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Fathoming fragile X in fruit flies

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Fragile X syndrome (FraX) is the most common inherited mental retardation disease. It is caused by mutation of the *fragile X mental retardation 1* (*fmr1*) gene. The FMR1 protein (FMRP) is a widely expressed RNA-binding translational regulator with reportedly hundreds of potential targets. Recent work has focused on putative roles of FMRP in regulating the development and plasticity of neuronal synaptic connections. The newest animal model of FraX, the fruit fly *Drosophila*, has revealed several novel mechanistic insights into the disease. This review focuses on *Drosophila* FMRP as (i) a negative regulator of translation via noncoding RNA, including microRNA and adaptor BC1 RNA-mediated silencing mechanisms; (ii) a negative regulator of microtubule cytoskeleton stability; and (iii) a negative regulator of neuronal architectural complexity.

Fragile X syndrome (FraX), one of the most common genetic diseases, is an X-linked disorder with a conservative prevalence of 1 in 4000 males and 1 in 8000 females. FraX is one of a group of neurological diseases caused by unstable trinucleotide expansion, which includes Huntington's disease and at least seven other neurodegenerative disorders [1,2]. A distinguishing feature of FraX is that the trinucleotide expansion is in the $5'$ regulatory sequence of the gene, causing hypermethylation and subsequent transcriptional silencing. FraX can also arise from point mutations or deletions in coding sequence, demonstrating that loss of function is causative. The *fragile X mental retardation 1* (*fmr1*) gene was identified in 1991 [3,4], and a mouse knockout model was established in 1994 [5]. Since then it has been shown that the FMR1 protein (FMRP) is an RNA-binding protein that associates with polyribosomes and can function as a translational suppressor [1,6,7]. Recent microarray studies suggest that FMRP has hundreds of putative RNA targets (432 in mouse) [8,9]; however, only a handful of the encoded proteins have been demonstrated to be regulated *in vivo* [9,10]. Owing to postsynaptic DENDRITIC SPINE (see Glossary) structural abnormalities in human patients and mouse *fmr1* knockouts [7,11,12], investigation has focused almost exclusively on putative defects in synaptic development and plasticity [7,11,13–15]. Despite these leads, two factors have greatly hindered our ability to tackle the disease. First, the list of putative FMRP target mRNAs is extremely long [8,9], and the specificity and physiological relevance of these interactions remain elusive. Second, the neurological

phenotypes of *fmr1* knockout mice are subtle, at both behavioral and cellular levels, which has made it difficult to assess the role FMRP *in vivo*.

In response to these limitations, a *Drosophila* FraX model was developed by mutating the homologous *Drosophila fmr1* (*dfmr1*) gene [16], also known as *dfxr* [17]. Systematic characterization of mutant phenotypes has been performed by multiple laboratories including ours [18–21]. *Drosophila* FMRP (dFMRP) displays close homology with mammalian FMRP, including conserved structure, RNA-binding properties, tissue and subcellular expression patterns and a conserved functional role as a translational suppressor [16,17,22], suggesting that the *Drosophila* model can complement and expand studies in mice. Importantly, the *Drosophila* FraX model offers distinct advantages. First, *dfmr1* mutants appear to display more prominent phenotypes than mouse *fmr1* knockouts, greatly facilitating analyses. This might be due to the presence of three closely related genes in mammals (*fmr1*, *fxr1* and *fxr2*), whereas *Drosophila* contains only a single gene [16,17]. Second, *Drosophila* has an array of relatively simple assays for neuronal structure and function [23]. This includes the ability to uniquely label single, identifiable mutant neurons *in situ* for precise analyses of neuronal structural development. Third, *Drosophila* is the only forward genetic system that contains an *fmr1* homolog. Thus, this system uniquely enables classic genetic screens for enhancers and suppressors of *dfmr1*-dependent mutant phenotypes, and a direct means to test the significance of putative molecular interactions *in vivo*.

FraX and FMRP have been the subject of excellent recent reviews [1,7,12,15,24–26], but these reviews have focused primarily on the human disease and progress in the mouse knockout model. This review focuses on the novel insights arising from the *Drosophila* model, with comparison to mammalian studies, and their possible implications for our fight against FraX disease.

Glossary

Dendritic spines: small ($\approx 1\,\mu$m) mushroom-shaped protrusions from dendrites that form the postsynaptic sites of excitatory synapses.
G quartet: a secondary structure within a mRNA in which four guanines form hydrogen bonds in a symmetrical square planar arrangement.
microRNA (miRNA): a single-stranded, noncoding RNA that complements a coding message and represses translation.
RNA BC1: a small noncoding RNA that binds fragile X mental retardation protein (FMRP) and associates with FMRP target mRNAs via sequence-pairing to act as a translational suppressor.
RNA interference (RNAi): a mechanism in which small double-stranded RNAs whose sequence complements a native message triggers mRNA degradation.

