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Molecular and Genetic Analysis of the *Drosophila* Model of Fragile X Syndrome

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Abstract

The *Drosophila* genome contains most genes known to be involved in heritable disease. The extraordinary genetic malleability of *Drosophila*, coupled to sophisticated imaging, electrophysiology and behavioral paradigms, has paved the way for insightful mechanistic studies on the causes of developmental and neurological disease as well as many possible interventions. Here, we focus on one of the most advanced examples of *Drosophila* genetic disease modeling, the *Drosophila* model of Fragile X Syndrome, which for the past decade has provided key advances into the molecular, cellular and behavioral defects underlying this devastating disorder. We discuss the multitude of RNAs and proteins that interact with the disease-causing *FMR1* gene product, whose function is conserved from *Drosophila* to human. In turn, we consider *FMR1* mechanistic relationships in non-neuronal tissues (germ cells and embryos), peripheral motor and sensory circuits, and central brain circuits involved in circadian clock activity and learning/memory.

1.1. Introduction

The genomic locus responsible for Fragile X Mental Retardation Syndrome (FXS) was mapped in 1991 to an unstable CGG trinucleotide repeat region at Xq27.3, which can rapidly expand and lead to DNA hypermethylation and transcriptional silencing of the *fragile X mental retardation-1* (*FMR1*) gene (Kremer et al., 1991; Pieretti et al., 1991; Poustka et al., 1991; Yu et al., 1991). A *FMR1* knockout mouse model of the disease was quickly generated to investigate cellular and behavioral phenotypes (Consortium, 1994; Kooy et al., 1996). This model has proven absolutely vital to advancing our understanding of FXS, but nevertheless has some limitations. First, the long generation time and high colony maintenance costs of mice significantly slow the rate of experimental analyses. Second, the identification of genes that interact with *FMR1* via forward genetic interaction screens is impractical in mice. Third, even with a candidate gene approach, creating multiply mutant mice to probe *FMR1* interactions is time consuming and laborious. Finally, many *FMR1* knockout mouse phenotypes are subtle and highly dependent on genetic background, and thus relatively difficult to analyze. Owing to these limitations, there was a clear need for an

additional genetic model to enhance our understanding of the FXS disease state. *Drosophila* was the obvious candidate model to fill in many of these gaps; with inexpensive maintenance and rapid generation time, excellent suitability for forward genetic screening and the ability to readily interrogate multiple genetic elements in single animals. Importantly, *Drosophila* has well-defined neural circuits driving a range of complex behaviors defective in FXS patients, including circadian patterned activity and learning formation coupled to robust memory consolidation (Dykens et al., 1988; Elia et al., 2000; Miano et al., 2008). The *Drosophila* genome contains a highly-conserved *FMR1* gene, *dfmr1*, which was targeted for mutation by mobilization of transposable P-elements located in the 5' portion of the locus (Wan et al., 2000; Zhang et al., 2001). The *Drosophila* FXS model was established in 2001 by imprecise P-element excision to produce *dfmr1* null mutants with robust phenotypes (Zhang et al., 2001). Subsequently, a wide array of *dfmr1* alleles and transgenes have been created to facilitate FXS research in this classic genetic model (Dockendorff et al., 2002; Lee et al., 2003; Morales et al., 2002).

