Fragile X mental retardation protein has a unique, evolutionarily conserved neuronal function not shared with FXR1P or FXR2P

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SUMMARY
Fragile X syndrome (FXS), resulting solely from the loss of function of the human fragile X mental retardation 1 (hFMR1) gene, is the most common heritable cause of mental retardation and autism disorders, with syndromic defects also in non-neuronal tissues. In addition, the human genome encodes two closely related hFMR1 paralogs: hFXR1 and hFXR2. The Drosophila genome, by contrast, encodes a single dFMR1 gene with close sequence homology to all three human genes. Drosophila that lack the dFMR1 gene (dfmr1 null mutants) recapitulate FXS-associated molecular, cellular and behavioral phenotypes, suggesting that FMR1 function has been conserved, albeit with specific functions possibly sub-served by the expanded human gene family. To test evolutionary conservation, we used tissue-targeted transgenic expression of all three human genes in the Drosophila disease model to investigate function at (1) molecular, (2) neuronal and (3) non-neuronal levels. In neurons, dfmr1 null mutants exhibit elevated protein levels that alter the central brain and neuromuscular junction (NMJ) synaptic architecture, including an increase in synapse area, branching and bouton numbers. Importantly, hFMR1 can, comparably to dFMR1, fully rescue both the molecular and cellular defects in neurons, whereas hFXR1 and hFXR2 provide absolutely no rescue. For non-neuronal requirements, we assayed male fecundity and testes function. dfmr1 null mutants are effectively sterile owing to disruption of the 9+2 microtubule organization in the sperm tail. Importantly, all three human genes fully and equally rescue mutant fecundity and spermatogenesis defects. These results indicate that FMR1 gene function is evolutionarily conserved in neural mechanisms and cannot be compensated by either FXR1 or FXR2, but that all three proteins can substitute for each other in non-neuronal requirements. We conclude that FMR1 has a neural-specific function that is distinct from its paralogs, and that the unique FMR1 function is responsible for regulating neuronal protein expression and synaptic connectivity.

INTRODUCTION
Fragile X syndrome (FXS) is the most common cause of inherited mental retardation and the leading known genetic cause of autism (Clifford et al., 2007; Cohen et al., 2005; Fisch et al., 2002; Hagerman et al., 2005; Rogers et al., 2001). The X chromosome-linked disorder is caused by loss of function of a single gene, fragile X mental retardation 1 (FMR1), most frequently by expansion of CGG repeats (>200 repeats) in the 5’ regulatory region causing hypermethylation that results in transcriptional silencing (Heitz et al., 1992; Oberle et al., 1991; Pieretti et al., 1991). In addition to mental impairment, FXS patients also display a wide range of social interaction problems characterized by poor eye contact, hyperactivity, attention deficit and obsessive-compulsive behaviors (Boccia and Roberts, 2000; Cornish et al., 2001; Fryns et al., 1984; Torrioli et al., 2008), and hypersensitivity to sensory stimuli (Fryns, 1984; Hessl et al., 2001). Other physical anomalies include elongated face, prominent ears and enlarged male testes (Chudley and Hagerman, 1987; Giangreco et al., 1996; Moore et al., 1982). These non-neurological symptoms testify that the FMR1 gene performs important functions in non-neuronal tissues. Indeed, the FMR1 product (FMRP) is expressed ubiquitously, albeit with elevated expression in the brain and testes (Agulhon et al., 1999; Devys et al., 1993). Although FXS is a monogenic disease, the wide range of clinical symptoms strongly indicates that FMRP is involved in the regulation of multiple modulatory factors.

FXS has been investigated extensively in both vertebrate and invertebrate genetic model systems (Bassell and Warren, 2008; Gatto and Broadie, 2009b). In all systems, FMRP has five well-defined functional domains: two RNA-binding KH domains in the middle region (KH1, KH2; KH=heterogeneous nuclear ribonucleoprotein K homology) (Siomi et al., 1993), an RNA-binding RGG box in the C-terminal region (containing repeats of an Arg-Gly-Gly motif) (Darnell et al., 1999), a non-classical nuclear localization signal (NLS) and a nuclear export signal (NES) (Eberhart et al., 1996; Zhang and Broadie, 2005). Consistent with its ability to bind to RNA, FMRP regulates transcript trafficking and functions as a negative regulator of translation (Dictenberg et al., 2008; Estes et al., 2008; Laggerbauer et al., 2001; Mazroui et al., 2002). In vertebrates, FMRP is part of a three-member family that includes two other similar proteins: fragile X-related protein 1 (FXR1P) and 2 (FXR2P). The autosomally encoded paralogs are expressed in a very similar tissue and cellular profile to FMRP, including the subcellular distribution in neurons, with only slight differences (Agulhon et al., 1999; Bakker et al., 2000). For example, FXR1P is expressed more abundantly in cardiac and skeletal muscle compared with FMRP and FXR2P (Bakker et al., 2000; Mientjes et al., 2004). Moreover, all three proteins show ultrastructurally overlapping expression, can be co-immunoprecipitated and can associate with the same protein partners (Bakker et al., 2000; Ceman et al., 1999; Christie et al., 2009; Schenck et al., 2001; Zhang et al., 1995). Both hetero- and homo-dimerization of the
FMRP/FXR1P/FXR2P family has been proposed to occur (Ceman et al., 1999; Christie et al., 2009; Tamanini et al., 1999b; Zhang et al., 1995).

Only the loss of FMR1 causes FXS, and loss of FXR1 or FXR2 has not been linked to any disease state. However, the mouse Fxr1 knockout is lethal shortly after birth owing to defects in cardiac and skeletal muscle development (Mientjes et al., 2004), whereas both Fmr1 and Fxr2 knockouts, as well as double knockouts, are adult viable. At least some FXS-like phenotypes are exhibited in Fxr2 knockout mice (Bontekoe et al., 2002), whereas Fmr1 knockouts recapitulate many FXS symptoms including learning defects, hyperactivity, sensory hypersensitivity, social deficits and macroorchidism (Chen and Toth, 2001; Dobkin et al., 2000; McNaughton et al., 2008; Slegtenhorst-Egedeman et al., 1998). At a cellular level, Fmr1 knockouts exhibit elevated levels of brain protein synthesis (Qin et al., 2005) and an accumulation of developmentally arrested postsynaptic spines (Comery et al., 1997; Nimchinsky et al., 2001). Interestingly, the double knockout of Fmr1 and Fxr2 results in augmented defects, including exaggerated behavioral phenotypes in open-field activity, prepulse inhibition of the acoustic startle response, contextual fear conditioning and circadian arrhythmicity (Spencer et al., 2006; Zhang et al., 2008), and worsened cellular phenotypes, including further enhanced long-term depression (LTD) (Zhang et al., 2009). These data predict that the paralogs have overlapping functions and/or compensate for each other. However, the expression levels of Fxr1/2 are unaltered in Fmr1 null mice and, similarly, levels of FMRP and FXR1P are unaltered in Fxr2 null mice (Bakker et al., 2000; Bontekoe et al., 2002). Recent work has shown that kissing-complex RNA (kRNA) interference with the KH2 domain is able to displace FXR1P and FXR2P from polyribosomes as it does for FMRP (Darnell et al., 2009); however, FMRP has a unique ability to recognize G-quadruplexes, suggesting that the FMRP RGG box domain function may not be duplicated in the two paralogs. Thus, despite co-expression, co-molecular complex formation and phenotypic interactions between these three gene family members, evidence of their distinctive versus overlapping roles remains elusive.