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Biochemical features of FMRP

In mammals, FMRP and its two autosomal homologs, FXR1P and FXR2P, can form homodimers or heterodimers [27]. Although the three homologs share >60% amino acid identity, the functions of FMRP cannot be compensated by its two homologs, because FraX is still manifested while the two homologs are expressed normally. The *fmr1* gene family is represented by only a single member in the *Drosophila* genome (*dfmr1*), and is not present in either *Caenorhabditis elegans* or yeast genomes. Mammalian FMRP is expressed in most if not all cell types, with particular enrichment in nervous system and testes. The protein is cytoplasmic and predominantly in the cell body, but presumably shuttles between nucleus and cytoplasm because it contains nuclear localization (NLS) and nuclear export (NES) signals (Figure 1). FMRP rarely exists in isolation, but rather associates with FXR1P, FXR2P and other proteins and mRNAs to form a messenger ribonucleoprotein (mRNP) particle that exceeds 660 kDa [28]. FMRP associates with actively translating polyribosomes in an RNA-dependent manner, although the percent associated varies substantially depending on tissues analyzed and protocols employed [10,29–31]. Indeed, the polyribosomal, rather than cytoplasmic, mRNA profile is altered in human FraX cell lines and *fmr1* knockout mouse brains [1,8,10,32].

In general, the N-terminal third of FMRP interacts with proteins, whereas the C-terminal two-thirds of FMRP binds to RNA (Figure 1a). Putative interacting protein partners and mRNA targets have been identified from various tissues and cells by different methods (Table 1). Using the N-terminal 218 amino acids as a bait to screen a mouse cDNA library, several FMRP-interacting proteins were identified, including a nuclear FMRP-interacting protein (NUFIP) [33], an 82 kDa FMRP-interacting protein (82-FIP) [34], and two cytoplasmic FMRP-interacting proteins (CYFIP1 and CYFIP2) [35]. An interesting feature of these FMRP-interacting proteins is that many of them independently bind RNA, including FXR1P, FXR2P, NUFIP, 82-FIP, nucleolin [36] and a Y box binding protein [YB1 (mouse), p50 (rabbit)] [37], which adds a layer of complexity to direct FMRP RNA binding.

In microarray screens, FMRP bound 432 of 11 067 mRNAs ($\approx$4%), and in polyribosome association shift assays $\approx$2% (251/11 000) of mRNAs changed profiles [8]; many of the targets were identified in common. FMRP has four putative RNA-binding motifs: an N-terminal domain (NDF) [38], two K homology domains (KH1 and KH2) and an RGG box (a cluster of arginine and glycine residues) (Figure 1a). The 3D structure of the KH domain has been resolved [39], but the RNA target sequence remains undefined. Point mutation I304N in the FMRP KH2 domain generates the most severe form of FraX mental retardation [40]. However, this mutation has been found
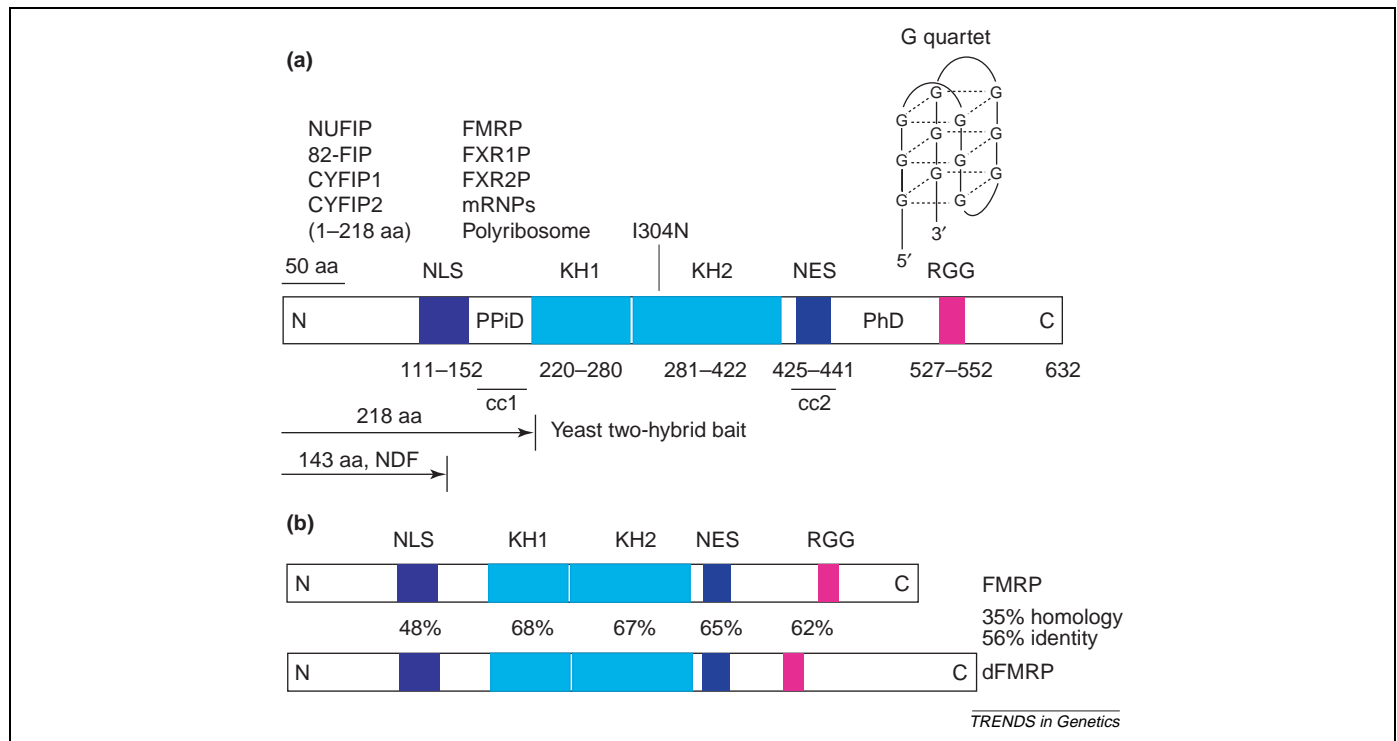

