**Drosophila** FMRP regulates microtubule network formation and axonal transport of mitochondria

Aiyu Yao1, Shan Jin1,2, Xinhai Li1, Zhihua Liu1, Xuehua Ma1, Jing Tang1 and Yong Q. Zhang1,*

1Key Laboratory of Molecular and Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China and 2College of Life Sciences, Hubei University, Wuhan, Hubei 430062, China

Received May 18, 2010; Revised and Accepted October 1, 2010

Fragile X syndrome, the most common form of inherited mental retardation, is caused by the absence of the fragile X mental retardation protein FMRP. The RNA-binding FMRP represses translation of the microtubule (MT)-associated protein 1B (MAP1B) during synaptogenesis in the brain of the neonatal mouse. However, the effect of FMRP on MTs remains unclear. Mounting evidence shows that the structure and the function of FMRP are well conserved across species from *Drosophila* to human. From a genetic screen, we identified *spastin* as a dominant suppressor of rough eye caused by *dfmr1* over-expression. *spastin* encodes an MT-severing protein, and its mutations cause neurodegenerative hereditary spastic paraplegia. Epistatic and biochemical analyses revealed that *dfmr1* acts upstream of or in parallel with *spastin* in multiple processes, including synapse development, locomotive behaviour and MT network formation. Immunostaining showed that both loss- and gain-of-function mutations of *dfmr1* result in an apparently altered MT network. Western analysis revealed that the levels of α-tubulin and acetylated MTs remained normal in *dfmr1* mutants, but increased significantly when *dfmr1* was over-expressed. To examine the consequence of the aberrant MTs in *dfmr1* mutants, we analysed the MT-dependent mitochondrial transport and found that the number of mitochondria and the flux of mitochondrial transport are negatively regulated by *dfmr1*. These results demonstrate that dFMRP plays a crucial role in controlling MT formation and mitochondrial transport. Thus, defective MTs and abnormal mitochondrial transport might account for, at least partially, the pathogenesis of fragile X mental retardation.

**INTRODUCTION**

Fragile X syndrome, the most common form of inherited mental retardation, is caused by the absence of the RNA-binding fragile X mental retardation protein FMRP. Although the *in vivo* functions of FMRP have been under intensive investigations in the last two decades, it remains unclear how the absence of FMRP leads to mental retardation (1–3). Multiple independent lines of evidence indicate that FMRP regulates microtubules (MTs). First, FMRP has been shown to bind and suppress the translation of MT-associated protein 1B (MAP1B) mRNA (4–6). MAP1B stabilizes MTs. In the neonatal mouse brain, FMRP suppresses the expression of MAP1B during synaptogenesis, and the elevated expression of MAP1B in *Fmr1* knockout mice leads to increased MT stability (7). Secondly, fractionation and western analysis showed that FMRP is associated with polymerized MTs (8,9). Thirdly, the *Drosophila* homologue of FMRP (dFMRP) suppresses the expression of Futsch, the fly homologue of MAP1B, in the nervous system (10,11). Together, these data establish an important role for FMRP in regulating MTs. However, evidence showing abnormal MTs associated with loss of FMRP *in vivo* has been scarce.

In addition to regulating MTs, FMRP has been shown to be an adaptor between FMRP-containing RNA granules and motor proteins. FMRP was found to be in the same protein complex with kinesin heavy chain in mouse brain homogenates (12). Specifically, FMRP interacts with the C-terminus of KIF3C, and disruption of the KIF3C function impedes the transport of FMRP-containing RNA granules in primary
cultured neurons (8). More recently, Dictenberg et al. (13) reported that the C-terminal domain of FMRP interacts directly with kinesin light chain (KLC), the cargo-binding subunit of the kinesin-5 motor subunit of the KIF5 holoenzyme. In Drosophila, dFMRP associates with both kinesin heavy chain and dynein heavy chain, and knockdown of either of the two motors blocks the transport of dFMRP granules (14). These data together indicate a requirement of motor function for the transport of FMRP-positive cargos. However, a possible effect of FMRP on motor-mediated intracellular transport has not been closely examined.

From a genetic screen, we identified spastin as a dominant suppressor of the rough eye caused by dfmr1 over-expression. spastin encodes an MT-severing protein, and mutations of spastin result in neurodegenerative hereditary spastic paraplegia (15–18). Epistatic and biochemical analyses revealed that dfmr1 acts upstream of or in parallel with spastin in multiple processes, including neuromuscular synapse formation, locomotive control and MT network formation. Immunohistochemical analysis showed an apparently altered MT network in both loss- and gain-of-function dfmr1 mutants. Moreover, the number of mitochondria and the flux of mitochondrial transport in axons are negatively regulated by dfmr1. Thus, dfmr1 is required for both MT formation and mitochondrial transport, which offers novel insights into the pathogenesis of fragile X mental retardation.

RESULTS

spastin mutants or RNAi knockdown suppresses rough eye induced by dfmr1 over-expression

To better understand the functions of dFMRP in vivo, we took advantage of the powerful genetics available in Drosophila to identify dfmr1 interacting genes. Over-expression of dfmr1 in the compound eye by the UAS-Gal4 system (19) led to a mild rough eye (compare Fig. 1A and B), consistent with earlier reports (10,20). Eye-specific over-expression of dfmr1 was achieved by crossing GMR-Gal4 with EP3517 that carries a UAS element inserted in the 5′ regulatory region of dfmr1 (Fig. 1B). Modification of the rough eye indicates a putative dfmr1 interacting gene. From the genetic screen, we found that spastin5.75 nulls with the coding exons completely deleted (16) dominantly suppressed the rough eye caused by dfmr1 over-expression (Fig. 1C; the effect of homozygous spastin null mutants on the dfmr1 over-expression rough eye cannot be scored because they are adult lethal). spastin RNAi knockdown also suppressed the rough eye phenotype (Fig. 1D). Conversely, over-expression of spastin and dfmr1 together enhanced the rough eye caused by over-expression of dfmr1 or spastin alone (Table 1). To rule out any influence of the genetic background on the interaction between dfmr1 and spastin, we examined the interaction in another independent system. Ectopic over-expression of dfmr1 under the direct control of the sevenless enhancer also resulted in a rough eye phenotype [Fig. 1E and (20)]. Again, spastin5.75 mutants dominantly and strongly suppressed the rough eye (compare Fig. 1E and F). Additionally, ubiquitous over-expression of dfmr1 by act-Gal4 was lethal, but spastin RNAi knockdown rescued the lethality (Table 1). It is worth pointing out that spastin RNAi knockdown showed a stronger effect in suppressing the lethality of ubiquitous dfmr1 over-expression than heterozygous mutants (Table 1). This is