The single Drosophila FMR1 gene (dFMR1) product (dFMRP) is highly homologous to all three human family members: 35% identity/56% similarity compared with hFMRP, 37% identity/65% similarity compared with hFXR1P and 36% identity/65% similarity compared with hFXR2P. The N-terminal region has a higher homology (dFMRP:hFMRP 50% identity, 84% similarity), with the C-terminal being relatively divergent (Zhang et al., 2001). Importantly, dFMRP displays a highly conserved structure in all defined functional domains: KH1, 68% identity; KH2, 67% identity; RGG box, 62% identity; NLS, 48% identity; NES, 65% identity (compared with hFMRP). With the exception of FMR1 exons 11/12, for which there are no corresponding FXR1/2 sequences, the sizes of exons 1-10 and 13 are nearly identical in FMR1, FXR1 and FXR2 (Kirkpatrick et al., 2001). Non-mammalian FMR1 orthologs similarly lack exons 11 and 12. These comparisons imply that the mammalian gene family probably arose by duplication from a common ancestor similar to the dFMR1 gene. Consistently, dFMRP displays conserved RNA-binding domains; tissue and subcellular expression patterns; and functional roles in mRNA trafficking and negative translational regulation (Banerjee et al., 2007; Epstein et al., 2009; Estes et al., 2008; Reeve et al., 2005; Zhang et al., 2001; Zhang et al., 2005). Moreover, Drosophila that lack the dFMR1 gene (dfmr1 knockouts) closely recapitulate FXS symptoms in a wide range of molecular, cellular and behavioral phenotypes (Bolduc et al., 2008; Dockendorff et al., 2002; Gatto and Brodie, 2009a; McBride et al., 2005; Pan et al., 2004). These striking similarities between Drosophila and mammalian FMRP suggest a well-conserved function, but beg the question of why mammals have an expanded three-member protein family.

In this study, we investigate the functional conservation of the entire fragile X gene family by expressing each of the three human genes in the Drosophila FXS model with tissue-specific drivers. A wide-ranging series of phenotypic tests at the molecular, cellular and ultrastructural levels were selected to survey function in the nervous system and non-neuronal tissue. A wild-type dFMR1 transgene was used as the positive control and each human gene was investigated in two independent transgenic lines, all with targeted expression driven in either neurons or germ cells within the dfmr1 null mutant background. The results show that FMR1 has an evolutionarily conserved function in the Drosophila central and peripheral nervous system that is not possessed by either FXR1 or FXR2. When all three human genes are targeted to Drosophila neurons, only human FMR1 is able to restore brain protein levels in the dfmr1 null mutant, and it is just as effective as the native dFMR1. Similarly, only human FMR1 is able to restore normal synaptic architecture in dfmr1 null neurons. FXR1 and FXR2 completely lack this ability to compensate. By contrast, all three human genes are equally competent at replacing dFMR1 function in non-neuronal tissue. When each gene is targeted to the testes, they all fully restore male fecundity and rescue testes spermatid axoneme defects. These results indicate a unique, evolutionarily conserved role for FMR1 in neuronal mechanisms and a broader, shared role for FMR1, FXR1 and FXR2 in non-neuronal tissue.

RESULTS
Transgenic constructs with targeted pan-neuronal expression
Humans have a three-member gene family composed of the highly similar hFMR1, hFXR1 and hFXR2 genes (Zhang et al., 1995). The three gene products associate with ribosomes in large complexes that are thought to cooperatively mediate transport of neural mRNAs to specific intracellular locations and inhibit their translation until signaled (Ceman et al., 1999; Christie et al., 2009; Dictenberg et al., 2008; Khandjian et al., 2004; Siomi et al., 1996; Zhang et al., 1995). In Drosophila, the single FMR1 gene (dFMR1) probably represents an ortholog of the common ancestor of hFMR1 and its two paralogs. This speculation suggests that the functions of the three-member gene family may subdivide the roles of dFMR1, in addition to any newly evolved functions that each gene may serve. The dFMR1 gene has similar sequence homology to all three human genes, so it is not clear which of the human genes, if any, may be the true homolog. To address these questions, we engineered transgenic human cDNA constructs for hFMR1, hFXR1 and hFXR2, as well as wild-type dFMR1 as a positive control, and expressed each with tissue-specific drivers in the Drosophila FXS model. The generation and testing of these transgenic tools is illustrated in Fig. 1.

The cDNA constructs that were engineered for dFMR1 and each human family gene member were sub-cloned downstream of the
Evolutionary conservation of the human FMR gene family

upstream activating sequence (UAS) promoter sequence (5X UAS) (Fig. 1A). A MYC epitope tag was added at the amino terminus of each transgene to track protein expression. The tagged transgenes could then be targeted to specific tissues using the pUAST/GAL4 expression system. Each construct was microinjected into w1118 genetic background control embryos (Fig. 1B). Multiple stably integrated genomic lines for each transgene were isolated and self-perpetuating stocks were generated. Third chromosome transformants were recombined into the dfmr1 null (dfmr150M) background and a stock was produced with TM6-GFP serving to balance the recombined UAS transgene chromosome (Fig. 1B). In order to assay the rescue of neuronal phenotypes, all transgenic lines were crossed with a stock line that was homozygous for the father-GAL4 driver alone (negative control) and UAS-dfmr1 positive control were discarded, and two independent insertion lines with comparable expression for each transgene were selected for detailed analyses. Western blot analyses of brain protein extracts show comparable levels of MYC epitope tag expression across all of the selected transgenic genotypes (Fig. 1C,D). Endogenous dFMRP expression is ubiquitous within neurons and relatively uniform between neurons throughout the wild-type Drosophila brain (Fig. 2A). We therefore selected elav-GAL4 as the best described pan-neuronal driver to mimic this expression (Gatto and Broadie, 2009a).

Transgenic lines with high and low elav-GAL4 driven expression compared with the UAS-dfmr1 positive control were discarded, and two independent insertion lines with comparable expression for each transgene were selected for detailed analyses. Western blot analyses of brain protein extracts show comparable levels of MYC epitope tag expression across all of the selected transgenic genotypes (Fig. 1C). The protein sizes of each transgene are roughly equivalent, albeit with the dfmr1 product being slightly larger; and the hFMR1 product being slightly smaller, than the other transgenes. We were careful to select lines that did not overexpress the transgenes relative to endogenous dfmr1 (supplementary material Fig. S1). We confirmed that elav-driven transgene expression is widespread throughout neurons (Fig. 1D). Anti-MYC labeling of brains from all four transgenic lines showed comparable transgene expression levels and distribution across genotypes. Importantly, the UAS-dfmr1 positive control was indistinguishable from the three human transgenes in brain expression profile (Fig. 1D). These lines were therefore selected to systematically test their ability to rescue a wide range of dfmr1 null mutant phenotypes.