Figure 1. Structural features of mammalian and *Drosophila* fragile X mental retardation protein (FMRP). **(a)** Interacting partners and functional domains of FMRP. The N-terminal one-third of FMRP is mainly responsible for protein–protein interactions, the C-terminal two-thirds for protein–RNA interactions. NLS, nuclear localization signal; NES, nuclear export signal; KH1 and KH2, pre-mRNA-binding heterogeneous nuclear ribonucleoprotein K homology domains (the point mutation I304N in the KH2 domain that gives rise to an aggravated mental retardation is indicated); RGG box, a motif rich in arginine and glycine that binds a G quartet (the RGG box binds the G quartet encoding the RGG box itself [44,45]); CC, coiled coil domain involved in protein–protein interactions (CC1 associates with FMRP, FXR1P and FXR2P, CC2 associates with ribosomes [73]); PPiD, protein–protein interaction domain; PhD, a domain phosphorylated by kinase [22,74]; NDF, N-terminal domain of FMRP, also involved in dimerization and RNA binding [38]. NUFIP, 82-FIP, CYFIP1 and CYFIP2 interact with the N-terminal 218 amino acids; FMRP, FXR1P, FXR2P, mRNPs and polyribosomes interact with PPiD. The PPiD and KH domains are essential for FMRP association with polyribosomes, whereas the RGG box and PhD domain are dispensable [75]. It is noteworthy that although Siomi *et al*. originally found that CC2 is involved in association with ribosomes [73], Mazroui *et al*. concluded that PPiD and KH domains are required for the same interaction [75]. **(b)** Structural comparison of FMRP and *Drosophila* FMRP (dFMRP). The percentage of identity between functional domains and the overall homology and identity of FMRP and dFMRP are indicated. dFMRP has similar overall homology to FMRP, FXR1P and FXR2P [17].

Table 1. Protein and RNA partners of fragile X mental retardation protein (FMRP)

Proteins associated with FMRP	Tissue	Method[a]	Refs
FXR1P[b]	In vitro	BA	[27,76]
FXR2P[b]	Human HeLa cell	y2-h, BA, IP	[27]
CYFIP1, CYFIP2	HeLa and fly S2 cell	y2-h, BA, IP	[35,65]
82-FIP[b]	Monkey COS cell	y2-h, BA, IP	[34]
NUFIP[b]	COS and HeLa cell	y2-h, BA, IP	[33]
Nucleolin[b]	Mouse brain, L-M cell	IP	[36]
YB1 (mouse) p50 (rabbit)[b]	Mouse L-M cell	IP	[37]
Pur α[c]	Mouse brain	IP	[77]
mStaufen	Mouse brain	IP	[77]
Myosin Va	Mouse brain	IP	[77]
Ribosomal proteins L5[b] and L11	Fly S2 cell	IP, BA	[31]
VIG[b]	Fly S2 cell	IP	[51]
RNAi and/or miRNA components			
AGO1, AGO2	Human and fly cell	IP, BA	[31,51,53]
Dicer	Human and fly cell	IP, BA	[31,53]
Dmp68 (p68 RNA helicase)	Fly S2 cell	IP, BA	[31]
RNAs associated with FMRP			
FMRP, dFMRP	Human and fly brain	IP, BA	[6,17]
MiRNAs	Human and fly cell	IP	[31,53]
BC1 (mouse) BC200 (primate)	Mouse brain	IP, BA	[10]
Futsch (Drosophila) MAP1B	Fly and mouse brain	IP	[10,17]
Arc (also known as Arg3.1)	Mouse brain	IP	[10]
α-CaMKII	Mouse brain	IP	[10]
Myelin basic protein	In vitro	BA	[42]
GTPase Rac1	Whole fly	IP	[21]
Pickpocket1	Whole fly	IP, BA	[52]
5S RNA	Fly S2 cell	IP	[31]

A list of putative RNA targets of FMRP uncovered by systematic approaches by different groups [8,9,32,44] is not shown in the table.
[a]Abbreviations: BA, in vitro binding assay; IP, co-immunoprecipitation; y2-h, yeast two-hybrid interaction assay.
[b]Indicates an RNA-binding protein.
[c]The association of Pur α and FMRP complex is RNA mediated.

in only a single human patient, and experimental tests of its RNA-binding ability have been inconsistent [28,41–43]. The KH2 domain might also be involved in association with ribosomes because the I304N mutation leads to dissociation [28] or homo-oligomerization [43] of FMRP. The target sequence of the RGG box is the G QUARTET, contained in numerous putative mRNA targets (Figure 1) [8,44,45]. The majority of G-quartet-containing mRNAs have an altered polysome association in FraX patient cells [44]. G quartet recognition is an intrinsic property of monomeric FMRP, but it is not known whether the G quartet binding is modulated by the presence of FMRP-interacting proteins [45]. It is likely that a combination of G quartet, other sequence-specific target sites and interacting RNA-binding proteins specify the subset of mRNAs subject to FMRP regulation.