![Figure 1. spastin mutants and RNAi knockdown suppress the rough eye phenotype induced by dfmr1 over-expression.](http://hmg.oxfordjournals.org/)

Table 1. spastin mutants and RNAi knockdown suppress rough eye or lethality induced by dfmr1 over-expression

<table>
<thead>
<tr>
<th>Transgenes or alleles</th>
<th>GMR-Gal4</th>
<th>GMR-Gal4, EP3517</th>
<th>sev-dfmr1</th>
<th>act-Gal4; EP3517</th>
</tr>
</thead>
<tbody>
<tr>
<td>spastin5.75/+ (KZ)</td>
<td>na</td>
<td>S</td>
<td>S</td>
<td>N</td>
</tr>
<tr>
<td>spastin17-7/+ (KZ)</td>
<td>na</td>
<td>S</td>
<td>S</td>
<td>N</td>
</tr>
<tr>
<td>spastin RNAi (KB)</td>
<td>N</td>
<td>S</td>
<td>na</td>
<td>S</td>
</tr>
<tr>
<td>UAS-spastin (KZ)</td>
<td>R</td>
<td>E</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>UAS-spastin (KB)</td>
<td>R</td>
<td>E</td>
<td>na</td>
<td>na</td>
</tr>
</tbody>
</table>

KZ and KB indicate stocks from K. Zinn (16) and K. Broadie (17), respectively. Over-expression of dfmr1 or spastin by GMR-Gal4 produced a rough eye. Co-over-expression of spastin and dfmr1 enhanced the rough eye, whereas spastin RNAi knockdown or mutants suppressed the rough eye caused by over-expression of dfmr1 driven by GMR-Gal4 or under the control of the eye-specific sevenless promoter [sev-dfmr1 (20)]. spastin RNAi knockdown also rescued the lethality caused by the ubiquitous over-expression of dfmr1 driven by act-Gal4. E, enhance; N, no effect; na, not applicable or not assayed; R, rough eye; S, suppress.
understandable as the RNAi knockdown probably decreases the expression of spastin by more than 50%. Taken together, the robust genetic interactions between dfmr1 and spastin suggest that spastin is required for the pathogenesis caused by dfmr1 over-expression.

**dfmr1 and spastin convergently regulate synaptic growth on both pre- and post-synaptic side**

Earlier work showed that dfmr1 regulates synaptic growth negatively through repressing the expression of the MT-associated protein Futsch (10). Consistently, dfmr1 mutants show an increased number of Futsch-positive cytoskeletal loops within synaptic boutons, further supporting a role of dfmr1 in regulating MTs (21). spastin null mutants also show over-grown neuromuscular junction (NMJ) synapses (16), similar to that in dfmr1 null mutants (10). We therefore examined the interaction between dfmr1 and spastin at NMJ synapses to further understand how dfmr1 and spastin regulate synaptic development.

We first confirmed that the NMJ synaptic terminals in dfmr1 and spastin null mutants were overgrown as reported (Fig. 2) (10,16). To quantify this phenotype, we examined simple NMJ4 synapses that were double-labelled with the neuronal membrane marker anti-horseradish peroxidase (HRP) and the synaptic vesicle marker anti-cysteine string protein (CSP). Complete loss of dfmr1 and spastin caused a significant increase in synaptic branching (5.43 ± 0.36 branches for dfmr1, 7.06 ± 0.64 for spastin and 3.48 ± 0.25 for wild-type; Fig. 2I), bouton number (46.10 ± 2.17 for dfmr1; 54.94 ± 3.14 for spastin, P < 0.001; 36.40 ± 1.87 for wild-type; Fig. 2J) and synaptic area (169.51 ± 4.83 μm² for dfmr1, 195.83 ± 9.15 μm² for spastin and 132.89 ± 6.25 μm² for wild-type; Fig. 2L). The synaptic overgrowth phenotype of spastin mutants was stronger than that of dfmr1 mutants (compare Fig. 2C and B), although the average bouton size in the spastin and dfmr1 mutants and the wild-type was all similar (Fig. 2K).

To determine the genetic interaction between dfmr1 and spastin, we first generated and examined double mutants. The synaptic branch and the synaptic area of dfmr1 spastin single mutants were comparable to those of spastin mutants (P > 0.05; Fig. 2I and L); however, there were more boutons (77.10 ± 3.91 for double mutants versus 54.94 ± 3.14 for spastin mutants; Fig. 2J) and smaller boutons (2.66 ± 0.12 μm² for double mutants versus 3.66 ± 0.16 μm² for spastin mutants; Fig. 2K) in the double mutants than those in spastin and dfmr1 single mutants, suggesting that dfmr1 and spastin act in parallel to regulate synaptic growth. To further understand the interaction between dfmr1 and spastin, we over-expressed dFMRP pre- or post-synaptically in a spastin null background. Presynaptic over-expression of dFMRP driven by the pan-neuronal elav-Gal4 (Fig. 2E) led to fewer (76% of wild-type) and larger (150% of wild-type) boutons than the wild-type, consistent with an earlier report (10). Remarkably, presynaptic over-expression of dFMRP (dfmr1NOE) in the spastin null background completely rescued the bidirectional

![Figure 2](http://hmg.oxfordjournals.org/)