Fig. 1. Generation of transgenic constructs with targeted neuronal expression. (A) The four UAS transgenic constructs generated and tested in this study. The positive control is wild-type dfmr1, and the three human genes are hFMR1, hFXR1 and hFXR2. All cDNA transgenic constructs are tagged with a MYC epitope in the pUAST (5X UAS) expression vector to follow protein expression. In all assays, two independent transgenic lines for each human transgenic construct were analyzed. (B) The embryonic transformation and genetic crossing scheme that was used to introduce each stably integrated UAS transgene into the dfmr1 null mutant background and then drive expression with the pan-neuronal GAL4 driver elav-GAL4. (C) Western blot analyses of transgenic protein expression for the dfmr1 line (control) and two independent lines of hFMR1, hFXR1 and hFXR2 (denoted as a/b). Expression from brain extracts (1–2 day-old adults) was tested with an anti-MYC antibody against the epitope tag common to all four transgenes (see A). Lines were selected for comparable transgene expression. The loading control is α-tubulin. (D) Brain immunohistochemistry for transgene expression of the dfmr1 line (control) and the three human lines (hFMR1, hFXR1 and hFXR2). Drosophila adult brains (1–2 days old) were probed with anti-MYC to detect the transgene epitope tag. Comparable transgene expression occurs in all conditions. Bar, 100 μm.
post-eclosion [0-7 hours after eclosion (AE)] (Fig. 2B). Protein levels per animal were 16.6±0.45 μg in wild-type controls compared with 20.2±0.47 μg in the null mutant (P<0.01, n=8). The positive transgenic control, elav-GAL4-driven UAS-dFMR1 in the null mutant background, displayed brain protein levels of 16.4±0.61 μg, which was 23% lower than the dfmr1 null mutant, and showed complete rescue to wild-type control levels (P<0.01, n=8) (Fig. 2B). Two independent transgenic lines of all three human genes were assayed for brain protein levels. Both UAS-hFXR1 lines (light and dark bars in Fig. 2B) showed exactly the same effect of lowering brain protein levels by 28% compared with the dfmr1 null mutant, restoring proteins to levels that were indistinguishable from the wild-type control (15.83±0.56 μg, 15.8±0.4 μg; n=8, P<0.001). By contrast, elav-GAL4-driven neuronal expression of the two human paralogs, UAS-hFXR1 and UAS-hFXR2, maintained brain proteins at levels that were comparable to dfmr1 null mutants, with no indication of rescue. For UAS-hFXR1, the two independent lines showed levels of 21.2±0.43 μg and 21.1±0.63 μg (Fig. 2B). For UAS-hFXR2, the protein levels were 19.34±0.61 μg and 20.1±0.66 μg. There was no significant difference between any of these four lines relative to each other or compared with the dfmr1 null condition. All four hFXR1 and hFXR2 transgenic lines maintained highly significantly (P<0.001) elevated brain protein levels compared with the wild-type controls.

These results demonstrate that only hFMR1, and not its paralogs hFXR1 and hFXR2, can rescue the hallmark elevation of brain protein levels in the dfmr1 null mutant back to the control state. hFMR1 was just as effective as dFMR1 in restoring normal brain protein levels, indicating a completely conserved function in this fundamental role. The fact that hFXR1 and hFXR2 lack this function may be predicted by the fact that these proteins have not been shown to act as negative translational regulators (Laggerbauer et al., 2001).

Only FMR1 restores brain circuit synaptic architecture

At a cellular level, the hallmark defect in FXS patients and disease models is inappropriate synaptic connectivity (Braun and Segal, 2000; Bureau et al., 2008; Comery et al., 1997; Gatto and Broaddie, 2008; Hanson and Madison, 2007; Tessler and Broaddie, 2008). In both mouse and Drosophila models, synaptic architecture also appears immature or developmentally arrested. We first examined synaptic connectivity in the central brain, based on well-established dfmr1 phenotypes. dfmr1 null mutants exhibit strikingly abnormal circadian rhythm patterns, with a complete loss of rhythmicity in the absence of environmental entrainment (Bushey et al., 2009; Dockendorff et al., 2002; Inoue et al., 2002; Sofola et al., 2008). Although Fmr1 knockout mice show only mild impairments, the Fmr1/Exr2 double knockout is likewise entirely arrhythmic (Zhang et al., 2008). In Drosophila, circadian activity is controlled by well-defined clock circuitry, in which the small ventrolateral (sLNv) neurons are over-elaborated and extend beyond their normal processes are over-elaborated and extend beyond their normal
Evolutionary conservation of the human FMR gene family

In the Drosophila FXS model, the glutamatergic neuromuscular junction (NMJ) synapse is extremely well characterized (Gatto and Broadie, 2008; Pan et al., 2008; Zhang et al., 2001). The size and accessibility of this synaptic arbors, particularly suited to a systematic investigation. dfmr1 null mutants, wild-type controls, and both are very significantly elevated compared with the wild-type control (45.1±1.3; n≥10; P<0.001). Thus, both Drosophila and human FMR1 similarly and completely rescue the synaptic defect in the clock neurons, whereas neither human paralog exerts any detectable compensatory function.

Fig. 3. Only hFMR1 rescues clock neuron synapse arbors in dfmr1 null mutants. (A) Representative images of small ventrolateral (sLNv) clock neurons in the adult brain labeled with anti-PDF. The low-magnification image on the left shows the bilaterally symmetrical sLNv projections into the protocerebrum (Fig. 3A). At the point of axonal defasciculation, the processes split into a localized array of small synaptic projections at the dorsal horn and into the protocerebrum. These projections are bilaterally symmetrical on the two sides of the brain (Fig. 3A; middle and right panels). dfmr1 null animals exhibit a highly significant (n≥10; P<0.001) increase in the number of PDF boutons compared with controls (Fig. 3B). Wild-type terminals contain a mean of 45.1±1.3 boutons compared with 67.6±2.0 in the dfmr1 null mutants. Thus, the mutant condition shows a ~50% increase in PDF-positive synaptic boutons (Fig. 3C).

Neuronal expression of wild-type dFMR1 completely rescues the synaptic overgrowth characterizing the null mutant, and the terminals become clearly more restricted in extent and refined in the number of synaptic boutons (Fig. 3B). The expression of wild-type dFMR1 results in the differentiation of 45.1±1.3 boutons, a number that is indistinguishable from wild-type controls and that is rescued significantly (n=10; P<0.001) when compared with the dfmr1 null condition (Fig. 3C).

Next, each human transgene was expressed in turn to evaluate rescue of the sLNv synaptic arbors in dfmr1 null mutants. First, the expression of hFMR1 was assayed in neurons and showed complete rescue of the overgrowth defect (Fig. 3B). Targeted hFXR1 resulted in 42.6±1.2 PDF-positive boutons in the sLNv arbor, a rescue as complete as the native Drosophila gene (Fig. 3C). Next, the two paralogs were assayed in turn. At a qualitative level, sLNv synaptic terminals appeared to be as overgrown with hFXR1 or hFXR2 expression as in the purely null mutant state (Fig. 3B). Indeed, quantification of the number of PDF boutons failed to show any significant rescue by either of these transgenes. hFXR1 expression resulted in 67.0±2.6 boutons and hFXR2 expression similarly resulted in 68.5±1.2 boutons (Fig. 3C). Neither value is significantly different from the dfmr1 null mutant (67.6±2.0), and both are very significantly elevated compared with the wild-type control (45.1±1.3; n≥10; P<0.001). Thus, both Drosophila and human FMR1 similarly and completely rescue the synaptic defect in the clock neurons, whereas neither human paralog exerts any detectable compensatory function.

Only FMR1 restores neuromuscular junction synaptic architecture

In the Drosophila FXS model, the glutamatergic neuromuscular junction (NMJ) synapse is extremely well characterized (Gatto and Broadie, 2008; Pan et al., 2008; Zhang et al., 2001). The size and accessibility of this synaptic arbors, combined with the wealth of synaptic markers and structural information, make this terminal particularly suited to a systematic investigation. dfmr1 null mutants display defects on many levels of NMJ synaptic architecture, including grossly elevated synaptic area, increased synaptic branching, and the formation of supernumerary synaptic boutons (Gatto and Broadie, 2008; Pan et al., 2008; Zhang et al., 2001). Most strikingly, developmentally arrested mini (or satellite) boutons accumulate in the absence of dFMRP function (Gatto and Broadie, 2008), which probably represents an early stage of normal bouton maturation (Beumer et al., 1999; Gorczyca et al., 2007; Ruiz-Canada et al., 2004). To compare synaptic development, we co-labeled junctions with presynaptic [horseradish peroxidase (HRP)] membrane marker and postsynaptic [DLG (Discs Large)] scaffold marker antibody probes to quantify all of these features in dfmr1 null mutants, wild-type controls, elav-GAL4-driven (presynaptic) UAS-dFMR1 positive controls, and all three UAS human transgenes in the dfmr1 null mutant background.

