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Fragile X mental retardation protein modulates the fate of germline stem cells in *Drosophila*

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Fragile X syndrome, a common form of inherited mental retardation, is caused by the loss of fragile X mental retardation protein (FMRP). FMRP, which may regulate translation in neurons, associates not only with specific mRNAs and microRNAs (miRNA), but also with components of the miRNA pathway, including Dicer and Argonaute proteins. In *Drosophila*, *dFmr1* is also known to be involved in germ cell and oocyte specification; however, the question of whether *dFmr1* is required for controlling the fate of germline stem cells (GSCs) has gone unanswered. Here we show that *dFmr1* is required for both GSC maintenance and repressing differentiation. Furthermore, we demonstrate that in *Drosophila* ovary, *dFmr1 protein* interacts with Argonaute protein 1 (AGO1), a key component of the miRNA pathway. Thus *dFmr1* could modulate the fate of GSCs, likely via the miRNA pathway. Our results provide the first evidence that FMRP might be involved in the regulation of adult stem cells.

INTRODUCTION

Fragile X syndrome, the most common form of inherited mental retardation with an estimated prevalence of one in 4000 males and one in 8000 females, is typically caused by a massive CGG trinucleotide repeat expansion within the 5′-untranslated region (UTR) of the fragile X mental retardation 1 gene (*FMR1*), which results in transcriptional silencing of *FMR1* (1–4). The identification of other mutations (e.g. deletions in patients with the typical phenotype) has confirmed that *FMR1* is the only gene involved in the pathogenesis of fragile X syndrome (5–7); loss of the *FMR1* product, fragile X mental retardation protein (FMRP), causes fragile X syndrome.

FMRP and its autosomal paralogs, the fragile X-related proteins FXR1P and FXR2P, constitute a well-conserved, small family of RNA-binding proteins (fragile X-related gene family) that share more than 60% amino acid identity and contain two types of RNA-binding motifs: two ribonucleoprotein K homology domains (KH domains) and a cluster of arginine and glycine residues (RGG box) (8,9). FMRP is known to form a messenger ribonucleoprotein (mRNP) complex that associates with translating polyribosomes. It has been proposed that FMRP plays a role in synaptic plasticity via the regulation of mRNA transport and translation (10). Furthermore, the cumulative work of several groups now suggests that FMRP may regulate the translation of its mRNA through miRNA interactions. In one likely scenario, once FMRP binds to its specific mRNA ligands, it then recruits the RNA-induced silencing complex (RISC) along with miRNAs and facilitates recognition between miRNAs and their mRNA ligands. Thus, FMRP could modulate the efficiency of translation of its mRNA targets using miRNAs (11). It has also been shown that the loss of *Fmrp* can alter the proliferation and differentiation of embryonic neural stem cells in mice (12); however, up to now the role of FMRP in the maintenance and fate specification of stem cells has not been explored.

Unlike their mammalian counterparts, the fly genome harbors a single *Fmr1* gene homolog, also referred to as *dFmr1* or *dfxr* (*dFmr1* here, per FlyBase annotation). Sequence comparisons show a high level of similarity between the functional domains of fly and mammalian *Fmrp*, with an overall 56% similarity and 35% identity (13,14). Given the power of *Drosophila* genetics to elucidate biological pathways, over the last few years, the fruit fly has proved enormously useful for exploring the physiological roles of FMRP. Whereas homozygous *dFmr1* mutant