Drosophila FMRP is homologous to mammalian FMRP, in addition to FXR1P and FXR2P, in all defined structural domains (Figure 1b). Several laboratories have shown that dFMRP shares common biochemical features, including both RNA binding and protein–protein interactions [16,17,22,25]. Because there are three fragile X family members in mammals, dFMRP presumably mediates functions of the entire family. Transgenic rescue of dfmr1 phenotypes by human FMRP, FXR1P or FXR2P is still needed to determine to what extent dFMRP and FMRP are conserved functionally.

FMRP as a translational repressor
Using different experimental systems, several groups independently determined that FMRP acts as a negative regulator of translation [10,43,45–47]. Laggerbauer et al. showed that FMRP, but not FXR1P or FXR2P, suppresses translation of various mRNAs including a reporter luciferase mRNA, the survival of motor neuron (SMN) mRNA, and its own message in cell-free rabbit reticulocyte lysate (RRL) and also in Xenopus laevis oocytes [43]. FMRP reportedly interferes with the assembly of 80S ribosomal complexes, suggesting a possible mechanism in which FMRP regulates translation at the initiation level [43]. Similarly, Li et al. showed that FMRP inhibits translation in the RRL system of target mRNAs such as myelin basic protein mRNA, although via a direct RNA-binding mechanism [46]. More recent work has demonstrated that FMRP suppresses translation of reporter genes in co-transfected STEK cells [47].

The first evidence that FMRP acts as a translation repressor in vivo came from work in Drosophila [17]. dFMRP associates with its own message in addition to futsch mRNA, which encodes the Drosophila homolog of microtubule-associated protein 1B (MAP1B) [48]. The level of Futsch protein in the nervous system is increased (approximately twofold) in dfmr1 null mutants [17]. Conversely, the level of Futsch and MAP1B is significantly decreased if dfmr1 is overexpressed in neurons [17]. Although the regulation mechanism remains unknown, it is possible that the MICRORNA pathway is involved, as the 3′ untranslated region (UTR) of futsch contains a K box and a GY box, which are complementary to miRNAs [49] (also see below). It was subsequently shown that mammalian FMRP also acts as a translational suppressor of MAP1B, in addition to α-Ca^{2+}/calmodulin-dependent protein kinase II (α-CaMKII) and activity-regulated cytoskeleton-associated protein (Arc) [10]. FMRP association with MAP1B, α-CaMKII and Arc messages is mediated via a noncoding adaptor RNA BC1 [50], through direct base pairing, thereby providing a specificity mechanism for the FMRP–mRNA interaction [10]. Although downregulation or subcellular mislocalization of several other proteins were reported in knockout mice [9,32], these data demonstrate that both mammalian and Drosophila FMRPs can act as translational repressors, on an overlapping subset of target mRNAs.

FMRP in the microRNA and RNAi pathway
New evidence from Drosophila suggests that dFMRP acts as part of a microRNA-mediated silencing mechanism. The dFMRP-associated mRNP complex in Drosophila S2 cells contains Argonaute2 (AGO2) and Dicer (a double-stranded specific RNase III); both are essential parts of the

RNA INTERFERENCE (RNAi) and microRNA (miRNA) pathways [31,51]. Also in *Drosophila* S2 cells, dFMRP is required for efficient RNAi of a reporter gene [51]. Moreover, dFMRP and AGO2 interact to downregulate the level of *pickpocket1* mRNA, a sensory neuron subtype-specific sodium channel involved in larval locomotion, confirming a potential role of dFMRP in the RNAi pathway for at least a subset of mRNAs [52].

Jin *et al.* extended these studies to mammals, showing that FMRP also associates with miRNAs and components of the miRNA pathways including Dicer and a putative translation initiation cofactor eIF2C2, the mammalian ortholog of AGO1 [53]. Moreover, in *Drosophila*, AGO1 is crucial for dFMRP function in the nervous system [53]. Although RNAi and miRNA pathways share crucial common components, including Dicer and RNA-induced silencing complex (RISC), their biological consequences are different; RNAi leads to transcript cleavage, whereas miRNA leads to translation repression. It was proposed that FMRP suppresses translation specifically via the miRNA pathway; this was based on (i) the absence of apparent abnormal mRNA degradation in FraX patients and *fmr1* knockout mouse brain [8,32], and (ii) the FMRP complex association with miRNA-specific components such as AGO1. It remains to be determined which mRNA targets are regulated via the miRNA pathway, as opposed to translational repression via the adaptor RNA BC1, direct binding of the G quartet or both.