Labeling for HRP delineates the innervating presynaptic neuron (red) and anti-DLG reveals the postsynaptic domain (green) of the target muscle (Fig. 4A). The elav-GAL4-driven UAS-dFMR1 positive transgenic control fully rescued both the enlarged junctional area and increased synaptic branching that characterize
the dfmr1 null condition (Fig. 4B,C). To quantify synaptic area, the junction delimited by DLG expression was measured (control, 264±13 μm²; dfmr1 null, 359±11 μm²; n≥10, P<0.001) (Fig. 4B). Presynaptic dFMR1 expression completely restored junctional area to control levels (267±6 μm²; n≥10, P<0.001). To quantify branching, HRP-labeled synaptic projections with more than two boutons were counted (control, 2.2±0.14; dfmr1 null, 3.2±0.1; n≥10, P<0.001) (Fig. 4C). Presynaptic dFMR1 expression completely restored synaptic branching from the elevated mutant level back to wild-type control levels (2.2±0.14 branches; n≥10, P<0.001).

Strikingly, hFMR1 was equally able to completely restore synaptic junctional area and arbor branching to wild-type levels (262±6 μm² area, 2.18±0.16 branches; n≥10, P<0.001) (Fig. 4B,C). By sharp contrast, the two paralogs, hFXR1 and hFXR2, were totally unable to restore synaptic area in the null mutant (hFXR1, 352±5.5 μm²; hFXR2, 353±4.1 μm²; n≥10) (Fig. 4B). Similarly, both hFXR1 and hFXR2 failed to restore normal synaptic branch number in the mutant (3.3±0.17 branches and 3.4±0.28 branches, respectively; n≥10) (Fig. 4C). Thus, only hFMR1 has a conserved function in maintaining gross synaptic architecture, and hFXR1 and hFXR2 completely lack this ability.

dFMRP plays a key role in limiting synaptic bouton number and regulating the normal rate of bouton differentiation. To quantify the number of mature type Ib boutons, HRP/DLG co-labeled varicosities that were >2 μm in minimal diameter were counted within individual synaptic arbors (Fig. 5A). dfmr1 null mutants exhibit a very significantly increased number of synaptic boutons compared with controls (dfmr1 null, 29.1±0.7; control, 19.7±0.5; n≥10, P<0.001) (Fig. 5B). Presynaptic elav-GAL4-driven expression of the UAS-dFMR1 positive control completely rescued bouton number back to control levels (19.8±0.6; n≥10, P<0.001) (Fig. 5B). Strikingly, hFMR1 was equally able to completely rescue synaptic bouton number to the wild-type array (20±0.5 boutons; n≥10, P<0.001) (Fig. 5B). Conversely, the two paralogs, hFXR1 and hFXR2, were totally incapable of restoring the elevated bouton number in the dfmr1 null mutant (27±1.0 and 28±1.2 boutons, respectively; n≥10) (Fig. 5B). A particularly key feature of the null mutant phenotype is the accumulation of small, immature mini-boutons (Fig. 5A; arrows). These boutons were elevated tenfold in the dfmr1 null mutant compared with genetic controls (dfmr1 null, 4.9±0.4; control, 0.46±0.14; n≥10, P<0.001) (Fig. 5C). The positive control dFMR1 was able to strongly rescue the mini-bouton phenotype.
number back to wild-type levels (0.78±0.1; n≥10, P<0.001) (Fig. 5C). With the same efficiency, hFMR1 also restored mini-bouton numbers to control levels (0.94±0.17; n≥10, P<0.001) (Fig. 5C). However, the two paralogs, hFXR1 and hFXR2, were totally incapable of restoring the elevated mini-bouton number, which remained just as elevated as in the dfmr1 null mutant condition (4.5±0.36 mini-boutons and 4.4±0.4 mini-boutons, respectively; no significant difference from dfmr1 null; n≥10) (Fig. 5C). These findings further delineate a specific role for FMR1 in the control of synaptic architecture and bouton maturation that cannot be compensated for by its FXR paralogs.

hFMR1, hFXR1 and hFXR2 all restore male fecundity and spermatogenesis

FXS patients display a range of non-neuronal symptoms, the most prominent of which is impaired testicular development in males (Lachiewicz and Dawson, 1994; Nistal et al., 1992; Turner et al., 1980). Both mouse and Drosophila disease models are similarly characterized by enlarged testes and reduced testicular function (Slegtenhorst-Eegdeman et al., 1998; Zhang et al., 2004). dfmr1 null males exhibit severely reduced fertility owing to immotile sperm (Zhang et al., 2004). We utilized these defects as a sensitive assay for the non-neuronal roles of the three human genes. Each transgene was driven in the male germline with a nos GAL4 driver line (nos-GAL4) (Fig. 6A). Males of each genotype were mated to virgin wild-type females and the number of resulting progeny was assessed. Nine male genotypes were tested: wild-type control, dfmr1 null mutant, and the UAS-dFMR1 positive control and two independent lines each for the three human transgenes in the dfmr1 null mutant background.

We first confirmed that nos-GAL4 drives expression in the testes, as described previously (Fig. 6C) (Schulz et al., 2004). Using the common MYC epitope tag on all four transgenes, similar transgene expression was present in all cases, with the highest expression observed in germline stem cells and lower expression seen throughout the spermatagonia, as expected (Fig. 6C). We then carried out male brooding tests with the wild-type, dfmr1 null and all four transgenic lines. All male genotypes where paired with three w1118 virgin females, allowed to mate for 9 days, and the progeny counted for a further 9 days. Wild-type control males produced an average of 138.5±1.3 progeny under these conditions (n=8 trials) (Fig. 6B). By sharp contrast, the dfmr1 null mutant was completely sterile in these trials, producing no viable progeny. The positive transgenic control of UAS-dFMR1 driven by nos-GAL4 in the null background completely rescued the male fecundity defect (135.3±1.5 progeny, n=8 trials) (Fig. 6B). To our surprise, all three
human genes were similarly capable of completely rescuing mutant male performance to control levels. The hFMR1 gene restored the number of progeny to 136.5±2.0 per male. The two paralogs, hFXR1 and hFXR2, similarly restored the mutant male output to 135.1±1.0 and 139.3±1.1 progeny, respectively (Fig. 6B). Thus, all three human genes fully and equally compensate for the loss of dFMRP in the testes, indicating that they share the conserved function required for male fecundity.

Loss of male fecundity in the Drosophila FXS model is caused by defects in sperm tail microtubule organization, which renders the dfmr1 null sperm immotile (Zhang et al., 2004). In wild-type testes, the spermatid axoneme contains a 9+2 microtubule configuration of nine outer doublets and a single central pair (Fig. 7A). As the axoneme develops, accessory proteins are added to this core microtubule structure, giving the axoneme its characteristic pinwheel cross-section (see control inset in Fig. 7A). In dfmr1 null spermatids, the central pair of microtubules is routinely lost (Fig. 7A), whereas the outer ring microtubule doublets are often deranged (Fig. 7B). The central pair is required for the motility of the sperm tail. This microtubule defect is not caused by misregulation of the microtubule-associated protein 1B (MAP1B) homolog futsch, a key cause of microtubule defects in neurons (Hummel et al., 2000; Zhang et al., 2001), as futsch is not detectably expressed outside of the nervous system. Therefore, this function represents a clearly non-neuronal role for dFMR1 utilizing an independent molecular mechanism.