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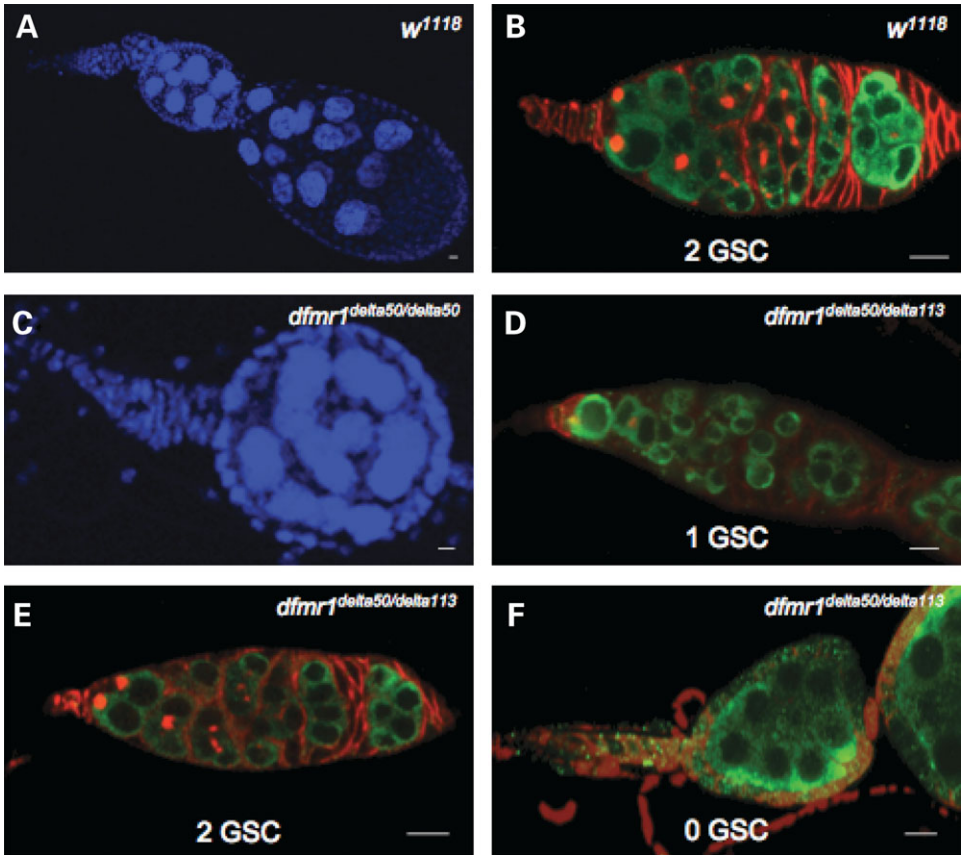

Figure 1. Germline stem cells (GSCs) maintenance is defective in female *dfmr1* mutants. (**A**, **C**) The loss of *dFmr1* leads to defects in the early germ cell development. Both wild-type and *dfmr1* mutant ovaries stained with Hoechst are shown. Wild-type ovarioles contain a normal germarium (A) and some of *dfmr1* mutant ovarioles have empty germaria and a few egg chambers, indicating that *dFmr1* may play a role in germ cell development. Ovaries collected from wild-type and *dfmr1* mutants at Day 7 after eclosion were stained with anti-*Vasa* (green) and anti-*Hts* (red) antibodies. (**B**) A typical wild-type germarium carries two GSCs in the tip of the germarium; fusomes in GSCs were localized at an anterior position between GSCs and cap cells or basal cells of the terminal filaments. (**D**) $dfmr1^{Delta50/Delta113}$ mutant germarium contains one GSC. (**E**) Another type of germarium contains two GSCs in the tip of $dfmr1^{Delta50/Delta113}$ mutant ovaries. This type of germarium exists with very low frequency (Table 1). (**F**) An example of empty germaria in $dfmr1^{Delta50/Delta113}$ mutant ovaries. Scale bar represents 10 μm.

adult flies appear morphologically normal, they display abnormalities in behavior, synaptogenesis and spermatogenesis, phenotypes that resemble some of those observed in fragile X patients (13,15–20). In *Drosophila* ovary, a very small population of germline stem cells (GSCs) is maintained in a well-defined microenvironment (i.e. the stem *niche* at the tip of each ovariole). This environment provides an attractive system for investigating the regulatory mechanisms that determine stem cell fate [see review (21) for details] (21,22). Previous studies have shown that *dFmr1* is involved in germ cell and oocyte specification; however, whether *dFmr1* is required for controlling the fate of GSCs has remained unknown (23,24).