When considering this model, it is critical to note that the *Drosophila* genome contains only a single *FMR1* gene, whereas mammalian genomes contain three highly related genes: *FMR1* and two paralogs (*FXR1* and *FXR2*). The three gene products have been suggested to have overlapping functions, although loss of *FMR1* function solely results in FXS, and *FXR1/2* mutations are not linked with any disease state (Cavallaro et al., 2008; Coffee et al., 2010; Darnell et al., 2009; Mientjes et al., 2004; Siomi et al., 1995; Zhang et al., 1995). The *dfmr1* gene product, *Drosophila* Fragile X Mental Retardation Protein (dFMRP), is approximately equally identical to all three mammalian gene products (~35% identity, ~60% similarity), and shows particularly high sequence conservation (~70% identity) in critical protein-protein and RNA-binding domains (Kirkpatrick et al., 2001). Consistently, murine and *Drosophila* FMRP have been shown to be RNA-binding proteins with conserved functions in multiple mRNA regulative processes, including transcript stability, localization and repression of mRNA translation (De Diego Otero et al., 2002; Feng et al., 1997; Lagerbauer et al., 2001; Weiler et al., 2004; Xu et al., 2008; Zalfa et al., 2007; Zalfa et al., 2003; Zhang et al., 2007). Importantly, transgenic expression of all three human gene family members in the *Drosophila* FXS model shows that only the disease-linked gene, *FMR1*, is able to rescue molecular and cellular defects (Coffee et al., 2010). Human *FMR1* completely rescues all requirements in the *Drosophila* disease model, just as effectively as the native *Drosophila* gene, whereas *FXR1/2* completely fail to provide any detectable rescue of *dfmr1* loss of function phenotypes in the nervous system. Interestingly, however, both human *FXR* paralogs are able to rescue non-neuronal defects in spermatid development and male fecundity in the *Drosophila* FXS model (Coffee et al., 2010). These findings confirm the evolutionary conservation of *FMR1* function in the nervous system, show that *FXR1/2* are not required for this conserved *FMR1* neuronal function, and suggest *FXR1/2* have diverged to assume non-neuronal roles that are not essential to the behavioral and cognitive defects of the disease state. These results validate the single gene *Drosophila* knockout as a model of the FXS human disease caused by exclusive loss of *FMR1* function.

Drosophila has been used for more than a century to dissect the molecular genetic bases of developmental processes, making this model system a particularly appealing venue to study FXS developmental defects. A tremendous amount of information on genes involved in

Drosophila development is readily available, and the availability of mutations in these developmental genes allows for experimentation with combinatorial mutations to identify *dfmr1* interactions. In addition, *Drosophila* has been used for many decades to systematically study the nervous system. The availability of mutations in neurological genes, coupled to excellent *in vivo* imaging, electrophysiological and behavioral analyses, makes *Drosophila* a powerful system to specifically investigate FXS neurological defects. This chapter will discuss how *Drosophila* genetic interaction tests and forward genetic screens have been harnessed to identify dFMRP developmental and neurological roles. Multiple stages of *Drosophila* development (embryo, larva, pupa and adult) have been used to investigate dFMRP neuronal and non-neuronal requirements. Our discussion will begin by considering non-neuronal roles of dFMRP, and then move on to focus the bulk of our attention on dFMRP roles within the nervous system that are the heart of FXS neurological dysfunction.

1.2. dFMRP roles in non-neuronal development

Although dFMRP is most highly enriched in neurons, it is also widely expressed in other tissues during development where it has important functions (Schenck et al., 2002; Wan et al., 2000). In particular, the role of dFMRP in the formation of *Drosophila* germ cells has been investigated at several levels. Following fertilization, syncytial blastoderm nuclei rapidly divide, and nuclei fated to form germ cells migrate to the posterior pole of the embryo. Maternal mRNAs and proteins localized at the pole trigger cellularization of these pole cells, which then function as progenitors to the germ line. This process of pole cell cellularization is disrupted in *dfmr1* null embryos lacking maternal dFMRP (Deshpande et al., 2006). Mutant embryos show a significant reduction in the overall number of embryonic pole cells and a consistent reduction in the number of germ cells present in the embryonic gonads. Interestingly, as with many *dfmr1* phenotypes, the penetrance of this defect is highly variable, with some embryos containing near wildtype numbers of pole cells and others containing virtually zero pole cells (Deshpande et al., 2006). One proposed mechanism for this *dfmr1* defect is misregulation of the cytoskeleton. Cytoskeletal contractile rings around nuclei are required for proper pole cell cellularization, and myosin-binding Annilin and actin-binding Chickadee/Profilin are both grossly mislocalized in *dfmr1* null pole cells (Deshpande et al., 2006). Interestingly, the *chickadee/profilin* transcript has been characterized as a direct mRNA binding target of dFMRP (Reeve et al., 2005). A manifestation of this binding interaction could be mRNA mislocalization or inappropriate translation, which may account for the unstable cellularization process in *dfmr1* mutant embryos. However, other processes also appear misregulated. Maternally contributed gene products normally suppress transcriptional activity in pole cells during the blastoderm stage, with transcription subsequently activated later during gonad development (Leatherman and Jongens, 2003). This early quiescent state is absent in some *dfmr1* embryos, as activated RNA polymerase II can be detected in the blastoderm pole cells (Deshpande et al., 2006). Moreover, the disruption of pole cell formation may also hinge on the regulation of microRNA pathways (Megosh et al., 2006). dFMRP associates with maternally contributed PIWI (P-element induced wimpy testis) and the RNA helicase Dicer-1 proteins, both involved in RNA silencing mechanisms. Their loss of function similarly reduces the number