To assess the function of the human gene family, we compared spermatid ultrastructural differentiation in all three UAS human transgenes in the dfmr1 null mutant background. Wild-type controls exhibited a consistent 9+2 microtubule array in cross-sections of the mature bundled spermatid tails in the testes (Fig. 7B). By contrast, dfmr1 axoneme abnormalities included variably skewed and malformed outer doublets, and the central pair of microtubules were often missing completely or, occasionally, only one microtubule in the central pair was present (Fig. 7B). In wild-type controls, only 7.1±1.2% (n=751) of spermatid axonemes lacked a detectable central pair of microtubules, whereas there was a sevenfold increase, to 49.3±2.5%, in dfmr1 null mutants that were missing the central pair (n=1152, P<0.001) (Fig. 7C). The two paralogs were also fully proficient at rescuing the defect in this setting, with only 2.1±0.7% of hFXR1 spermatid axonemes in the dfmr1 null mutants (n=588, P<0.001) (Fig. 7C). To our surprise, all three genes in the human gene family (hFMR1, hFXR1 and hFXR2) were also able to fully restore axoneme microtubule architecture to levels that were comparable to wild-type controls (Fig. 7A). hFMR1 expression produced sperm axonemes that were indistinguishable from wild type, with only 7.1±1.9% missing the central microtubule pair (n=641, P<0.001) (Fig. 7C). The two paralogs were also fully proficient at rescuing the defect in this setting, with only 2.1±0.7% of hFXR1 spermatid axonemes (n=1043, P<0.001) and 4.5±1.2% of hFXR2 spermatid axonemes (n=1382, P<0.001) missing the central pair (Fig. 7C). In this non-neuronal assay of function, these results clearly demonstrate complete redundancy in the function of all three human genes in successfully restoring both male fecundity and the underlying spermatid differentiation defects of dfmr1 null mutant animals.

DISCUSSION

Fragile X syndrome (FXS) is caused solely by a loss of the FMR1 gene product. However, humans also have two highly similar gene family paralogs, FXR1 and FXR2, whose function remains comparatively unexplored. The three gene products have been identified as part of the same molecular complex in neurons and other cells, but are clearly not functionally redundant in the FXS disease condition (Bakker et al., 2000; Ceman et al., 1999; Schenck et al., 2001; Zhang et al., 1995). Both homo- and hetero-dimerization within the gene family may occur, although homodimerization may be more common in vivo (Tamanini et al., 1999b). FMRP and FXR2P can be found in complexes lacking FXR1P, indicating a possible unique interactive or redundant functional overlap of at least these two proteins (Christie et al., 2009; Tamanini et al., 1999b). The expression of FXR1 and FXR2 appears to remain...
unchanged in FXS patients (Agulhon et al., 1999) and in the mouse FXS model (Bakker et al., 2000), and mutations in FXR1 or FXR2 are not associated with FXS or any other disease condition. Nevertheless, FXR1 is clearly essential because mouse Fxr1 and zebrafish fxr1 knockouts are lethal shortly after birth, owing to cardiac and muscle defects (Mientjes et al., 2004; Van’t Padje et al., 2009), showing that FXR1 has taken on a unique function in vertebrate muscle. This is consistent with the elevated expression of FXR1 in muscles (Mientjes et al., 2004). It is not clear what role FXR1 may play in neurons, where the protein is present at a lower level. By contrast, mouse Fmr1 and Fxr2 single knockouts display clear neuronal phenotypes (Bontekoe et al., 2002; Comery et al., 1997; Hoogeveen et al., 2002), and the Fmr1/Fxr2 double knockout exhibits exaggerated behavioral and neural circuit defects (Spencer et al., 2006; Zhang et al., 2008; Zhang et al., 2009). These findings show that FXR2, at least, plays a role in neurons with functional consequences overlapping with FMR1 requirements. However, it is not at all clear whether this link reveals an interaction between FMR1 and FXR2 in the same mechanism, or partial compensation that is permitted because of functional overlap between these two proteins.

dFMR1 may resemble the common ancestral gene of the vertebrate gene family. dFMR1 shows high sequence homology, domain conservation and functional properties to hFMR1 (Zhang et al., 2001; Zhang et al., 2004), but is just as similar to hFXR1 and hFXR2. To validate and further develop the Drosophila FXS model, it was crucial to determine the evolutionary conservation of dFMR1 relative to the three human genes. For the last decade, the repeatedly posed question has been whether the Drosophila FXS model studies the role of FMR1, FXR1 or FXR2, or some combination of all three. Given that FXS is caused solely by the loss of FMR1, does this gene play a unique function in the nervous system? If so, is the study of dFMR1 a good model for this disease-dependent hFMR1 requirement? To answer these questions, we expressed each of the three human genes independently in the Drosophila FXS model and tested for their functional rescue of a carefully selected, diverse range of null mutant phenotypes. Specifically, we selected the core molecular and cellular phenotypes that were distributed over the widest range of tissues: the brain, neuromusculature and testes. Our findings reveal three important conclusions: (1) hFMR1 replaces all dFMR1 functions, indicating complete functional conservation; (2) FXR1 has a unique function in the Drosophila peripheral and central neurons, as a translational regulator sculpting synaptic connections, which cannot be compensated for by either hFXR1 or hFXR2; and (3) the entire human gene family can fully replace the dFMR1 requirement in the testes, demonstrating a fundamentally different mechanistic requirement in non-neuronal cells.

The hallmark molecular requirement for FMRP is as a negative regulator of translation (Laggerbauer et al., 2001; Schutt et al., 2009; Zalfa et al., 2003; Zhang et al., 2001). FMRP is present in actively translating polyribosomes and inhibits the translation of mRNA targets (Khandjian et al., 2004; Napoli et al., 2008; Reeve et al., 2005; Schutt et al., 2009; Stefani et al., 2004; Yang et al., 2009). In the
absence of dFMRP, total protein levels are elevated in the Drosophila brain, particularly during the late developmental stages of synaptogenesis and during early synaptic refinement in newly eclosed animals (Tessier and Broadie, 2008). This is consistent with the Fmr1 knockout mouse, which also exhibits increased protein synthesis in the brain (Qin et al., 2005). This increase in protein levels is predicted because dFMRP, like mouse FMRP, has been established as a negative regulator of protein translation (Costa et al., 2005; Reeve et al., 2005; Zhang et al., 2001). Using targeted neuronal expression, only dFMR1 and hFMR1 can restore elevated brain protein levels back to the wild-type condition in the Drosophila FXS brain. Human FMRP is just as effective as the native fly protein in limiting brain protein expression. Whereas FMR1 is both necessary and sufficient for this mechanism in neurons, hFMR1 and hFXR2 are completely unable to rescue this phenotype. Therefore, despite the high functional domain conservation, the FXR paralogs are unable to compensate for FMR1 in the mechanism of protein regulation in neurons.

In mammals, FMR1 and FXR2 work synergistically (or redundantly) to regulate circadian rhythmicity, with a dramatic impairment only in the double knockout condition (Zhang et al., 2008). Although each single mutant animal shows a significant shift of circadian periodicity, the double knockouts are completely arrhythmic and fail to entrain to light. This phenotype is nearly identical to the dfmr1 null defect in circadian activity, which has been known for many years (Dockendorff et al., 2002; Inoue et al., 2002). These results suggest that FMR1 and FXR2 may cooperate, or be functionally redundant, within the circadian clock neural circuit in a mechanism that is shared between mammals and flies. In Drosophila, the central brain clock circuit is particularly well characterized (Chang, 2006; Helfrich-Forster, 2005; Nitabach and Taghert, 2008). Much attention has focused on the small ventrolateral clock neurons, which secrete the neuropeptide PDF and are sufficient for maintaining circadian rhythms (Grima et al., 2004; Renn et al., 1999; Stoleru et al., 2004). In dfmr1 null animals, it has long been known that these neurons produce over-elaborated and over-extended synaptic arbors in the protocerebrum (Gatto and Broadie, 2009a; Morales et al., 2002; Reeve et al., 2005; Sekine et al., 2008). Among the human gene family, only hFMR1 was able to rescue the synaptic defect in this central circuit. Indeed, hFMR1 was just as proficient as the native dFMR1, indicating full functional conservation of FMR1 function between flies and humans. By contrast, the expression of hFXR1 and hFXR2 in the clock circuit had absolutely no effect on the null mutant phenotype. Therefore, it is likely that the behavioral impairment seen in mammals between FMR1 and FXR2 is a consequence of effects on complementary pathways that function in the same readout. In any case, it is clear that, at least in this circuit, the evolutionarily conserved role in the refinement of central synaptic connections is possessed only by FMR1 and not by its two paralogs.