Figure 2. dfmr1 and spastin convergently regulate synaptic growth on both pre- and post-synaptic side. Confocal images of NMJ4 synapses of abdominal segment A3 were labelled with anti-CSP in red and FITC-conjugated anti-HRP in green to reveal synaptic vesicle and neuronal membrane, respectively. Representative NMJ synapses of different genotypes are shown: (A) wild-type (WT); (B) dfmr1MOE; (C) spastin5; (D) dfmr1MOE spastin5 double mutant; (E) pre-synaptic neuronal over-expression of dfmr1 (dfmr1NOE; elav-Gal4; UAS-dfmr1+/+); (F) neuronal over-expression of dFMRP in the spastin homozygous null background (elav-Gal4; UAS-dfmr1 spastin5;spastin5); (G) post-synaptic muscular over-expression of dfmr1 (dfmr1NOE; C57-Gal4/dfmr1) and (H) muscular over-expression of dfmr1 in the spastin null background (UAS-dfmr1 spastin5;C57-Gal4 spastin5). The scale bar represents 10 μm. (I–L) Quantification of synaptic morphological features: branch number (I), bouton number (J), bouton size (K) and synaptic area (L). The scale bar represents 10 μm. (I–L) Quantification of synaptic morphological features: branch number (I), bouton number (J), bouton size (K) and synaptic area (L). n > 19 for all genotypes assayed. Comparisons were made between each genotype with the wild-type control unless indicated otherwise. ∗P < 0.05, ∗∗P < 0.01, ∗∗∗P < 0.001; error bars indicate SEM.
NMJ phenotypes of the dfmr1NOE and spastin mutants to the wild-type (3.50 ± 0.30 for branch number, 33.60 ± 1.6 for bouton number, 4.08 ± 0.19 μm² for bouton size and 135.37 ± 7.90 μm² for synaptic area; $P > 0.05$ for all four parameters compared with the wild-type; Fig. 2I–L). Post-synaptic over-expression of DFMRP also led to fewer (60% of wild-type) and larger boutons (173% of wild-type) than the wild-type. Interestingly, these synaptic defects were also restored to wild-type by homozygous spastin5.75 null mutants (34.75 ± 2.26 for bouton number and 4.10 ± 0.23 μm² for bouton size; $P > 0.05$ for both parameters compared with wild-type; Fig. 2J and K). Together, these results demonstrate that dfmr1 acts in parallel with spastin to inhibit synaptic growth.

**spastin mutants rescue the locomotion impairment caused by muscular over-expression of dfmr1**

Given that dfmr1 and spastin interact in the eye development and NMJ synaptic growth (Figs 1 and 2), we sought to investigate whether both genes interact in controlling larval locomotive behaviour. We compared the coordinated behaviours of different animals using the larval roll-over assay, which involves turning a crawling larva to a totally inverted position and then recording the time the animal takes to flip right itself to the normal position. dfmr11MOE homozygous and hemizygous null mutants displayed faster roll-over than the wild-type (6.17 ± 0.56 s for homozygous mutant versus 14.7 ± 1.1 s for wild-type; Fig. 3). spastin5.75 null mutants (6.82 ± 0.47 s), such as dfmr11MOE null mutants, rolled over more quickly than the wild-type. dfmr1 spastin double mutants showed a phenotype similar to that of the dfmr1 or spastin single mutants. However, over-expression of dfmr1 in muscles by C57-Gal4 (dfmr1MOE) strongly impaired the coordinated locomotion, with roll-over time increased to 227.6% (33.32 ± 2.95 s). The dfmr1MOE animals stayed upside down for much longer and appeared to be struggling during a period of continuous, spasitic muscle contractions that did not lead to the turning behaviour, whereas wild-type animals showed smooth, cooperative locomotion and turned over quickly. However, animals with dfmr1 over-expression in the neuronal system induced by pan-neuronal elav-Gal4 (dfmr1NOE) rolled over normally, like the wild-type (13.05 ± 1.56 s for dfmr1NOE). As a control, C57-Gal4, elav-Gal4 and UAS-dfmr1 transgenic flies alone without dFMRP over-expression showed normal locomotion (Fig. 3).

Remarkably, the locomotion impairment caused by muscular over-expression of dfmr1 was fully rescued by heterozygous (7.0 ± 1.64 s for dfmr1MOE+; $P < 0.001$ compared with dfmr1MOE, but $P > 0.05$ compared with the wild-type) and homozygous spastin null mutants (13.21 ± 1.18 s for dfmr1MOE+; $P < 0.001$ when compared with dfmr1MOE, but $P > 0.05$ when compared with the wild-type; Fig. 3). Reciprocally, the faster roll-over of spastin homozygous mutants was rescued completely by over-expressing dfmr1 in neural (14.22 ± 1.62 s) and muscular systems (13.21 ± 1.18 s). A possible explanation behind the reciprocal rescue of the locomotive defects is that spastin mutants may suppress the MT defects caused by dfmr1 over-expression (discussed subsequently). Taken together, the similar locomotion defects of dfmr1 and spastin mutants and the reciprocal rescuing effect of dfmr1 over-expression in the spastin null background indicate that dfmr1 and spastin work in parallel in regulating larval locomotion.

**dfmr1 regulates formation of the MT network**

The above-mentioned genetic analysis showed that dfmr1 acts in parallel with or upstream of spastin, which encodes an MT-severing protein. Additionally, dfmr1 suppresses the expression of the MT-associated protein Futsch (10), but direct evidence demonstrating abnormal MTs in dfmr1 mutants has so far been scarce. We therefore sought to determine whether dfmr1 affects MTs. In the large multi-nucleated muscle cells from a wild-type third instar larva, a remarkable MT meshwork was revealed by anti-α-tubulin staining, with an obvious enrichment of MTs around the nucleus (Fig. 4A) (16,17,22). dfmr1MOE null mutants showed increased perinuclear MT density but reduced MT intensity in the central area among nuclei (Fig. 4B), whereas over-expression of dfmr1 in muscles driven by C57-Gal4 led to prominent thick parallel MT bundles with no enrichment of perinuclear MTs (Fig. 4C). The MT defects in dfmr1 mutants were rescued to wild-type by a genomic transgene [w+; dfmr1] (Fig. 4D), demonstrating that the phenotypes are specifically caused by dfmr1 mutations. To better define the MT defects, the intensity of α-tubulin staining in the perinuclear and central area was quantified (Fig. 4K–M). Compared with the wild-type, the intensity of the MT network in the perinuclear area was increased significantly in dfmr1 mutants (200.22 ± 6.40 for
dfmr1 mutants versus $186.26 \pm 2.33$ for the wild-type), whereas the MT intensity in the central area was decreased significantly ($37.04 \pm 4.43$ for dfmr1 mutants versus $57.00 \pm 6.04$ for the wild-type) (Fig. 4K–M). In addition, the MT network was uneven and tangled in dfmr1 mutants (Fig. 4B). Conversely, when dfmr1 was over-expressed in muscles, the perinuclear MT intensity was decreased ($129.53 \pm 8.34$ versus $186.26 \pm 2.33$), whereas the central area MT intensity was increased significantly ($121.89 \pm 15.56$ for dfmr1MOE versus $57.00 \pm 6.04$ for the wild-type) (Fig. 4K–M). These results demonstrate that dfmr1 regulates MT network formation.