Here, we use *Drosophila* GSCs as a model to show that FMRP can modulate the fate of stem cells. We demonstrate that *dFmr1* is required for both GSC maintenance and repressing differentiation. Furthermore, we show that *dFmrp* interacts with Argonaute protein 1 (AGO1), a key component of the miRNA pathway, to modulate the fate of GSCs. These results support the notion of a conserved role for translational control in the regulation of stem cell function, revealing a new function for FMRP in stem cells.

RESULTS

The loss of *dFmr1* leads to defects in germline stem cell maintenance and/or establishment

Given that the GSC in *Drosophila* ovary is a well-defined system for studying the regulatory mechanisms that determine stem cell fate, we chose to explore the potential role of dFmr1 in GSCs. A typical *Drosophila* ovary is composed of 16–20 ovarioles. Each ovariole consists of an anterior functional unit, called germarium, and a linear string of differentiated egg chamber posterior to the germarium. At the tip of the germarium, GSCs normally divide asymmetrically to ensure that one daughter cell remains attached to the niche cells for self-renewal, while the other is displaced from the niche, becoming a cystoblast (CB) that initiates differentiation and sustains oogenesis (25). In a previous study, two null alleles of *dfmr1* were generated, namely $dfmr1^{Delta50}$ and $dfmr1^{Delta113}$, both of which exhibited sterility in males and weak fertility in females (13,26). Using *Hoechst* staining for nuclei, we found that seven-day-old germaria from adult $dfmr1^{Delta113}$ mutants displayed morphological abnormalities (Fig. 1A and C).

Table 1. Phenotypic assay for *dfmr1* mutant flies

	Two or three germline stem cells	One germline stem cell	Zero germline stem cell/cysts only	Empty (no cysts)
WT Day 2	78/78 (100%)	0 (0%)	0 (0%)	0 (0%)
WT Day 7	123/123 (100%)	0 (0%)	0 (0%)	0 (0%)
$dfmr1^{Delta113/Delta113}$ Day 2	24/112 (21.4%)	51/112 (45.5%)	34/112 (30.4%)	3/112 (2.7%)
$dfmr1^{Delta113/Delta113}$ Day 7	9/74 (12.2%)	25/74 (33.8%)	34/74 (45.9%)	6/74 (8.1%)
$dfmr1^{Delta113/Delta50}$ Day 2	22/126 (17.5%)	58/126 (46.0%)	43/126 (34.1%)	3/126 (2.4%)
$dfmr1^{Delta113/Delta50}$ Day 7	8/136 (5.9%)	34/136 (25%)	78/136 (57.4%)	16/136 (11.8%)

Approximately, 50% of *dfmr1* mutant germaria were morphologically normal when compared with wild-type. Nevertheless, whereas approximately 40% of germaria appeared normal, they were attaching with only fewer egg chambers (one or two), rather than the normal six or seven. In some cases, the germaria were completely empty. This phenotype hinted at defects in GSC maintenance and/or establishment.

dFmr1 is required for both germ stem cell maintenance and repressing primordial germ cell differentiation