The hallmark cellular defect in FXS patients and genetic disease models is the over-proliferation of synaptic connections, many of which appear to be immature (Grossman et al., 2006; Irwin et al., 2001). Although most research has focused on postsynaptic dendritic spines, apposing presynaptic bouton specializations obviously accumulate in parallel. In the Drosophila FXS model, both presynaptic boutons and postsynaptic dendrites are overgrown and over-elaborated in the absence of dFMR1, and we have demonstrated that this is a cell-autonomous requirement with neurons (Gatto and Broadie, 2008; Pan et al., 2004). Our previous studies of the well-characterized NMJ synaptic arbor have established a solely presynaptic requirement for dFMR1 in governing terminal area, synaptic branching and the formation of synaptic boutons (Gatto and Broadie, 2008). dfmr1 null synapses display an increase in terminal area, synaptic branching and supernumerary synaptic boutons. As in the central brain, our work here demonstrates that only dFMR1 and hFMR1 are able to curb growth and restore normal synaptic architecture in the null mutant. By sharp contrast, the hFXR paralogs do not possess this ability to any detectable degree. Thus, FMR1 has the unique ability to sculpt synaptic connections also in the context of the Drosophila peripheral nervous system.

A defining feature of the overgrown synaptic connections arising in the absence of dFMR1 is that they appear to be structurally immature. The NMJ is characterized by the accumulation of so-called ‘mini’ or ‘satellite’ boutons in dfmr1 null mutants (Gatto and Broadie, 2008). These immature boutons represent a developmentally arrested state of an otherwise-normal stage of bouton maturation (Ashley et al., 2005; Beumer et al., 1999; Dickman et al., 2006; Torroja et al., 1999). In the absence of dFMR1, there is a 50% increase in the number of structurally mature boutons, but a striking tenfold elevation in the abundance of these immature mini-boutons. Only the transgenic introduction of dFMR1 and hFMR1 can overcome this developmental arrest, restoring the normal number of mature synaptic boutons and eliminating the accumulation of mini-boutons. By contrast, hFXR1 and hFXR2 exhibit no restorative activity in synaptic bouton differentiation. Although the functional domains appear similar between all members of the human gene family, as well as dFMR1, it has not yet been established whether FXR1P and FXR2P bind to the same target mRNAs as FMRP and, if so, whether they regulate them in the same fashion. Indeed, differential binding and regulative activities have been proposed (Cavallaro et al., 2008; Tamanini et al., 1999a). In the Drosophila context, we have shown here that only FMR1 has a detectable role in regulating neuronal protein expression. Clearly neuronal expression of either hFXR1 or hFXR2 is not sufficient to remodel synaptic structure at two very different classes of synapse, or to maintain the normal program of synaptic differentiation. We therefore conclude that FMR1 has a unique function in mRNA regulation that is required for the proper development and differentiation of synaptic connections.

The crucial breadth of this study came from investigating a key non-neuronal FXS phenotype: the role of FMR1 in testes development and the maintenance of male fecundity. Male FXS patients have enlarged testes and reduced fecundity accompanied by spermatogenesis defects (Lachiewicz and Dawson, 1994; Nistal et al., 1992; Turner et al., 1980). The Drosophila FXS model similarly exhibits enlarged testes and decreased fecundity caused by defects in spermatid maturation resulting in immotile sperm (Zhang et al., 2004). Normal mature sperm tails present the ‘9+2’ microtubule configuration of nine outer doublets and a single, specialized central pair. In dfmr1 mutant spermatids, the central pair of microtubules is often lost completely, whereas the outer ring of microtubule doublets is occasionally disordered. To our initial amazement, all
Evolutionary conservation of the human FMR gene family

three human family genes (FMR1, FXR1 and FXR2) are equally capable of fully providing the normal configuration. Each gene, when driven by a germline promoter, completely restores the null male mutant fecundity and rescues all aspects of the testes development defects. Ultrastructural analyses show the normal ‘9+2’ microtubule architecture in all cases. Thus, in contrast to the FMR1-specific role in neurons, FMR1 and its two FXR paralogs show complete functional overlap and competency in this non-neuronal context. These results suggest the startling conclusion that FMR1 functions in a fundamentally different way in the nervous system compared with the testes.

It is important to note that these experiments were performed using the longest cDNA constructs of each gene, and thus may not take into account the function of unique splice isoforms. At least FMR1 and FXR1 are expressed as differential isoforms, with some transcripts expressed more strongly in some tissues than others (Davidovic et al., 2006; Denman and Sung, 2002; Huang et al., 1996; Khandjian et al., 1998; Kirkpatrick et al., 1999; Sittler et al., 1996). The fact that the full-length FMR1 construct rescues all phenotypes indicates that the FMR1 transcript heterogeneity is dispensable, at least at the level of the phenotypes assayed here. However, the same may not be true for FXR1. Note that we have not expressed transgenes postsynaptically in muscle, and therefore cannot rule out some trans-synaptic mechanism by which muscle FXR1 could potentially alter dfmr1 null NMJ phenotypes. This seems unlikely, however, given that all NMJ structural phenotypes are fully rescued with presynaptic dFMR1 (Gatto and Broaddie, 2008). In regards to FXR2, our findings are surprising because previous studies suggest that FXR2 may have some redundancy with FMR1 within neurons. Nevertheless, in our dispersed array of neural assays, no FXR2 function was detected, suggesting that neuronal roles for FXR2 appear to be mammal specific. The primary conclusion is that the much greater complexity of the mammalian nervous system appears to require unique functions that are mediated at least by FXR2. These mechanisms are sufficiently similar that they can somehow impinge on FMR1 function.

METHODS

Drosophila stocks and genetics

All Drosophila stocks were maintained at 25°C on standard cornmeal agar. The control genotype was w+118; elav-GAL4/+ (neuronal assays) and w+/118; nos-GAL4/+ (germline assays). The null mutant genotype was homozygous dfmr150M (Zhang et al., 2001) with a single copy of the two GAL4 driver lines: dfmr150M; elav-GAL4/+ and dfmr150M; nos-GAL4/. For brain staining, the genotypes UAS-GFP/++; OK107/++; UAS-GFP/++; dfmr150M/++; OK107/++; were used to identify the mushroom body with GFP antibodies (Fig. 2). As described below, the four transgenic UCN constructs were UAS-MYC-HA-dFMR1 (positive control) and the three human transgenes: UAS-MYC-hFMR1, UAS-MYC-hFXR1 and UAS-MYC-hFXR2. Third chromosome recombinants were recombined with the dfmr150M allele by conventional genetic techniques. The GAL4 driver lines elav-GAL4 [P(GawB)elavC155]; [P[w(+mC)=GAL4-elav.L]/CyO] and nos-GAL4 [P[w(+mC)=GAL4-nos.NGT40]; [P[w(+mC)=GAL4::VP16-nos.UTR[MVD2] were obtained from the Bloomington Drosophila Stock Center (Bloomington, IN).