of pole cells and, in some cases, results in a complete loss of pole plasm (Megosh et al., 2006). It is possible that loss of dFMRP may disrupt PIWI/Dicer-1 association, which would be predicted to misregulate miRNAs involved in pole cell formation. This hypothesis needs to be tested experimentally, but could provide a mechanistic basis for dFMRP involvement during pole cell cellularization.

Following cellularization, a mid-blastula transition (MBT) occurs when maternally contributed gene products cease to be utilized and zygotic transcription is activated; requiring new regulation of mRNA synthesis and degradation (Tadros and Lipshitz, 2005). At this stage, maternally-contributed dFMRP appears necessary to regulate specific zygotic mRNAs (Monzo et al., 2006). In particular, dFMRP binds *trailerhitch* mRNA and activates its translation, and Trailerhitch protein levels are reduced and mislocalized in *dfmr1* null embryos (Monzo et al., 2006). This result is surprising given that dFMRP characteristically acts as a translational repressor, and thus direct targets are usually increased in *dfmr1* loss of function mutants. Also surprising is that this reduction of protein coincides with an increase in *trailerhitch* mRNA levels in *dfmr1* nulls. Nevertheless, loss of function *trailerhitch* mutants exhibit cleavage furrow defects similar to *dfmr1* nulls (Monzo et al., 2006). Trailerhitch and dFMRP proteins co-sediment in ribonucleoparticles, although they are not thought to interact directly to control MBT cellularization. In contrast, the RNA-binding translational regulator Caprin, which also associates with dFMRP at the MBT, does coordinately control common mRNA targets (Papoulas et al., 2010). Although *caprin/dfmr1* double mutants exhibit a normal spindle apparatus, embryos show premature entry into mitosis due to misregulation of mutual downstream targets, including Cyclin B and Frustart (FRS) (Papoulas et al., 2010). The transcripts of these cell cycle regulators are bound by both Caprin and dFMRP, but the proteins are inversely regulated during early MBT: Cyclin B is upregulated and FRS is downregulated in the absence of Caprin/dFMRP. Interestingly, expression changes are transient, with normal levels of each protein returning by late MBT (Papoulas et al., 2010). Consistently, elevated Cyclin B causes premature entry into mitosis at an early stage, within minutes of cellularization, but has no effect at later stages (Royou et al., 2008). Thus, the role of dFMRP in the developing embryo is also likely transient. The mammalian homolog of Caprin has been implicated in regulating specific subsets of mRNAs involved in synaptic plasticity (Shiina et al., 2005; Solomon et al., 2007). Thus, while the dFMRP/Caprin interaction was identified in embryos, a similar interaction could exist in the nervous system. The association with Caprin highlights the idea that dFMRP participates in different ribonucleocomplexes, which may possess specific functions dictated by the precise composition of each particle.

Additional dFMRP mRNA targets may also contribute to defects during embryogenesis. In particular, 3 subunits of the 8-subunit CCT (Chaperonin containing TCP) complex are direct dFMRP mRNA targets in MBT embryos (Monzo et al., 2010). The CCT complex is an ATP-dependent chaperone which functions in protein folding of a large range of substrates, most notably actin and tubulin (Dekker et al., 2008; Yam et al., 2008). The 3 targeted subunits are misexpressed in *dfmr1* mutants in varying ways; CCT7 is upregulated, CCT4 is downregulated and CCT3 is inappropriately posttranslationally modified (Monzo et al., 2010). Interestingly, all subunits not identified as dFMRP mRNA targets also appear unchanged at the protein level. It is not clear how these changes to specific complex subunits

affect the overall CCT function, but the complex does not appear to properly form in *dfmr1* nulls, suggesting that chaperone activity is likely compromised. Consistently, disruption of the CCT complex with loss of function subunit mutants leads to cellularization defects (Monzo et al., 2010). Combining these mutations with the *dfmr1* null further exacerbates the phenotype, suggesting direct interaction between dFMRP and the complex. While the complete substrate identities of CCT are not known, the septin Peanut, which is known to be required for furrow formation, is one target that is mislocalized in both CCT mutants and even more so in *dfmr1* mutants (Adam et al., 2000; Deshpande et al., 2006; Neufeld and Rubin, 1994). Thus, another mechanism by which dFMRP affects cellular processes may be indirectly through protein folding programs essential for proper protein expression. Certainly more analysis into the functions of these proteins in embryonic development and elsewhere is warranted to explore the full extent of this intriguing mechanism.