To address whether *dFmr1* is required for GSC maintenance, we examined the number of GSCs in germaria from *dfmr1* mutant flies at different ages. The ovaries from both newly eclosed and seven-day-old *dfmr1* mutant females were tested by staining with anti-*Vasa* and anti-*Hts* antibodies. *Vasa* staining can specifically visualize all germ cells during oogenesis, while *Hts* is preferentially rich in fusome, a germ cell-specific organelle that is morphologically spherical in primordial germ cells (PGC) and GSCs/cystoblasts, but branched in differentiated cysts. GSCs can be reliably recognized at the tip of the germarium by their position of direct contact with cap cells or base cells of the terminal filament and the anterior localization of spherical fusomes (also called spectrosomes) (21). In wild-type females, a typical germarium has an average of two to three GSCs (Fig. 1B). In contrast, as shown in Table 1, the newly eclosed *dfmr1* homozygous germaria contained an average of 0.88 ($dfmr1^{Delta113}$/$dfmr1^{Delta113}$) and 0.81 ($dfmr1^{Delta113}$/$dfmr1^{Delta50}$) GSCs per germarium. Among them, 17.5–21.4% have two GSCs, 45.5–46% contain one GSC and 30.4–34.1% have only differentiated cysts or an empty germarium. In the seven-day-old *dfmr1* homozygous germaria, the number of GSCs was reduced to an average of 0.58 ($dfmr1^{Delta113}$/$dfmr1^{Delta113}$) and 0.37 ($dfmr1^{Delta113}$/$dfmr1^{Delta50}$) per germarium, suggesting that the loss of *dFmr1* causes either the progressive loss of GSCs or a defect in GSC maintenance (Fig. 1D–F). To further exclude the possibility that the observed phenotype was due to genetic background, we examined the number of GSCs in different combinations of *dfmr1* null allele with deficiency line *Df(3R)BSC38*. We observed that the newly eclosed *dfmr1* homozygous germaria contained an average of 0.99 ($n = 57$) [$dfmr1^{Delta113}$/*Df(3R)BSC38*] and 1.01 ($n = 83$) [$dfmr1^{Delta50}$/*Df(3R)BSC38*] GSCs per germarium, whereas, in the same condition, wild-type (w^{1118}) germaria contained an average of 2.6 ($n = 99$) GSCs per germarium. These results together suggest that *dFmr1* is required for GSC maintenance.

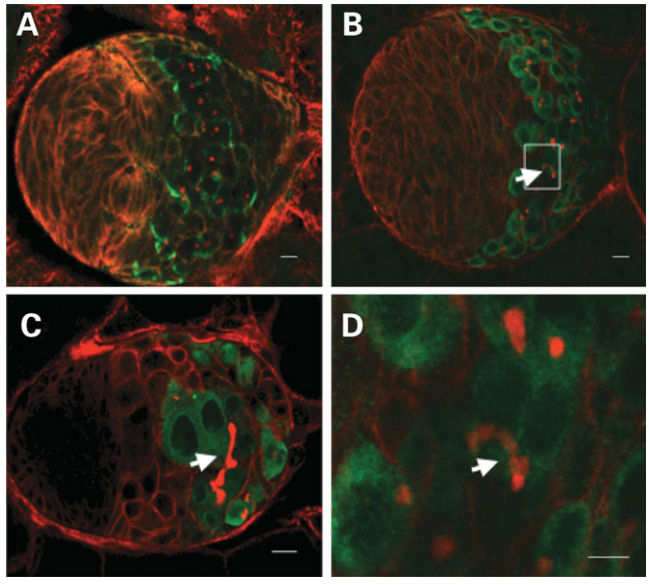

Figure 2. *dFmr1* is required for repressing primordial germ cells (PGCs) differentiation. Gonads collected from wild-type and $dfmr1^{Delta50}$ female larvae at the LL3 (late third larval) stage were stained with anti-*Vasa* (green) and anti-*Hts* (red) antibodies. (**A**) Wild-type PGCs carry spherical fusomes, and dividing PGCs carry associated spherical fusomes. (**B**) A PGC cluster (16-cell cyst) with a highly branched fusome in a $dfmr1^{Delta50}$ mutant gonad. (**C**, **D**) A typical 4-cell cyst was observed in $dfmr1^{Delta50/Delta113}$ mutant gonad. Scale bar represents 10 μm.

To address whether *dFmr1* is involved in GSC establishment, we examined PGCs from *dfmr1* gonads. PGCs are the precursors of germ cells and are known to be controlled by similar signaling pathways as adult GSCs (27). During the three larval stages, the number of PGCs is simply multiplied from 12 cells to more than 100 cells, but without further differentiation into the female gonad (28). To determine whether PGCs can differentiate in a *dfmr1* background, we used anti-*Hts* and anti-*Vasa* antibodies to stain wild-type and *dfmr1* mutant gonads. As shown in Figure 2A, most wild-type gonads from late third-instar larvae exhibited PGCs carrying a single spherical fusome, and some of the PGCs were dividing with two spherical fusomes associated between two PGC cells. However, we observed that 76.7% ($n = 43$) of *dfmr1* mutant gonads contained differentiated PGC clusters that were marked by branched fusomes (Fig. 2B–D). This suggests that *dFmr1* is required to repress PGC differentiation and is probably involved in the transition from PGCs to GSCs. Based on these findings, we conclude that *dFmr1* is required to repress both PGC and GSC differentiation.