Loss of FMRP: behavioral consequences

In addition to cognitive defects, FraX patients display a range of behavioral symptoms including short attention span (100%), hyperactivity (90%), hypersensitivity to sensory stimuli (80%) and impaired motor coordination with gross motor clumsiness and poor fine motor skills [7,54]. Seizures occur in 15–25% of patients, although seizure frequency decreases with age. Similarly, the mouse knockout model most reliably displays hyperactivity, with one of the most robust phenotypes being seizures in response to audiogenic stimuli [7,12,15]. Other reproducible behavioral impairments include defects in motor functions and sensory integration [12]. By contrast, cognitive and learning phenotypes in the mouse *fmr1* knockout are mild. In particular, most studies reveal no defects in visuospatial learning performance dependent on hippocampal function, and the defects that have been reported are highly sensitive to genetic background [12,55].

Like human patients and mice mutants, *Drosophila fmr1* null mutants are viable, anatomically normal and display a wide repertoire of apparently normal behaviors. However, *dfmr1* mutants show significant locomotory defects including aberrant larval crawling [52] and impaired adult flight [17] (Table 2). More complex behaviors manifest stronger deficits, including an aborted courtship ritual [18] and disrupted circadian rhythm activities including eclosion, the synchronous emergence near dawn of an adult fly from the pupal case [18–20]. A severe *dfmr1* hypomorphic mutation ($\approx 5\%$ wild-type protein levels) [19,56] displays locomotor but not eclosion rhythm defects [19], indicating that the phenotypes are genetically separable. These rhythmic defects could be due either to an alteration of the clock or a perturbation of a downstream process. Crucial clock components *timeless* and *period* appear to oscillate normally [18,20], but the rhythmic cycling of at least one clock-controlled factor, cAMP response element binding (CREB) protein, is compromised in mutants [18]. Thus, the circadian defects appear to be manifest in downstream outputs of the clock, instead of the clock itself. Because the molecular mechanisms involved in the generation of circadian rhythms are highly conserved between *Drosophila* and mammals [18–20], it is reasonable to predict that FMRP has a similar role in mammals. Shortened periods of sleep and longer wakeful episodes have been reported in FraX patients [57]. It would be interesting to know if similar rhythmic defects are present in *fmr1* knockout mice.

It is not known whether *dfmr1* mutants display altered sensory responsiveness or have impaired learning or memory. One complication is that assays for cognitive defects commonly require flies moving to or from conditioning stimuli [58]. Because *dfmr1* mutants have significantly impaired locomotory behaviors (Table 2), it might be necessary to assay learning and memory using strategies to target removal of dFMRP from the mushroom body, the *Drosophila* brain center of learning and memory [59]. The lack of learning and memory assays remains a serious limitation in the development of the *Drosophila* FraX model.

Table 2. Phenotypic comparisons of fragile X patients and model animals[a]

	Human	Mouse	*Drosophila*	Refs
Cognitive	Cognitive deficits	Learning deficits	ND	[7,12]
Behavioral	Reduced motor ability	Reduced motor ability	Reduced motor ability	[12,17,60]
	Hyperactivity	Hyperactivity	Bouts of hyperactivity	[12,18]
	Sleep disorder	ND	Circadian rhythm defects	[8–20,57]
Testes	Macro-orchidism	Macro-orchidism	Macro-orchidism	[5,56]
	Sperm defects	Sperm defects	Sperm defects, sterile	[56]
Neuron structure	ND	Excess dendrites	Excess processes	[11,21,64]
	ND	ND	Excess branching	[18,64]
	ND	ND	Overgrowth	[63,64]
Synapse structure	Excess spine density, aberrant spines	Excess spine density, aberrant spines	Increased synapse number, aberrant synapse structure	[11,17]
Synapse function	ND	LTD increased	NMJ transmission up	[14,17]
		LTP decreased	CNS transmission down	[13,17]

[a]Abbreviations: CNS, central nervous system; LTD, long-term depression in hippocampus; LTP, long-term potentiation in cortex; macro-orchidism, enlarged testicles in postpubertal men; ND, not determined or present; NMJ, neuromuscular junction synapses.

Loss of FMRP: neuronal structural defects

No obvious gross neuronal structural defects are apparent in post-mortem FraX patients or *fmr1* knockout mouse brains [12]. The only morphological defects reported are at a fine-structural, subcellular level: an overabundance of postsynaptic dendritic spines that appear elongated and anatomically abnormal in both human patients and knockout mice (see below) [11,60,61]. This phenotype is exceptionally subtle and has been reported to be developmentally transitory and/or vary in its manifestation in different brain regions [7,62]. Similarly, most reports have observed no gross anatomical defects in *dfmr1* mutant *Drosophila* brains [17,18]. One recent study has reported gross fusion of the two lateral mushroom bodies across the brain midline caused by inappropriate neuronal crossing of the midline [63], but that result was not replicated in our studies [64]. However, as in mammals, *dfmr1* mutants display neuronal architecture defects at cellular and subcellular levels.