Given the strong genetic interaction between dfmr1 and spastin (Figs 1–3), and both dFMRP and spastin individually regulate MTs (Fig. 4A–D) (16–18,22,23), we therefore examined the interaction between dfmr1 and spastin in controlling MT formation. The MT network in the middle area among nuclei of a muscle cell is better visualized at higher resolution (Fig. 4E–J). Compared with the evenly distributed MT network with smooth MT fibres in wild-type muscle cells (Fig. 4E), dfmr1 mutants showed an unevenly distributed MT network with tangles and short MT fibres (Fig. 4F). Conversely, dfmr1 over-expression resulted in thick parallel MT bundles (Fig. 4G). spastin null mutants also exhibited...
short MT fibres (Fig. 4H). Interestingly, spastin homoyzous but not heterozygous null mutants fully rescued the thick parallel MT bundles in dfmr1\textsuperscript{MOE} cells to wild-type (Fig. 4I and J). Statistically, the MT breakpunts in the central area were increased significantly in dfmr1 mutants (18.00 ± 1.37 per 400 μm\textsuperscript{2} for dfmr1\textsuperscript{SOM} mutants versus 8.66 ± 1.53 for the wild-type) as well as in spastin\textsuperscript{575} null mutants (27.71 ± 3.35) (Fig. 4N). Conversely, when dfmr1 was over-expressed in muscles (dfmr1\textsuperscript{MOE}), the number of MT breakpoints was significantly decreased (0.25 ± 0.16). The reduced MT breakpoints in dfmr1 over-expressed animals were slightly rescued by heterozygous spastin mutation (1.90 ± 0.64), but completely rescued by homozygous spastin null mutation to wild-type (9.70 ± 1.36; P > 0.05) (Fig. 4N).

To determine the effect of the dfmr1–spastin interaction on MTs in neurons, we measured and compared the intensity of Futsch in the pre-synaptic terminals (Fig. 5). We found that the intensity of Futsch (the grey values of Futsch staining normalized to the Futsch-positive area reported by ImageJ) in dfmr1 null mutants was significantly increased compared with wild-type (87.57 ± 4.88 for dfmr1 mutants versus 73.88 ± 2.59 for wild-type, P < 0.05; Fig. 5F). Over-expression of dfmr1 in pre-synaptic neurons driven by elav-Gal4 led to a significantly decreased expression of Futsch (60.17 ± 1.87). The negative regulation of Futsch by dFMRP is consistent with previous findings (10,11,24). Similar to dfmr1 mutants, spastin mutants also showed an increased level of Futsch (86.69 ± 4.88). Interestingly, spastin mutants fully rescued the reduced intensity of Futsch in dfmr1\textsuperscript{NOE} animals (74.39 ± 2.98 for dfmr1\textsuperscript{NOE}, spastin mutants; Fig. 5F). To eliminate the effect of the uneven distribution of Futsch in the synaptic terminals (anti-Futsch stains strongly at the proximal end but weakly at the distal end of synaptic terminals) among different genotypes, we calculated and compared the intensity of Futsch normalized to the synaptic area demarcated by anti-HRP staining between different genotypes (Supplementary Material, Fig. S1). Apparently, the Futsch intensities normalized to the HRP-demarcated synaptic area matched well with the Futsch intensities normalized to the Futsch-positive area in all the genotypes except in spastin mutants (compare Supplementary Material, Figs S1 and 5F). The dfmr1–spastin interaction in regulating Futsch intensity in pre-synaptic terminals recapituclates that in other systems (Figs 1–4).

To better understand the genetic interaction between dfmr1 and spastin, we conducted biochemical assays. Co-immunprecipitation with anti-dFMRP of muscle lysates showed that dFMRP and spastin are not present in the same complex (data not shown). Western analysis of larval muscles showed that the expression level of dFMRP (85 kDa) remains normal as the wild-type when spastin expression is altered (Fig. 6A), demonstrating that spastin does not affect the expression of dfmr1. However, the expression level of spastin (90 kDa) was significantly increased to 3.1 times of the wild-type (P < 0.05, n = 4) when dfmr1 was over-expressed in muscles (dfmr1\textsuperscript{MOE}) driven by C57-Gal4, whereas loss of dFMRP did not affect the expression of spastin (Fig. 6B and C). The increased expression of spastin upon elevated expression of dfmr1 supports the epistasis of dfmr1 acting upstream of spastin, although the regulation mechanism is currently unknown.

**Figure 5.** spastin mutants rescued the reduced level of Futsch at NMJ synapses of dfmr1\textsuperscript{NOE}. (A–E) Representative images of NMJ4 synapses in the abdominal segment A3 of third instar larvae co-stained with anti-Futsch to detect MTs (red) and anti-HRP to label pre-synaptic membrane (green) in WT (A), dfmr1 null mutant (dfmr1\textsuperscript{SOM}) (B), neuronal over-expression (NOE) of dfmr1 driven by elav-Gal4 (C), spastin null mutant (spastin\textsuperscript{575}) (D) and dfmr1\textsuperscript{NOE}, spastin\textsuperscript{575} [neuronal over-expression of dfmr1 in homozygous spastin null background; (E)]. The arrows in (A)–(D) indicate the nerve innervation site. The scale bar in (D) represents 10 μm. (F) The intensity of Futsch staining normalized to the Futsch-positive area of the entire synaptic terminals starting from the innervation site was quantitatively analysed in different genotypes. n ≥ 11; *P < 0.05, **P < 0.001; error bars indicate SEM.