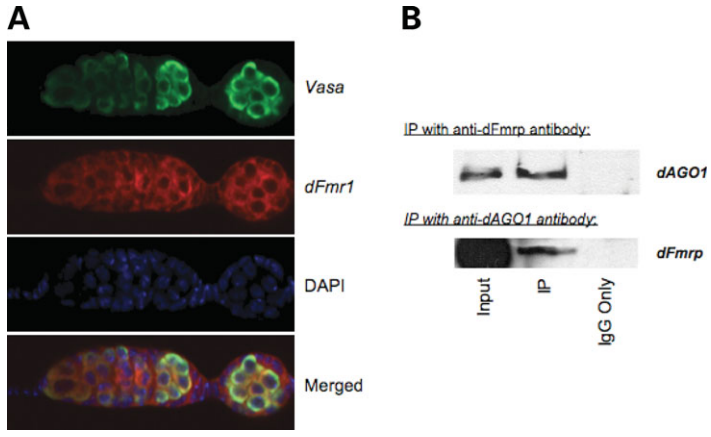

Figure 3. *dFmrp* is associated with Ago1 in *Drosophila* ovary. (**A**) *dFmrp* expression pattern in a wild-type germarium stained with anti-*dFmrp* and anti-*VASA* antibodies. Scale bar represents 10 μm. (**B**) *dFmrp* is associated with AGO1 in *Drosophila* ovary. *Ago1* could be co-immunoprecipitated with *dFmrp* from wild-type ovary lysates (top panel). *dFmrp* could be co-immunoprecipitated with *Ago1* from wild-type ovary lysates (bottom panel).

dFmr1 modulates the fate of germline stem cells as an extrinsic factor

The loss of GSCs in *dFmr1* mutant ovaries indicates a requirement for *dFmr1* in either GSCs or somatic cells (or both). To test how *dFmr1* modulates the GSC fate, we first examined the expression pattern of *dFmrp* in germarium. As shown in Figure 3A, using immunostaining with anti-*dFmrp* antibody, we found that *dFmrp* is ubiquitously expressed in both germ cells and somatic cells, suggesting that *dFmrp* could function in either cell type. To analyze whether *dFmrp* functions as a cell-autonomous factor for maintaining GSC fate, we used an FLP–FRT-mediated mitotic recombination technique to generate marked mutant GSCs, then calculated the life span of the marked mutant GSCs by quantifying their loss rate. The marked mutant GSCs were identified by a lack of GFP fluorescence in the nuclei and by their positions directly attaching to the base cells of the terminal filament or cap cells. The *dFmr1* loss-of-function alleles ($dfmr1^{Delta113}$ *and* $dfmr1^{Delta50}$) were used to generate marked mutant GSC clones for an analysis of *dFmr1* function in GSCs. The rates of Ubi–GFP-marked GSCs were measured at 2, 7 and 14 days after 5-day heat-shock treatment (AHST). As shown in Table 2, compared with wild-type (w^{1118}) control GSC clones, the marked clone rates of both $dfmr1^{Delta113}$ and $dfmr1^{Delta50}$ were not significantly reduced during the tested period. These findings suggest that *dFmr1* is required extrinsically for GSC maintenance (Fig. 4).