Additional layers to the complexity of dFMRP mechanistic functions have been revealed in germline cells. In particular, dFMRP binds to and repress the translation of the *Drosophila* cytoplasmic polyadenylation element binding protein (dCPEB) Orb (Costa et al., 2005). Orb functions in an autoregulatory feedback loop required for the translation of localized mRNAs in egg chambers and, as such, regulates its own expression to ensure efficient translational control (Tan et al., 2001). As predicted from their opposing molecular functions, mutations in *dfmr1* and *orb* antagonize each other: *dfmr1* null ovaries exhibit an increase in egg chambers, and this defect can be rescued by *orb* mutations. Likewise *orb* mutants show a defect in dorsal-ventral polarity, favoring ventralized eggs, which can be partially or completely rescued by reducing dFMRP expression (Costa et al., 2005). Thus, dFMRP antagonizes the Orb translational pathway in a dose-dependent manner. Parallels for this mechanism exist in the nervous system, where *Drosophila* Orb2 genetically interacts with *dfmr1*, although the consequence of this interaction on FXS related phenotypes is unknown (Cziko et al., 2009). However, mammalian CPEBs function in synaptic plasticity and memory formation, suggesting an intriguing possibility that Orb2 provides a similar role in *Drosophila* (Keleman et al., 2007). In parallel ovarian studies, microarray analysis identified the transcript of E3 ubiquitin ligase, *cbl*, as another potential binding target of dFMRP (Epstein et al., 2009). Indeed, *cbl* mRNA levels are elevated in *dfmr1* mutant ovaries, although Cbl protein levels are unaffected, suggesting that dFMRP may regulate the stability or trafficking of *cbl* mRNA as opposed to its translation. Just as with *dfmr1* and *orb* double mutants, loss of function *cbl* alleles either completely or partially rescue the aberrant egg chamber counts in combination with *dfmr1* null alleles (Epstein et al., 2009). Interestingly, loss of *dfmr1* seems to cause defects by altering germ cell proliferation, and rates of cell cycle progression, possible via increased expression of cyclin E. Null *dfmr1* germ cells overexpress cyclin E, are hyper-proliferative and progress through the cell cycle at a slower rate than controls (Epstein et al., 2009). Taken together, these studies reveal a surprising complexity of dFMRP-mediated mRNA regulation controlling the proliferative capacity of germline cells.

1.3. dFMRP roles in larval neuronal development

Clues about dFMRP function in oogenesis are providing direct insights into similar functions during early neuronal development. *Drosophila* neuronal stem cells (neuroblasts)

populate the nervous system through a series of asymmetric divisions occurring in two periods; in the embryo, to produce larval neurons, and in the larva through pupa, to produce adult neurons. The *dfmr1* null larval central nervous system manifests hyperproliferation from these stem cells (Callan et al., 2010). Null *dfmr1* neuroblast lineages exhibit excessive 5-bromo-2-deoxyuridine (BrdU) incorporation and produce a greater number of differentiated adult neurons per stem cell compared with controls. Although the length of the cell cycle in *dfmr1* null neuroblasts is not altered, a greater number of neuroblasts escape early quiescence in the absence of dFMRP, which leads to the increased production of differentiated neurons (Callan et al., 2010). Somewhat similarly, in the mouse FXS model, loss of FMRP leads to an increase in glial cell differentiation, although the penetrance of the phenotype is low (Hessl et al., 2004; Luo et al., 2010). There are also mild increases in cell numbers, in both *Drosophila* and mouse FXS models, but this defect may nevertheless have important ramifications for the human disease state. In some FXS patients, some brain regions appear larger than controls, which could be the result of increased cellular proliferation (Hoeft et al., 2010a; Hoeft et al., 2008; Hoeft et al., 2010b). Moreover, if FMRP differentially regulates the differentiation of specific subsets of progenitor cells, this could have consequences on neuronal connectivity and brain function. A more detailed description of the role of FMRP in stem cell maintenance and differentiation is described in another chapter.