The *Drosophila* neuromuscular junction (NMJ) is an exceptionally well-characterized synaptic arbor, the site of a great many mutant analyses [23], and was therefore selected as a starting point to examine cellular phenotypes in *dfmr1* mutants. Null mutants display structurally overgrown NMJs (increased branches, increased number of synaptic boutons and enlarged synaptic area), whereas *dfmr1* overexpression reduces growth at the NMJ (Figure 2 and Table 2) [53,65]. These data indicate that
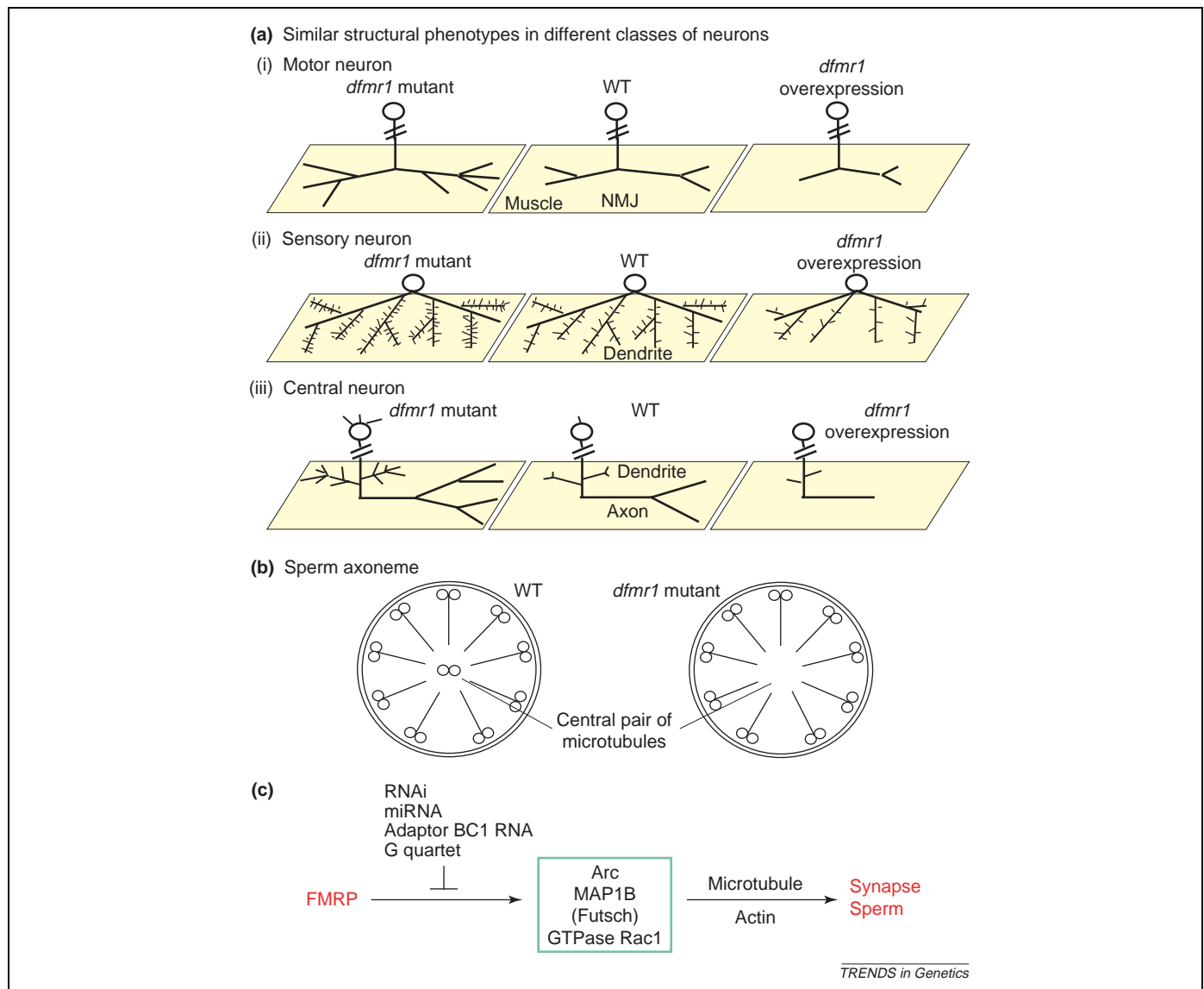

Figure 2. Functions of *Drosophila* fragile X mental retardation protein (dFMRP). **(a)** dFMRP is a global negative regulator of neuronal architectural complexity in motor, sensory and central neurons. (i) In motor neurons, neuromuscular junction (NMJ) synapses are overgrown and overbranched in *dfmr1* null mutants (left), but undergrown when *dfmr1* is overexpressed (right) [17]. (ii) In sensory neurons, terminal dendritic processes are overelaborated in *dfmr1* mutants (left) and simplified when *dfmr1* is overexpressed (right) [21]. (iii) In central interneurons, cells send out more processes, display overelaborated dendritic arbors and overgrown or overbranched axons in *dfmr1* mutants (left), but all processes are underelaborated and undergrown when *dfmr1* is overexpressed (right) [18,63,64]. **(b)** dFMRP is required to maintain stability of sperm axoneme microtubules. The wild-type sperm axoneme has nine pairs of outer ring microtubules plus one central pair of microtubules. The *dfmr1* mutant sperm progressively loses its central pair of microtubules during spermatogenesis, while keeping other structures, including the outer ring microtubules, intact [56]. **(c)** Our model proposes that dFMRP regulates neuronal and sperm development via a common cytoskeleton-based mechanism. dFMRP downregulates the translation of MAP1B (and its *Drosophila* homolog Futsch), Arc [10,17] and probably GTPase Rac1 [21,65] by noncoding RNAs (miRNA and BC1 RNA) and/or direct interaction pathways. This alters microtubule stability thus compromising cellular structure and function.

dFMRP acts as a negative regulator of NMJ synaptic differentiation, consistent with mammalian brain studies. Similar growth phenotypes in the peripheral sensory neurons of *dfmr1* mutants were reported [21]. Null mutants display increased growth and branching of sensory dendrites, especially the terminal dendritic processes (Figure 2a), whereas *dfmr1* overexpression reduces dendritic processes. Thus, in the peripheral nervous system, loss of dFMRP leads to overelaborated synaptic and dendritic processes, whereas excess dFMRP oversimplifies the structures. These data show that dFMRP acts as a negative regulator of neuronal complexity.