To further explore whether *dFmr1* is involved in controlling the rate of GSC division, we examined the ability of *dFmr1* mutant GSC producing cysts. At Day 14 after heat-shock induction, a wild-type GSC clone produced an average of 2.9 ($n = 35$) germline cysts, similarly, for $dfmr1^{Delta113}$ and $dfmr1^{Delta50}$ mutant GSCs, averages of 2.4 and 2.9 cysts were produced per mutant GSC, respectively, suggesting that *dFmr1* is not involved in controlling the rate of GSC division.

dFmr1 biochemically and genetically interacts with *Ago1* in *Drosophila* ovary

The necessity of *dFmr1* for GSC maintenance indicates a novel mechanism for GSC fate determination. Extensive studies of FMRP in both *Drosophila* and mammals suggest that FMRP functions as a translational repressor (11). Recently, FMRP was also shown to be associated with the RISC in both mammals and *Drosophila* (29–31). In *Drosophila*, genetic studies have suggested that *Ago1*, as a key component of the miRNA pathway, is critical for *dFmrp* function in neural development and synaptogenesis (29). These data indicate that FMRP could use the miRNA pathway to regulate the translation of its specific mRNA targets. To explore how *dFmr1* is involved in GSC maintenance, we examined whether *dFmr1* interacts with *Ago1* in the *Drosophila* ovarian GSC system. We performed co-immunoprecipitation (IP) experiments using anti-*Ago1* antibody and ovary lysates. As shown in Figure 3B, *dFmrp* (approximately 85 kDa) could be co-immunoprecipitated with *Ago1* from ovary lysates. To confirm that the association is specific, we performed a reciprocal IP experiment, and *Ago1* (approximately 120 kDa) could be co-immunoprecipitated with *dFmrp* as well. These results suggest that *Ago1* is specifically associated with *dFmrp* in *Drosophila* ovary.

To further test whether *dFmr1* genetically interacts with *Ago1* for the maintenance of germline cells, we quantified the number of GSCs in *dfmr1* ($dfmr1^{Delta113}/dfmr1^{Delta50}$) mutant and $Ago1^{l(2)k08121}/+;$ $dfmr1^{Delta113}/dfmr1^{Delta50}$ mutant germaria. Interestingly, we found that the loss of one copy of *Ago1* could enhance the *dfmr1* phenotype in GSCs. For example, in three-day-old *dfmr1* germaria, the average number of GSCs was 0.73 ($n = 58$), whereas for age-matched *dfmr1* homozygotes, which have lost one copy of *Ago1* ($Ago1^{l(2)k08121}/+;$ $dfmr1^{Delta113}/dfmr1^{Delta50}$), the average number of GSCs was 0.60 ($n = 44$), suggesting that *Ago1* and *dFmr1* may potentially function in the same genetic pathway to coordinate the regulation of GSC fate. These combined results indicate that *dFmrp* may function together with *Ago1* to modulate the fate of GSCs.

DISCUSSION

Fragile X syndrome, the most common cause of inherited mental retardation, results from the loss of functional FMRP

Table 2. Clonal analyses of *dFmr1* deficiency in germline stem cells

Genotypes	*Control-1 hs-flp; FRT82B, ubi-gfp/FRT82B*			*dfmr1^{Delta50} hs-flp; FRT82B, ubi-gfp/FRT82B, dfmr1^{Delta50}*			*Control-2 hs-flp; FRT82B, ubi-gfp/FRT82B*			*dfmr1^{Delta113} hs-flp; FRT82B, ubi-gfp/FRT82B, dfmr1^{Delta113}*		
Days after stopping heat shock	2	7	14	2	7	14	2	7	14	2	7	14
Percentage of marked germline stem cells	31.8	32.9	28.7	19.8	16.0	16.9	26.7	23.9	22.2	27.8	25.8	26.2
Relative percentage of marked germline stem cells	100	100.3	90.3	100	80.8	85.4	100	89.5	83.1	100	92.8	94.2
Total number of examined germaria	225	167	87	157	268	112	176	167	189	162	144	202