**Figure 6.** Elevated expression level of spastin when dfmr1 is over-expressed. (A) The expression level of dFMRP is un-altered in loss- and gain-of-function mutants of spastin. Actin was used as loading control. MOE indicates muscular over-expression of spastin driven by C57-Gal4. (B) The expression level of spastin is significantly increased when dfmr1 is over-expressed in muscles (MOE). The band sizes for dFMRP and spastin are 85 and 90 kDa, respectively. (C) Statistical analysis of spastin expression in loss- and gain-of-function mutants of dfmr1. n = 4; *P < 0.05, error bars indicate SEM.
Total and acetylated α-tubulins are increased when *dfmr1* is over-expressed

To further understand the effect of *dfmr1* on MT, we examined the status of tubulins in different genotypes by fractionation followed by quantitative western analysis. Tubulins are present in two forms: soluble, unpolymerized tubulins and precipitable polymerized MTs. As shown in Figure 7, the total tubulins detected by an antibody against α-tubulin were increased significantly (by 46%) in animals with *dfmr1* over-expressed in muscles driven by *C57-Gal4* compared with the wild-type (Fig. 7A and C). The increased level of α-tubulin was observed in both soluble and precipitable fractions of *dfmr1* over-expressing animals (Fig. 7C), whereas *dfmr1* mutants showed normal levels of α-tubulins (Fig. 7C). To confirm the results, we determined the level of acetylated α-tubulins in the three genotypes. Acetylated α-tubulin is a marker for polymerized stable MTs (25). We found that the level of acetylated α-tubulins in *dfmr1* over-expressing animals was also significantly higher than that in the wild-type (1.6-fold greater than that of wild-type, Fig. 7B and D), concurrent with the increased level of α-tubulin. On the contrary, *spastin* null mutants showed reduced levels of acetylated α-tubulins (Fig. 7B and D). Interestingly, homozygous *spastin* mutants and *dfmr1*MOE animals reciprocally and completely rescued the altered expressions of acetylated α-tubulin to wild-type, whereas heterozygous *spastin* mutants had a weaker rescuing effect than the homozygous mutants (Fig. 7B and D). The level of total α-tubulin changed in accordance with that of acetylated α-tubulin in *dfmr1*MOE and *dfmr1*MOE *spastin* genotypes (data not shown). These results together further support the genetic interaction that *dfmr1* acts in parallel with *spastin*.

dfmr1 negatively regulates the number of mitochondria in axons and synaptic terminals

Given that *dfmr1* regulates MTs (Figs 4 and 7) and mitochondria transport is dependent on MTs (26), we sought to investigate whether MT-dependent transport of mitochondria was disrupted in *dfmr1* mutants. To this end, we first examined the axonal distribution of mitochondria labelled by a green fluorescent protein (GFP) tag (mito-GFP) in different genotypes. The mito-GFP was constructed by fusing the N-terminal 31 amino acids (a mitochondria-targeting sequence) from the human cytochrome *c* oxidase subunit VIII to the N-terminus of GFP (27). The expression of mito-GFP driven by the motor neuron-specific *D42-Gal4* labels mitochondria brightly in axons (Fig. 8A) (27–29). To our surprise, we found that the number of axonal mitochondria was inversely correlated to the expression levels of *dfmr1* (Fig. 8A–C). In *dfmr1*ΔM homozygous mutants, the number of mito-GFP-labelled mitochondria was increased significantly by 63.4% (23.78 ± 1.56/100 μm² for mutants versus 14.55 ± 0.72/100 μm² for the wild-type; Fig. 8G). *dfmr1*ΔM/Df(3R)BSC621 mutants also showed an increased number of mitochondria, similar to that in the homozygous mutants (data not shown), indicating that the increased number of mitochondria is caused specifically by *dfmr1* mutations. Conversely, neuronal over-expression of *dfmr1* led to a decrease in the number of mitochondria (10.83 ± 0.83/100 μm²; Fig. 8G). Quantification of the area of mitochondria in axons showed trends similar to those of the number of mitochondria in different genotypes (compare Fig. 8H with G). As a control, the axonal distribution of synaptic vesicle precursors labelled by antibody staining against synaptotagmin (Syt) or CSP showed wild-type pattern in *dfmr1* mutants; similarly, the staining pattern for Bruchpilot, a synaptic active zone marker, was also normal when *dfmr1* was mutated or over-expressed (data not shown), indicating that *dfmr1* specifically affects the number of mitochondria but not other organelles in axons.

We further examined mitochondria at NMJ synapses to confirm the altered number of mitochondria in *dfmr1* mutants. As shown in Figure 8D, wild-type NMJ synapses contain GFP-labelled mitochondria as reported (29,30). However, the number of mitochondria was increased markedly in *dfmr1* mutants, but reduced when *dfmr1* was over-expressed by *D42-Gal4* (Fig. 8D–F). Due to the aggregation of mitochondria at the NMJ terminals of the three different genotypes. Similar to that observed in axons (Fig. 8G and H), the percentage of mitochondrial area at NMJ terminals was increased significantly to 24.6% in *dfmr1* mutants, but decreased to 7.0% when *dfmr1* was over-expressed compared with 14.3% in the wild-type (Fig. 8I). The changes in the number of mitochondria in the soma of motor neurons were similar to those in axons and NMJ synapses when the expressions of *dfmr1* were altered (data not shown). The analysis of GFP-tagged mitochondria in both axons and NMJ synapses showed that the number of mitochondria is negatively regulated by *dfmr1*.

dFMRP affects flux and processivity of mitochondrial transport in axons

The results presented above showed disrupted MTs (Figs 4 and 7) and altered numbers of mitochondria in axons and NMJ synapses (Fig. 8). To examine a possible role for *dfmr1* in MT-based axonal transport of mitochondria, we conducted live imaging of motile mitochondria labelled with GFP in different genotypes. Mitochondrial transport displays salutatory bidirectional movement, in which moving mitochondria frequently start, stop and change direction (Supplementary Material, Movie S1), and we followed the commonly used parameters to describe mitochondrial transport (28). Flux is defined as the number of mitochondria moving into the bleached area per minute. Anterograde flux of mitochondria was significantly increased in *dfmr1* mutants (5.91 ± 0.11 for mutants versus 5.2 ± 0.22 for wild-type), but reduced when *dfmr1* was over-expressed compared with the wild-type (4.38 ± 0.25 for *dfmr1*NOE) (Fig. 9D). Similarly, retrograde flux was increased significantly in *dfmr1* mutants (5.28 ± 0.19 for mutants versus 3.9 ± 0.10 for the wild-type), but was reduced in *dfmr1*NOE animals (2.27 ± 0.2; Fig. 9D: Supplementary Material, Movies S2 and S3). The bidirectional changes in the transport flux are positively correlated with alterations in the numbers of mitochondria observed in both loss- and gain-of-function *dfmr1* mutants (Fig. 8). Flux represents both the abundance of moving organelles and their
net velocity. To distinguish between the two possibilities, we quantified the speed of moving mitochondria. The velocity for both anterograde and retrograde transport in loss- and gain-of-function dfmr1 mutants was comparable to that of wild-type (data not shown). These results show that dfmr1 negatively regulates the flux of mitochondrial transport in axons.