Despite the accessibility of these peripheral neurons, the function of dFMRP in the central brain remains a primary interest. Although data arising from published brain studies have been somewhat inconsistent, a common theme consistent with peripheral nervous system (PNS) phenotypes is emerging. Dockendorff *et al.* examined the morphology of lateral neurons, which control circadian rhythms, to reveal more axonal branches and more synaptic arborizations in *dfmr1* mutants [18] (Figure 2a). Similarly, Morales *et al.* independently reported neurite overextension and mistargeting of axons, apparently caused by overgrowth [20]. In the mushroom body learning center, β lobe neurons also show overgrowth to cause midline crossover [63]. More recently, we have completed mosaic analyses in the mushroom body by MARCM (mosaic analyses with a repressible cell marker) [59], which permits detailed structural reconstruction of individual mutant neurons at single cell resolution [64]. This study revealed that dFMRP negatively regulates growth and elaboration of both axons and dendrites in all three classes of mushroom body neurons (Figure 2a and Table 2). Single mutant neurons display excess neurites arising from the cell body, supernumerary branching and expansion of the dendritic arbor, and excess branching and growth of axons. By contrast, dFMRP overexpression reduces process formation, dendritic branching and axonal growth [64]. Taken together, we conclude that dFMRP acts as a negative regulator of neuronal complexity throughout the nervous system, in both PNS and central nervous system (CNS), and in motor, sensory and central brain neurons.

Loss of FMRP: cytoskeleton misregulation during synaptogenesis

Several lines of evidence indicate that FMRP has a specific role at synapses, where neurons make contact and communicate. Although FMRP is predominantly localized in the soma of neurons, FMRP is also locally present at synapses, particularly in the postsynaptic compartment [66,67]. FMRP expression at synapses is increased by heightened activity and elevated neurotransmission, suggesting that FMRP is a synaptic activity-dependent translational regulator [66,67]. Protein synthesis-dependent synaptic plasticity is subtly altered in *fmr1* knockout mice; long-term depression (LTD), triggered by activation of metabotropic glutamate receptors, is enhanced in the hippocampus, whereas long-term potentiation (LTP) is reduced in the cortex (Table 2) [14,15]. Moreover, the longer, thinner and denser dendritic postsynaptic spines in knockout mice [60,62] and FraX patients indicate synaptic maturation and/or pruning defects [61], which might depend on local translational control of the targets of FMRP such as MAP1B, α-CaMKII and Arc, as discussed above.

At the *Drosophila* NMJ synapse, the level of dFMRP bidirectionally controls both synaptic growth and neurotransmission strength (Table 2 and Figure 2) [17]. What is the mechanism of this prominent synaptic role? As discussed above, dFMRP downregulates Futsch expression [17]. Direct genetic manipulations of *futsch* result in synaptic structural and functional phenotypes that inversely correlate with *dfmr1* phenotypes [17]. Given the long list of putative FMRP targets, it was a surprise that the *dfmr1*; *futsch* double mutants, selected to restore Futsch levels in an otherwise *dfmr1* null mutant, completely rescue both structural and functional defects in the NMJ and eye [17]. These results indicate that Futsch is an important physiological target of dFMRP regulation, and dFMRP controls synaptic development and function via Futsch-dependent microtubule regulation (Figure 2). Subsequent work has implicated dFMRP in regulation of the small GTPase Rac1 signaling pathway to remodel actin-based cytoskeleton during neuronal morphogenesis [65,68]. These *Drosophila* results are consistent with the most recent findings in mammals that MAP1B and Arc are upregulated in *fmr1* knockout mouse brain and more so in synaptoneurosomes [10]. In single-mutant *Drosophila* neurons, the absence of dFMRP impacts on synaptic differentiation, with obvious alteration in the abundance and distribution of synaptic vesicle pools [64]. Taken together, it is enticing to propose that misregulation of the actin-microtubule cytoskeleton underlies both neuronal and synaptic architectural abnormalities, and is a primary defect in FraX patients (Figure 2c).

Loss of FMRP: microtubule cytoskeleton misregulation during spermatogenesis

In addition to mental retardation, the hallmark symptom of FraX patients is macro-orchidism (i.e. enlarged testicles), which is also apparent in *fmr1* knockout mice (Table 2) [5]. FMRP, FXR1P and FXR2P are all enriched in mammalian testes, in various stages of spermatogenesis, suggesting that all three family members could be involved in testes development and/or spermatogenesis [69,70], although *fmr1* knockout mice maintain normal fecundity [5]. Similarly, dFMRP is also particularly enriched in *Drosophila* testes and developing spermatocytes [56]. It is therefore probable that FMRP and dFMRP play a similar role in spermatogenesis, a highly conserved process at molecular and cellular levels across species [71].