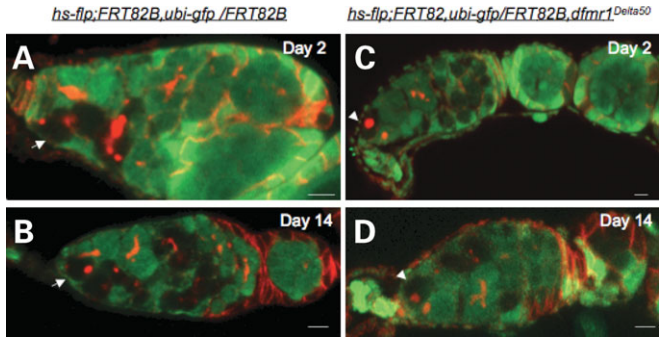

Figure 4. *dFmr1* modulates the fate of GSCs as an extrinsic factor. GSC clones were induced by heat-shock treatment in adult female flies. Ovaries from FRT control flies (**A**) and (**B**) and FRT, *dfmr1^{Delta113}* flies (**C**) and (**D**) were dissected at Day 2 and Day 14 after heat-shock treatment. GSC clones (indicated by arrows) were identified by the lack of GFP expression. The marked clone rate of *dfmr1^{Delta113}* was not significantly reduced during the tested period (Table 2).

(32). Since the *FMR1* gene was first cloned in 1991, most studies have been focusing in understanding how the loss of FMRP leads to mental retardation in the nervous system. FMRP is an RNA-binding protein and is known to bind to specific mRNAs and regulate their translation both *in vitro* and *in vivo*. FMRP is largely cytoplasmic, incorporated into large mRNP particles (11). It has been proposed that FMRP plays a role in synaptic plasticity via the regulation of mRNA transport and translation, particularly local protein synthesis in the dendrites (33). A growing body of work from several groups now suggests that the microRNA pathway is the major molecular mechanism by which FMRP regulates translation. In both *Drosophila* and mammals, FMRP, as well as its autosomal homologs in mammals, FXR1P and FXR2P, has been found to be a part of the RISC (29–31). However, it remains unclear what role, if any, FMRP plays in siRNA-mediated gene silencing. In the miRNA pathway, FMRP has been associated with miRNAs in both *Drosophila* and mammals, and the genetic interaction between dFmr1 and Ago1 has already been demonstrated in *Drosophila*. Therefore, FMRP is one component of the miRNA pathway involved in miRNA-mediated translational control. Recently, FXR1P has also been shown to work with AGO2 to activate the cell growth-dependent translation (34). Whether FMRP is involved in this process remains to be determined.

Whereas *Fmrp* has already been shown to alter the proliferation and differentiation of embryonic neural stem cells in mice, the role of FMRP in the maintenance and fate specification of stem cells had not been explored (12). In *Drosophila*, *dFmr1* is required for cyst formation and oocyte specification, potentially via the regulation of *Orb* mRNA translation (23). Our report provides the first evidence that in *Drosophila*, *dFmr1* as an RNA-binding protein is required for the maintenance of GSCs, which suggests that FMRP could be involved in the division and self-renewal of adult stem cells. The importance of translational regulation to the self-renewal and differentiation of stem cells has been recognized, due to the discovery of important roles for other RNA-binding proteins,

including *Pum* and *Nos*, in GSC regulation (22). Our findings provide further evidence that translational regulation plays pivotal roles in stem cell regulation.

The question becomes how *dFmr1* controls the maintenance of GSCs in *Drosophila*. Given that FMRP is a translational regulator, it is very likely that *dFmr1* protein could regulate the translation of specific mRNAs in GSCs that are critical for the differentiation of adult stem cells. Identifying those mRNAs regulated by *dFmrp* in *Drosophila* GSCs would be revealing and significant. Given that *dFmrp* is a component of the miRNA pathway involved in miRNA-mediated translational control, our present finding is also consistent with the recent discovery that the miRNA pathway plays critical roles in the maintenance of GSCs (35–37). On the other hand, earlier studies have demonstrated that *dFmrp/Fmrp* and the tumor suppressor protein *lethal (2) giant larvae* (Lgl) can form a functional complex in flies and mice (38). This *Fmrp/Lgl* complex is regulated by the PAR protein complex. Furthermore, both Lgl and the PAR protein complex have been implicated in the control of cell polarity, which is necessary for the self-renewal and differentiation of stem cells (39). So it is also possible that, besides regulating the translation of specific mRNAs in GSCs via the miRNA pathway, *dFmrp* may also work with Lgl and the PAR complex to control the maintenance of stem cells.