To determine whether dfmr1 controls additional transport parameters, we measured the length of time that mitochondria spent in forward runs (FRs), reverse runs (RRs) and stops. Although dfmr1 mutants showed normal axonal transport of mitochondria in both directions (Fig. 9E), dfmr1NOE led to significantly decreased FR time (46.7 ± 2.16% for dfmr1NOE versus 54.16 ± 1.50% for wild-type in anterograde transport and 50.99 ± 1.29% for dfmr1NOE versus 59.07 ± 2.9% for wild-type in retrograde transport), but increased stop time (46.9 ± 1.62% for dfmr1NOE versus 39.27 ± 0.99% for wild-type in anterograde transport and 43.45 ± 1.17% for dfmr1NOE versus 35.09 ± 1.28% for wild-type in retrograde transport) (Fig. 9E). In summary, dfmr1 mutants showed increased flux, whereas dfmr1NOE animals exhibited reduced flux in both directions. In addition, dfmr1NOE animals
shown increased stop time. These findings demonstrate that dfmr1 affects flux and processivity of mitochondrial transport in axons.

**DISCUSSION**

**dfmr1 acts upstream of or in parallel with spastin in multiple processes**

Previous studies identified several dfmr1 interacting genes such as futsch (10), cyfip (31) and ago1 (32). In the present study, we report spastin as a novel interactor of dfmr1. We identified robust genetic interactions between dfmr1 and spastin in multiple contexts. First, dfmr1 and spastin mutants have similar phenotypes such as overgrown NMJ synapses (Fig. 2), faster roll-over locomotion (Fig. 3), broken MT fibres (Fig. 4) and increased level of Futsch at synaptic terminals (Fig. 5), suggesting that the two genes act similarly in different processes. Secondly, the prominent dfmr1 over-expression phenotypes can be effectively rescued by heterozygous (Figs 1 and 3) and homozygous spastin null mutants (Figs 2–5), i.e. spastin is required for the dfmr1OE toxicity. Conversely, spastin null phenotypes can be rescued by dfmr1 over-expression (Figs 2–5), suggesting a novel strategy to interrupt the spastin-related pathogenesis. It would be informative to also examine the interaction in the reciprocal configuration, i.e. over-expression of spastin in a dfmr1 null background. Unfortunately, as we reported earlier (22), the dominance of spastin over-expression precluded the interaction assay. The rescue of dfmr1 over-expression phenotypes by spastin mutants (Figs 1–5) and the reciprocal rescuing effect (Figs 2–5) indicate that dfmr1 acts upstream of or in parallel with spastin. As spastin is sufficient to directly sever MTs (16,17,22), it is unlikely that spastin acts through dFMRP. Western analysis showed that the level of spastin was increased significantly when dfmr1 was over-expressed, whereas spastin had no effect on the expression of dfmr1 (Fig. 6), in support of the epistatic interaction of dfmr1 acting upstream of spastin. It is worth pointing out that although the requirement of spastin for the dfmr1OE toxicity is robust and consistent in the six systems we examined (Figs 1–5 and 7), the interaction does not apply to the mitochondrial transport (Supplementary Material, Fig. S2), indicating that the interaction is context-dependent. Thirdly, the dfmr1 spastin double null mutants showed stronger NMJ morphological defects than the single mutants (Fig. 2), supporting the point that the action of dfmr1 is parallel with that of spastin. Consistent with the parallel function between dfmr1 and spastin, we found no association between dFMRP and spastin by co-immunoprecipitation assay (data not shown). Taken together, the epistatic and biochemical analyses show that dfmr1 acts upstream of or in parallel with spastin.

**dfmr1 regulates MT network formation**

This study provides the first experimental evidence of an abnormal MT network when dfmr1 expression is altered in *Drosophila*. There were more perinuclear MTs, but reduced MTs with tangles and short MT fibres in areas distal to the nuclei of dfmr1 mutant muscle cells. On the contrary, dfmr1 over-expression resulted in thick parallel MT bundles with elevated expressions of α-tubulin and acetylated MTs.
(Figs 4 and 7). The mechanism by which dFMRP regulates the formation of the MT network is currently unknown. However, there are several possibilities. First, FMRP associates with MTs as detected by a fractionation assay (8,9), and the absence of FMRP might thus affect MTs directly or indirectly. Secondly, FMRP has been shown to interact directly with motor proteins such as KIF3C (8) and KLC (13). It is well known that motors such as kinesin-like protein at 61F (KLP61F) and dynein can bundle and slide MTs (33,34). Thus, dFMRP could regulate MTs by its interacting motors. Thirdly, FMRP represses the translation of MAP1B and is required for the accelerated decline of MAP1B during synaptogenesis in the neonatal mouse brain (7). The thick parallel MT bundles and increased α-tubulin expression in dfmr1NOE animals are similar to those in futsch mutants (24). Furthermore, dfmr1 interacts with futsch but not with spastin in regulating axonal transport (Supplementary Material, Fig. S2). However, Futsch is expressed only in the nervous system (35), whereas dFMRP is widely expressed in multiple tissues, including muscles, indicating that there might be targets other than Futsch by which dFMRP regulates MTs in non-neuronal cells. Last, but not the least, given that the episodic analysis revealed parallel functions between dfmr1 and spastin in multiple processes (Figs 1–5 and 7), it is conceivable that dfmr1 might regulate MTs in a manner similar to that of spastin or via spastin. In summary, dfmr1 might regulate MTs through distinct mechanisms involving different partners in different cellular contexts. It would be of interest to unravel the mechanism by which dFMRP regulates MTs.

**dfmr1 regulates the number and transport of mitochondria**

One surprising finding is that dfmr1 negatively regulates the number of mitochondria (Fig. 8). There are many reports of reduced numbers of mitochondria in axons or synaptic terminals when a gene is disrupted, but the opposite was rarely documented (36). Mitochondrial transport to synapse is tightly regulated to provide sufficient energy for synaptic transmission (37,38). Decreased synaptic transmission has been reported to be associated with reduced numbers of functional mitochondria (30,36,39). Consistently, the increased number of mitochondria (Fig. 8) is positively correlated with elevated neurotransmission at the NMJ synapses in dfmr1 mutants (10). The mechanism by which dfmr1 negatively regulates the number of mitochondria remains to be elucidated.