Quantitative analyses show that male *dfmr1* mutants are nearly sterile (<10% of wild-type fecundity) [56]. Late-stage spermatogenesis is specifically aberrant in *dfmr1* mutants. Fully elongated spermatid bundles develop, but no further advanced coiled spermatids appear at the base of mutant testes (Table 2) [56]. There is a highly specific disruption of sperm tail cytoskeleton

structure: mutants lose the central paired microtubules of the axoneme, although the outer ring microtubules remain intact (Figure 2b). The frequency of central pair microtubule loss becomes progressively greater as spermatogenesis progresses, demonstrating that dFMRP regulates microtubule stability [56]. A similar mechanism might operate in mammals, as evidenced by: (i) late stage of spermatogenesis of *fmr1* knockout mice is abnormal [56]; (ii) FXR1P is associated with sperm tail microtubules [70]; and (iii) double knockout mice of *fmr1* and *fxr2* are sterile (D. Nelson *et al.*, unpublished). To uncover dFMRP partners in spermatogenesis, a proteomics approach has identified several likely candidates, including heat-shock proteins and protein-folding chaperones [47]. The *in vivo* interaction between the candidates and dFMRP remains to be ascertained. Taken together with the above nervous system studies, these data suggest a common model in which FMRP regulates microtubule cytoskeleton stability in both spermatogenesis in testes and synaptogenesis in the nervous system (Figure 2c).

Outstanding questions and concluding remarks

Does FMRP act as a general regulator of neuronal architecture?

In *Drosophila*, FMRP acts as a regulator of cytoskeleton stability, and loss of dFMRP function in neurons results in inappropriate sprouting, branching and growth, causing gross changes in both axon and dendrite projections in motor, sensory and central neurons. It is not clear whether these phenotypes are more severe in flies than mammals, or if comparable assays have simply not been done. Greenough *et al.* reported excess dendritic processes in the *fmr1* knockout mouse [11], supporting the *Drosophila* results, but we find no other reports on gross neuronal structure in the mammalian literature. The commonly reported structural phenotype in mice, excessive abnormal dendritic spines, also supports the conclusion that FMRP acts as a negative regulator of neuronal complexity and synaptic differentiation. The question of whether this role is global, as shown in flies, or local to postsynaptic spines, as suggested in mammals, needs to be resolved. The significance of these different levels of neuronal architectural misregulation to the disease needs to be determined.

Does FMRP act as a local or global translational regulator?

In *Drosophila*, there is as yet no indication that FMRP is required locally at the synapse, whereas the mammalian work is focusing almost exclusively on local translation control in postsynaptic dendrites. In both systems, it is clear that FMRP resides almost entirely in the neuronal soma, with only a tiny fraction of the protein at synapses. Moreover, in both systems, the verified mRNA targets are globally present in neurons and either not restricted (Arc and α-CaMKII), or not present (MAP1B and Futsch), in postsynaptic spines. Moreover, many of the putative mRNA targets encode presynaptic rather than postsynaptic proteins, including MUNC-13, NAP-22, SEC-7 and RAB-6 [44]. The question of whether FMRP has a global role in translation regulation, as suggested in flies, or a local role in postsynaptic spines, as suggested in mammals, needs to be resolved. The significance of these different levels of translation misregulation to the disease needs to be determined.

Is FMRP required during a specific developmental window?

In mammals, FMRP is expressed abundantly during development, and numerous studies suggest that dendritic spine phenotypes could be transitory and/or reflect a defect in developmental pruning of excess processes. It is unclear whether there is a constitutive requirement for FMRP. This question has yet to be addressed in *Drosophila*, however, transgenic technologies are available to provide definitive answers. Conditional mutants need to be generated in which normal FMRP expression is maintained during development but later eliminated at maturity or, conversely, no FMRP is present during development with induced expression at maturity. These experiments will enable us to determine whether FMRP is required only during a specific developmental window, or whether introduction of FMRP at later stages can correct developmental defects. This information is crucial for designing therapeutic strategies.

Why the fly?

In the past three years, the *Drosophila* FraX model has provided novel insights into FMRP function and the molecular pathogenesis of FraX. Empowered with sophisticated genetic tools and experimental assays, *Drosophila* is well suited to reveal physiologically important partners of FMRP *in vivo*. Recent *Drosophila* modeling of other neurological diseases has revealed key molecular pathways and important genetic modifiers [72]. We believe that forward genetic screens for suppressors and enhancers of *dfmr1* based on (i) loss-of-function neuronal defects and sterility, and (ii) overexpression cell death and lethality will define the mechanisms of FMRP function and open new research avenues. Furthermore, as promising FMRP partners are identified in mammals, *Drosophila* is an excellent system to test hypothesized interactions *in vivo*. Looking ahead, the *Drosophila* model, combined with the mouse model, will continue to unfold the secrets of FMRP and its associated FraX, and should identify molecular targets or pathways for medical prevention or intervention in FraX in the near future.

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