Molecular techniques

Generation of the UAS-dFMR1 control line

The control UAS construct of the wild-type Drosophila FMR1 transgene was generated through three cloning reactions. First, the dfmr1 coding sequence was amplified from w118 total cDNA by PCR using the Expand Long Template PCR System (Roche, IN), according to the manufacturer's protocol, with the primers 5’-GCTCGAGAGATGAGACCTCTCGTGAGTCCGCTC-3’ and 5’-GTCTAGATGTGGCCGTACATTCAAGGACATC-3’. This sequence spans from the first base of the dfmr1 start codon through 155 bases into the 3’-UTR (untranslated region). Next, the product was double digested with Xhol and XbaI, and ligated into a similarly digested pUAS-T vector to create pUAS-dfmr1. A MYC-HA tag was created using the oligonucleotide 5’-GGAAATTCATGAGACAAACACCATATGCGAGATCCTGC-3’ and 5’-GTCTAGACTCGGAGCTATCCGGAATTAACCAACATC-3’ and 5’-GTCTAGACTCGGGACTATCCGGAATTAACCAACATC-3’. The product was double digested with EcoRI and NotI, and ligated into similarly digested pUAS-dfmr1. Finally, the dfmr1 DNA encompassing the 155 bp from the start of the 3’-UTR and the 76 bp after the end of the 3’-UTR was amplified from genomic DNA by PCR using the primers 5’-GTCTAGACACAACACACGCGCAGAAGCAAG-3’ and 5’-GTCTAGACACCGAGCTATCCGGAATTAACCAACATC-3’. The product was digested with XbaI and ligated into the similarly digested pUAS-dfmr1 containing the MYC-HA insert. The final plasmid pUAS-MYC-HA-dFMR1 was purified and confirmed by sequencing. The plasmid was microinjected into w118 embryos by Genetic Services, Inc. (Cambridge, MA). Transformants with stably integrated genomic inserts were identified and mapped to chromosomes using standard genetic techniques.

Generation of the UAS-hFMR1/hFXR1/hFXR2 lines

All three human gene family cDNAs in the pTL1 vector were kindly provided by Edouard W. Khandjian (URGHM, Centre de Recherche Hôpital St François d'Assise, Québec, Canada). The hFMR1 cDNA was double digested from the pTL1 vector with EcoRI and PstI, and subcloned into pBluescript II to provide the necessary restriction sites. The plasmid was microinjected into w118 embryos by Genetic Services, Inc. (Cambridge, MA). Transformants with stably integrated genomic inserts were identified and mapped to chromosomes using standard genetic techniques.
Western blot analyses
Western blots were performed as described previously (Tessier and Broadie, 2008). In brief, a pool of 4-6 heads was homogenized in 1× NuPage sample buffer (Invitrogen, Carlsbad, CA) supplemented with 40 mM DTT. The debris was pelleted by centrifugation at 11,700 × g at 25°C and samples were boiled for 5 minutes. Extracts were loaded onto a 4-12% Bis-Tris gel, electrophoresed and transferred to nitrocellulose. Membranes were rinsed once with NanoPure water, blocked for 1 hour in Odyssey blocking buffer (Li-Cor, Lincoln, NE) and probed for 12-16 hours at 4°C with primary antibodies. The antibodies that were used include: anti-dFMRP (1:3000; 6A15, Sigma, St Louis, MO), anti-α-Tubulin (1:400,000; B512, Sigma), anti-MYC [1:15; 9E10, Drosophila Studies Hybridoma Bank (DSHB), Iowa City, IA] and anti-MYC (1:1000; 71D10, Cell Signaling Technology, Danvers, MA). Blots were washed with 0.1% Triton-X 100 in PBS (PBST) and then probed for 1 hour at 25°C with secondary antibodies. Antibodies used include: Alexa Fluor 680-conjugated goat anti-mouse (1:10,000) and Alexa Fluor 680-conjugated goat anti-rabbit (1:10,000), both from Invitrogen-Molecular Probes (Carlsbad, CA). Blots were imaged using the Odyssey infrared imaging system (Li-Cor). Raw integrated intensities were calculated, with levels normalized to α-Tubulin.

Protein extraction and assay
Brain protein concentrations were determined as described previously (Tessier and Broadie, 2008). In brief, adult Drosophila heads (0-7 hours old) were rapidly frozen in liquid nitrogen and then stored at −80°C. Protein was extracted from 10-20 pooled heads by homogenizing in 8 M urea and 1% SDS supplemented with 1× complete protease inhibitor cocktail (Roche). The homogenate was incubated at 60°C for 1 hour. Protein concentrations were determined using a MicroBCA Assay (Pierce, Rockford, IL). All concentrations are reported as mean μg protein per head.

Immunohistochemistry
Adult brains, testes and third instar larvae were dissected and fixed for immunolabeling as described previously (Gatto and Broadie, 2008; Tessier and Broadie, 2008). In brief, all tissues were fixed for 40 minutes with 4% paraformaldehyde in PBS (pH 7.4). The preparations were then rinsed with PBS, blocked, and permeabilized with PBST containing 1% bovine serum albumin (BSA) for 1 hour at 25°C. Primary and secondary antibodies were diluted in PBST/BSA and incubated for 12-16 hours at 4°C, and 2 hours at 25°C, respectively. The primary antibodies used were: anti-dFMRP (1:500; 6A15, mouse, Sigma), anti-pigment dispersing factor (PDF) (1:250; rabbit, Sigma), anti-α-Tubulin (1:250; rabbit, Sigma), anti-GFP (1:50,000; clone 290, rabbit, Abcam, Cambridge, MA) and anti-MYC (1:15; 9E10, mouse, DSHB) (1:500; 71D10, mouse, Cell Signaling Technology). The secondary antibodies used were: Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 594-conjugated goat anti-rabbit IgG, both from Invitrogen-Molecular Probes. Preparations were mounted in FluoroMount G (EMS, Hatfield, PA). All fluorescent images were collected using an upright Zeiss LSM 510 META laser-scanning confocal microscope. Images were presented as maximum-intensity z-projections.

Clock neuron analyses
Brains from staged adult animals were dissected in standard saline and then fixed for 40 minutes with 4% paraformaldehyde in PBS, pH 7.4. Dissected brains were blocked and permeabilized with 0.2% Triton X-100 in PBS supplemented with 1% BSA for 1 hour at room temperature. The small ventrolateral (sLNv) clock neurons were labeled with anti-PDF antibody and stained with an Alexa Fluor secondary antibody (1:250; Invitrogen-Molecular Probes). Primary and secondary antibodies were diluted in PBST with 0.2% BSA and incubated overnight at 4°C and for 2 hours at room temperature, respectively. All fluorescent images were collected using a Zeiss confocal microscope. The total number of PDF-positive synaptic punctae (>1 μm diameter) were counted for each sLNv terminal projection on the right and left hemispheres of the brain, for each animal.

Neuromuscular junction structural analyses
The neuromuscular junction (NMJ) of wandering third instar larvae was quantified for structural features, as described previously (Gatto and Broadie, 2008). In brief, the muscle 4 NMJ of abdominal segment 3 (A3) was used for all quantification. Values were determined for both left and right A3 hemisegments, and then averaged for each animal (n=1). Synapse area was measured as the maximal cross-sectional area in a maximum projection of each collected z-stack. A synaptic branch was defined as an axonal projection with at least two synaptic boutons. The synaptic bouton classes that were defined included (1) type Ib (>2 μm diameter) and (2) mini/satellite (≤2 μm diameter and attached directly to a type Ib bouton). Each class of bouton is reported as the number per terminal. ImageJ software (http://rsweb.nih.gov/ij/) was used for the automated regional outline and area calculation.