We have demonstrated for the first time that dfmr1 affects axonal transport of mitochondria. The flux of both anterograde and retrograde transport is increased in dfmr1 mutants, but reduced in dfmr1NOE animals (Fig. 9). Additionally, in dfmr1NOE animals, there was reduced time in FRs but increased stop time in the mitochondrial transport of both directions, indicative of more ‘stop signs’ for axonal transport. The altered flux of mitochondrial transport in both loss- and gain-of-function dfmr1 mutants is positively correlated with the altered numbers of mitochondria (Figs 8 and 9). It has been shown that FMRF co-localizes with mRNA in granules (40,41). In RNA granule transport, FMRP associates with motor proteins such as KLC, kinesin heavy chain, KIF3C and dynein (42). Loss of dFMRP causes increased bidirectional and oscillatory movement of mRNA granules (42). Fluorescence recovery after photobleaching (FRAP) analysis showed that the fluorescence recovery rate of mRNA granules is reduced in dfmr1 mutants (42). Thus, FMRP acts as a processivity factor for mRNA transport and a molecular adaptor between RNA granules and motor proteins (8,42).

The transport of mitochondria depends on MTs (26) and so does the transport of FMRP-positive mRNA granules (40,41). MT-severing spastin regulates mitochondrial localization and transport; expressing mutant spastin in neurons results in abnormal perinuclear clusters of mitochondria (43). More recently, a reduction in the anterograde flux of mitochondrial transport was reported in cultured neurons from spastin mutant mice (44). We showed that the flux of both anterograde and retrograde mitochondrial transport was decreased in Drosophila spastin mutants, similar to that observed in dfmr1NOE animals (Fig. 9 and Supplementary Material, Fig. S2). Consequently, spastin mutants did not rescue the transport defects in dfmr1NOE animals (Supplementary Material, Fig. S2), although the rescue effect has been observed readily in multiple other contexts (Figs 1–5 and 7), indicating that the interactions between the two genes may work differently in different systems (axonial transport versus other processes) or that axonal transport requires special machinery not related to the dfmr1–spastin interaction. In contrast, futsch mutants showed reduced flux of mitochondrial transport, similar to that observed in dfmr1NOE animals, but opposite to that of dfmr1 mutants (Supplementary Material, Fig. S2). Interestingly, futsch mutants rescued the increased flux of mitochondrial transport in dfmr1 mutants (Supplementary Material, Fig. S2), which supports the previous finding that dfmr1 negatively regulates futsch (10,11,24). We therefore favour the hypothesis that dfmr1 might regulate mitochondrial transport by affecting MTs mainly through Futsch, although dfmr1 and spastin interact in other cellular contexts such as synapse growth, locomotive control and MT network formation.

**MATERIALS AND METHODS**

**Drosophila stocks and genetics**

*Drosophila* w1118 flies were used as the wild-type control. All flies were cultured in standard cornmeal medium at 25°C. The Drosophila stocks used in this study include the eye-specific GMR-Gal4, the muscle-specific C57-Gal4 and the pan-neuronal elav-Gal4, dfmr1-related stocks EP3517, UAS-dfmr1 and dfmr150M null allele with a large intragenic deletion were described previously (10). Transgenic flies carrying a genomic rescue construct P[w: dfmr1] on the second chromosome were from Siomi and co-workers (45). *spastin* hypomorph spastin17–7 and null allele spastin5.75, which has coding sequences completely deleted, were from Sherwood et al. (16). Multiple UAS-spastin and spastin RNAi lines were from Sherwood et al. (16) and Broadie and co-workers (17). *sev-dfmr1* was from Zarnescu et al. (20). D42-Gal4 UAS-mito-GFP/TM6B was from Saxton and co-workers (27). Chromosomal recombinants used for genetic interaction assay were made following conventional genetic techniques.
**Larval roll-over assay**

The larval roll-over assay was performed essentially as described previously (46). Before the assay, larval cultures and agar plates were kept at room temperature for 2 h to acclimatize. For each assay, an individual animal was placed onto a 1% agar plate and allowed to move freely for ~2 min. A test animal that moved in a straight line was rolled over by a soft brush to a completely inverted position, as indicated by the ventral midline facing up. The length of time that the animal took to totally right itself was recorded. Three consecutive assays were performed for each animal and then averaged. The data were analysed by two-tailed Student’s t-test.

**Immunostaining, confocal microscopy and quantitative analysis**

Dissection and antibody staining of wandering third instar larvae were as described (10,22). The primary antibodies used include anti-α-tubulin (1:1000; mAb B-5-1-2 from Sigma, St Louis, Missouri), anti-CSP [1:500; 6D6 from the Developmental Studies Hybridoma Bank (DSHB) at the University of Iowa], anti-Futsch (1:50; 22C10 from DSHB) and FITC and Texas red-conjugated anti-HRP (used at 1:100; Jackson ImmunoResearch, West Grove, Pennsylvania). The primary antibodies were detected using Alexa 488 or Alexa 568 conjugated goat anti-mouse IgG (1:1500; Invitrogen, Carlsbad, California). Nuclei were labelled by staining with 1.25 μg/ml propidium iodide (PI) at room temperature for 30 min. Images were collected with a Leica SP5 confocal microscope and processed using Adobe Photoshop.

NMJ phenotypes were quantified essentially according to published protocols (10,22). All images analysed were three-dimensional (3D) projections from complete Z-stacks through the entire NMJ4 of abdominal segment A3. A synaptic branch was defined on the basis of anti-HRP staining; the elaboration originating directly from the nerve entry point was defined as primary branches, and each higher order branch was counted only when two or more boutons could be discerned in the subsequent branch. Individual boutons were defined according to the discrete staining signal of anti-CSP (a synaptic vesicle marker). Bouton size was calculated by dividing the synaptic area by the number of boutons. The synaptic area was determined by measuring the area of anti-CSP staining of the whole synaptic terminal with ImageJ. At least 19 animals were analysed for each genotype.