In short, we have discovered a new role for *dFmrp* in stem cells. It will be important to determine whether in mammals FMRP can modulate stem cells, particularly neural stem cells, given the neurological phenotypes associated with fragile X syndrome. Studying the role of FMRP in stem cells will not only facilitate further understanding of the molecular pathogenesis of fragile X syndrome, but also improve our understanding of the role translational control plays in the maintenance and fate specification of stem cells in general, a prerequisite for any therapeutic application of stem cells for human diseases.

MATERIALS AND METHODS

Drosophila genetics

All flies were maintained under standard culture conditions. $dfmr1^{Delta113}$ and $dfmr1^{Delta50}$ (gifts from Dr Y. Zhang) were null *dfmr1* alleles as described previously (13). $Ago1^{k08121}$ has also been described (40). The deficiency line *Df(3R)BSC38* came from Bloomington Stock Center.

Immunohistochemistry and microscopy

Ovaries were prepared for reaction with antibodies as described previously (41). Polyclonal anti-*Vasa* antibody (Santa) was used at a 1:200 dilution, and monoclonal anti-*Hts* antibody was used at a 1:500 dilution. Secondary antibodies used were goat anti-mouse Alexa 568, goat anti-rabbit Alexa 488 and goat anti-rat Cy3 (Molecular Probes), all at 1:200. All samples were examined by Zeiss Microscope, and images were captured using the Zeiss Two Photon Confocal LSM510 META system. Images were further processed with Adobe Photoshop 6.0.

Phenotypic assay for quantification of germline stem cell maintenance in mutant adult ovaries

Ovaries isolated from wild-type and homozygous mutant flies of different ages were incubated with anti-*Hts* antibody, anti-*Vasa* antibody and DNA dyes to identify terminal filament cells, fusomes and germ cells. We scored as GSCs any *Vasa*-positive germ cells at the anterior position that appeared close to cap cells or to the basal cells of terminal filaments and also carried spherical fusomes at the anterior position or extending fusomes.

Germline clonal analysis

FLP–FRT-mediated recombination was used to generate *dFmr1* mutant GSC and PGC clones. To generate GSC clones, three-day-old females, w; *hs-flp; FRT82B,ubi-gfp/FRT82B,dfmr1*$^{-}$, underwent heat-shock treatment at 37°C for 60 min twice daily at 12 h intervals (w; *hs-flp; FRT82B, ubi-gfp/FRT82B* as control). After 4–5 days of heat-shock induction monitored by GSC clone efficiency of control, ovaries were dissected for quantification of GSC clones at Day 2, Day 7 and Day 14 of the post-clonal induction. The percentage of GSC clones measured at Day 2 was also calculated as the initial rate (100 of relative %). GSC clones were identified by a lack of GFP fluorescence in the nucleus and carrying anterior-positioned dot fusome (spectrosome).

Immunoprecipitation and western blot analysis

Fly ovaries were collected and homogenized in 1 ml ice-cold lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 30 mM EDTA, 0.5% Triton X-100) with 2× complete protease inhibitors. All further manipulations of the ovary lysates were performed at 4°C or on ice. Nuclei and debris were pelleted at 10 000*g* for 10 min; the supernatant was collected and precleared for 1 h with 100 μl recombinant protein G agarose (Invitrogen). Antibody against either *dFmr1* protein or *Ago1* was incubated with recombinant protein G agarose at 4°C for 2 h and washed three times with lysis buffer. The precleared lysates were immunoprecipitated with antibody-coated recombinant protein G agarose at 4°C overnight. The precipitated complexes were used for western blot analysis. For western blotting, antibody was used at a dilution of 1:1000. Anti-rabbit or -mouse secondary antibodies were horseradish peroxidase (Amersham Biosciences, formerly Amersham Pharmacia Biotech, Inc.) conjugated and detected by Enhanced ChemiLuminescence (Amersham Biosciences, formerly Amersham Pharmacia Biotech, Inc.).

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Conflict of Interest statement. The authors declare that they have no conflicts of interest.

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