1* mRNA (data not shown). *Fmr1* may inhibit *Rac1* mRNA translation in vivo, although the exact underlying molecular mechanism remains to be determined. Based on the observation that *Fmr1* can be found in DA neuron dendrites, our results also raise the possibility that *Fmr1* regulates the translation of its target mRNAs in dendrites, which may play an important role in regulating local protein synthesis and neuronal function (reviewed by Steward and Schuman, 2001). It would be interesting to dissect whether *Fmr1* functions in controlling neuronal morphology through an RNAi-based mechanism or in some other mRNA degradation/translation regulatory pathways.

We thank G. Dreyfuss, T. A. Jongens, T. Kornberg, the Bloomington Stock Center and Szeged Stock Center for antibodies and fly lines; S. Ordway and G. Howard for editorial assistance; Nhue Do for technical help; and Kathleen Anderson for manuscript preparation. We also thank Neal Sweeney and other Gao laboratory members for suggestions and comments on the manuscript. This work is supported by grants from the Alfred P. Sloan Foundation, the Esther A. and Joseph Klingenstein Fund, the Sandler Family Foundation, and the McKnight Endowment Fund for Neuroscience (F.-B.G.). B.A.B. is supported by a UCSF Neuroscience Graduate Program Predoctoral Fellowship.

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