The intensity of tubulin staining in different genotypes was quantified, as described previously (22). Specifically, muscle 2 in abdominal segment A4 was chosen for analysis because it has fewer tracheal branches to obscure the observation of MTs (22). For quantification of MTs detected by anti-α-tubulin staining in muscles, all images analysed were 3D projections of serial stacks through the muscle cell. The perinuclear area was defined as the coverage that spans a 5 μm diameter circular ring next to nuclei. The middle of the 10 × 10 μm square marked in Figure 4A was defined as the central area among nuclei. Anti-α-tubulin staining signals in the perinuclear or central area were calculated using ImageJ. The software reports the grey value of the selected area. MT breakpoints in the central areas (20 × 20 μm²) of muscle cells stained with anti-α-tubulin were counted and compared. Breakpoints were defined as the ends of an MT fibre. If both ends of an MT fibre were present in the analysed area, it was recorded as one breakpoint. Six to eight larvae of each genotype were analysed per experiment, and three repeats were conducted for quantification analysis.

For quantification of the fluorescence intensity of Futsch, all images of the whole NMJ4 elaborations of different genotypes were taken at identical settings without over-exposure. The intensity of Futsch in Figure 5 was presented as grey values normalized to the Futsch-positive area automatically calculated using ImageJ.

**Protein preparation and western analysis**

Third instar larvae were dissected in phosphate-buffered saline (PBS). All internal organs were removed, and the remaining fillet containing largely muscles was homogenized on ice in lysis buffer [100 mM KCl, 2 mM MgCl₂, 50 mM Tris (pH 7.5), 2 mM ethylene glycol tetraacetic acid, 2% (v/v) glycerol, 0.125% (v/v) Triton X-100, 100 μM pachitaxel (Invitrogen), 1% (v/v) dimethyl sulphoxide and 1% Protease Inhibitor Cocktail (Calbiochem, Darmstadt, Germany)]. After centrifugation at room temperature for 3 min at 500g, the supernatant was recovered and centrifuged at 4°C for 1 h at 100 000g. The supernatant was recovered and used to determine the amount of soluble tubulin. The pellet was suspended in lysis buffer to assess the precipitated, presumably polymerized, tubulin. Protein concentration was determined by the Bradford assay (Bio-Rad, Foster, California). Equal amounts of protein from different genotypes were mixed with 2× Laemmli buffer, resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The blots were first probed with mouse monoclonal antibodies against α-tubulin (Sigma, 1:30 000), acetylated-α-tubulin (Sigma, 1:30 000), actin (mAb1501 from Chemicon, Rosemont, Illinois; 1:50 000), dFMRP (6A15 from Sigma, 1:2000) or guinea pig polyclonal antibody against spastin (a gift from Sherwood, 1:1000) followed by incubation with HRP-conjugated corresponding secondary antibodies (Sigma, 1:10 000). The processed membrane was developed with the chemiluminescent HRP substrate (Millipore, Billerica, Massachusetts) to detect target proteins. To quantify the expression levels of α-tubulin, acetylated α-tubulin and spastin in different genotypes, the positive signals from multiple repeats were calculated using ImageJ software and normalized against that of the wild-type.

**Scanning electron microscopy (SEM) of the eye**

SEM was adapted from a published protocol (47). Specifically, adult flies were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M PBS (pH 7.2) for 2 h, followed by washing in PBS for 30 min. The fixed heads were removed, dehydrated in a graded ethanol series, followed by drying and sputter coating and finally examined with a HITACHIS-300N SEM instrument.
Live imaging, tracking and analysis of GFP-tagged mitochondrial transport in axons

The analysis of mitochondrial transport was performed essentially as described (28). Wandering third instar larvae carrying the mito-GFP marker driven by the motor neuron-specific D42-Gal4 were dissected quickly in HL6 solution and then imaged directly at room temperature with a Leica SP5 confocal microscope under a 40× water immersion objective. To allow tracking of long-range mitochondrial transport, a 40 μm region of the segmental nerve was photobleached with 488 nm light at full intensity before image collection. Image collections were made every 1 s on a single plane for 5 min and completed within 15 min after dissection. The movements of mitochondria into or through the photobleached region were tracked and analysed by ImageJ (28) and plugin MTrackJ (E. Meijering, Biomedical Imaging Group of Rotterdam, The Netherlands; http://www.imagescience.org/meijering/software/mtrackj/; last accessed on October 13). Motile mitochondria were tracked as long as each remained visible in consecutive frames for no less than 60 s. The x-y-t tracking coordinates of mitochondrial movements calculated by MTrackJ were exported to modified Excel-based software (28,29) for automated analysis.

For quantitative analysis, mitochondrial transport was described in a three-state system consisting of FRs, RRs and stops adapted from Russo et al. (29). The start of a run was defined as a minimal speed of 0.151 μm/s and the end of a run by a velocity no less than 0.12 μm/s. A stop was defined in the MTrackJ program as no motility, moving at <0.12 μm/s following a run or <0.151 μm/s before a run starts (28,29). The following motility parameters were analysed: net velocity of anterograde and retrograde transport and the percentage of time spent in runs and stops. Flux was calculated by counting the number of mitochondria that moved past the anterior (anterograde flux) and posterior (retrograde flux) boundaries of the photobleached area per minute for 5 min.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS
We thank N. Sherwood and K. Broadie for spastin mutants and transgenic UAS-spastin and spastin RNAi flies, M. Saxton for D42-Gal4 UAS-mito-GFP/TM6B, D. Zarnescu for sev-dfmr1, H. Siomi for dfmr1 genomic rescue P[w+; dfmr1] flies, Dan Wang for assistance in initial genetic screens for dfmr1 interactors and N. Sherwood for spastin antibodies. We are grateful to the Bloomington Stock Centre for fly stocks and the Developmental Studies Hybridoma Bank, University of Iowa for antibodies. Dr Q. Hu at the Institute of Neuroscience, Shanghai Institutes for Biological Sciences, assisted in the analysis of axonal transport. We thank Edouard Khandjian, Ming Guo, Qionglin Yang, Xun Huang and Lily Jan for helpful discussions on the manuscript.

FUNDING
This work was supported by grants from the National Science Foundation of China (NSFC; 30930033) and the Ministry of Science and Technology of China (2007CB947200) to Y.Q.Z. and from NSFC (30800324) to A.Y.

REFERENCES