Fecundity measurements
Transgene expression was driven in the male germline with a nanos-GAL4 driver line (nos-GAL4) (Schulz et al., 2004). Assays of male fecundity were performed as described previously (Zhang et al., 2004). In brief, for brooding tests, individual males (n=8) of each genotype were mated to virgin w1118 females (n=3) at 25°C, and mated animals were then removed from vials after 9 days. The adult progeny from individual vials were then counted for a subsequent 9 days.

Electron microscopy
Ultrastuctural analyses of Drosophila testes were performed as described previously (Zhang et al., 2004). In brief, testes from young adults (<24 hours post-eclosion) were dissected in PBS and fixed in 2% glutaraldehyde in PBS (pH 7.4) for 1 hour. For light imaging, testes were processed for anti-MYC labeling, as above. For electron microscopy, preparations were washed twice in PBS for 10 minutes and then incubated in tannic acid for 30 minutes to increase membrane contrast. Preparations were then transferred to 1% OsO4 in PBS for 2 hours and then washed three times in dH2O for 10 minutes each. Following secondary fixation, preparations were stained en bloc with aqueous 1% uranyl acetate for 1 hour, washed three times in dH2O, and then dehydrated through an ethanol series (50-100%). Finally, samples were passed through propylene oxide as a transition solvent using a 1:1 araldite:propylene oxide mixture. The solution was replaced with pure araldite and put under...
Evolutionary conservation of the human FMR gene family

Disease Models & Mechanisms

**TRANSLATIONAL IMPACT**

**Clinical issue**
The FMR gene family consists of fragile X mental retardation 1 (FMR1) and the closely related FXR1 and FXR2 genes. The FMR1 product (FMRP) is an RNA-binding, polyribosome-associated protein that regulates translation. Loss of FMRP function causes fragile X syndrome (FXS), the most common heritable form of mental retardation, and autism spectrum disorder. Neurological symptoms include hyperactivity, childhood epilepsy and cognitive dysfunction, and non-neurological symptoms include connective tissue abnormalities, ovarian failure in females, and enlarged testes in males. It has been proposed that FXR1 and FXR2 also have some function in FXS, but the relationship of these genes to neurological disease is not known.

In Drosophila, there is a single FMR1 gene (dFMR1) that has close homology to the human FMR1 genes. Elimination of dFMR1 from the fly results in phenotypes reminiscent of those observed in humans. Drosophila that lack the dFMR1 gene (dFmr1 null mutants) have altered synaptic structures that appear immature and are associated with inappropriate synaptic connectivity. In Drosophila, the protein encoded by dFMR1 suppresses neuronal protein synthesis and its loss results in the elevated expression of protein in the brain. Mutant flies also experience non-neuronal symptoms, and fecundity is reduced in mutant male flies. This system provides a tractable model to begin to elucidate the independent roles of the three human FMR1 genes.

**Results**
In this study, the authors introduce all three human FMR genes (FMR1, FXR1 and FXR2) into a well-characterized Drosophila FXS disease model. Only the FMR1 gene rescues neurological dysfunction. Flies expressing human FMR1 exhibit normal levels of protein in the brain, establish proper neuronal connections in the central brain circadian clock circuit, and exhibit complete synaptic differentiation. FMRP has a unique neuronal function that is independent of either FXR1P or FXR2P, and it cannot be replaced by FXR1P or FXR2P. In the fly tests, all three human genes are independently able to restore male fecundity and rescue defects in spermatogenesis. Thus, the requirement for FMR genes is different in neuronal versus reproductive tissues (or possibly all non-neuronal tissues), and this probably reflects tissue-specific functions of their resulting gene products.

**Implications and future directions**
This study demonstrates total functional conservation of the FMR family of genes, including FMR1, validating Drosophila as a relevant disease model for understanding this form of retardation and autism. The tissue-specific influence of FMRP on neurons indicates that its function is necessary for proper neurological function and that it is non-redundant with its gene family members. This Drosophila model will probably prove valuable for future structure-function analyses of human FMRP to define how the protein is regulated and which domains are important for its function in vivo.

**Statistics**
All statistical analyses were performed using GraphPad InStat 3 (GraphPad Software, San Diego, CA). Unpaired, nonparametric Tukey-Kramer multiple comparison tests were used to compare means and were applied in parallel to all control, dFmr1 null and transgenic construct lines. Significance levels in figures are represented as P<0.05 (*), P<0.01 (**) and P<0.001 (***) . All error bars represent standard error of the mean (s.e.m.) for independent trials.

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**COMPETING INTERESTS**
The authors declare no competing financial interests.

**AUTHOR CONTRIBUTIONS**
All four authors contributed directly to this study. R.L.C. designed some experiments, performed most of the experiments, analyzed data and co-wrote the manuscript. C.R.T. performed some of the experiments, helped analyze the data and edited the manuscript. E.A.W. performed all of the ultrastructural analyses, analyzed the electron microscopy data and edited the manuscript. K.B. conceived the study, designed the experiments, supervised all experimental work and co-wrote the manuscript.

**SUPPLEMENTARY MATERIAL**
Supplementary material for this article is available at http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.004598/-/DC1

Clinicai issue
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In Drosophila, there is a single FMR1 gene (dFMR1) that has close homology to the human FMR1 genes. Elimination of dFMR1 from the fly results in phenotypes reminiscent of those observed in humans. Drosophila that lack the dFMR1 gene (dFmr1 null mutants) have altered synaptic structures that appear immature and are associated with inappropriate synaptic connectivity. In Drosophila, the protein encoded by dFMR1 suppresses neuronal protein synthesis and its loss results in the elevated expression of protein in the brain. Mutant flies also experience non-neuronal symptoms, and fecundity is reduced in mutant male flies. This system provides a tractable model to begin to elucidate the independent roles of the three human FMR1 genes.

**Results**
In this study, the authors introduce all three human FMR genes (FMR1, FXR1 and FXR2) into a well-characterized Drosophila FXS disease model. Only the FMR1 gene rescues neurological dysfunction. Flies expressing human FMR1 exhibit normal levels of protein in the brain, establish proper neuronal connections in the central brain circadian clock circuit, and exhibit complete synaptic differentiation. FMRP has a unique neuronal function that is independent of either FXR1P or FXR2P, and it cannot be replaced by FXR1P or FXR2P. In the fly tests, all three human genes are independently able to restore male fecundity and rescue defects in spermatogenesis. Thus, the requirement for FMR genes is different in neuronal versus reproductive tissues (or possibly all non-neuronal tissues), and this probably reflects tissue-specific functions of their resulting gene products.

**Implications and future directions**
This study demonstrates total functional conservation of the FMR family of genes, including FMR1, validating Drosophila as a relevant disease model for understanding this form of retardation and autism. The tissue-specific influence of FMRP on neurons indicates that its function is necessary for proper neurological function and that it is non-redundant with its gene family members. This Drosophila model will probably prove valuable for future structure-function analyses of human FMRP to define how the protein is regulated and which domains are important for its function in vivo.

**Statistics**
All statistical analyses were performed using GraphPad InStat 3 (GraphPad Software, San Diego, CA). Unpaired, nonparametric Tukey-Kramer multiple comparison tests were used to compare vacuum at 25 mm Hg for 1 hour. Samples were put into fresh resin and placed into a 60°C oven overnight. Ultra-thin sections (55-65 nm) were obtained on a Leica (Wetzlar, Germany) UCT Ultratome microtome and transferred to formvar-coated grids. Grids were examined on a Philips CM10 transmission electron microscope at 80 V and images were captured with an AMT 2-megapixel camera. For quantification, sections were taken at a magnification of 19,000× or 25,000× and scored for axoneme microtubule morphology.