Apart from the above recent work, most effort has been focused on the function of dFMRP in late nervous system development, especially during synaptogenesis and synaptic refinement. In the *Drosophila* system, the larval neuromuscular junction (NMJ) is a particularly well-characterized and easily accessible glutamatergic synapse, which is an excellent model for glutamatergic synapse structural and functional development (Ball et al., 2010; Keshishian et al., 1996; Koh et al., 2000; Korkut et al., 2009; Rohrbough et al., 1999). Null *dfmr1* mutants exhibit a number of NMJ phenotypes. The neuronal branches innervating muscles are over-elaborated in *dfmr1* null mutants, indicating a role for dFMRP in repressing growth morphology (Zhang et al., 2001). Consistently, the number of synaptic boutons, sites of glutamate neurotransmitter release, is increased in mutant animals. These findings resemble synaptic defects in the mouse model and FXS patients. Indeed, the classical cellular hallmark of FXS patients is cortex neurons with supernumerary dendritic postsynaptic spines (Comery et al., 1997; Irwin et al., 2000; Irwin et al., 2001). Spines in patients and mice have been described as “long”, “thin” and “torturous”, and suggested to improperly mature in the FXS disease state. Consistently, *dfmr1* null NMJs exhibit an increased number of immature synaptic boutons referred to as “mini” or “satellite” boutons (Coffee et al., 2010; Gatto and Broadie, 2008). These satellite boutons are developmentally-arrested at an early stage when normal synapses have morphologically and functionally matured (Beumer et al., 1999; Dickman et al., 2006). Structural changes are accompanied by alterations in synaptic function (Gatto and Broadie, 2008; Repicky and Broadie, 2009; Zhang et al., 2001). Both spontaneous and evoked neurotransmission currents are increased in *dfmr1* null NMJs, and FM1-43 dye imaging confirms an elevated level of synaptic vesicle turnover (Gatto and Broadie, 2008; Zhang et al., 2001). Based on these core phenotypes, the *Drosophila* NMJ has been used as a tool to probe dFMRP function and dissect dFMRP interactions with other neuronal gene products.

One explanation for the synaptic function changes in *dfmr1* null NMJs is a change in neurotransmitter receptor composition. Glutamate receptors are the primary excitatory ionotropic channels located in the postsynaptic muscle juxtaposed to presynaptic sites of glutamate release (DiAntonio, 2006; Schuster et al., 1993). At the larval NMJ, GluRII AMPA-like receptors are expressed as tetrameric complexes containing 3 common subunits (GluRIIC, D and E) combined with either GluRIIA or GluRIIB subunits, to make two distinct classes of receptor (Featherstone et al., 2005; Qin et al., 2005). These receptor classes differ in their regulation, subcellular localization and functional conductance properties. Interestingly, the distribution of each receptor class is differentially altered in the absence of dFMRP (Pan and Broadie, 2007). Null *dfmr1* NMJs exhibit an increase in GluRIIA receptors and a concomitant decrease in GluRIIB receptors, though notably the overall number of total receptors does not change. Thus, dFMRP regulates the ratio of different GluR subclasses at a single synapse. In contrast, overexpression of dFMRP exclusively in the muscle using the targeted UAS-GAL4 expression system reduces expression of all GluRIIs at the NMJ (Pan and Broadie, 2007). Similar findings have been seen in the mouse model of FXS where loss of FMRP results in changes in AMPA receptor surface expression which may be dependent on both the precise brain region examined and particular upstream signaling events (Nakamoto et al., 2007; Soden and Chen, 2010; Suvrathan et al., 2010; Wang et al., 2010). Overexpression of dFMRP in the presynaptic neuron does not affect GluRII expression, but increases spontaneous glutamate release amplitude and frequency (Pan and Broadie, 2007; Zhang et al., 2001). Consistently, ultrastructural analysis reveals increased synaptic vesicle density around presynaptic active zones in *dfmr1* null terminals. These findings indicate both presynaptic and postsynaptic requirements for dFMRP function. Importantly, while most analysis in the mouse is focused on the postsynaptic requirement, presynaptic roles are also beginning to be defined which share many similarities to those identified in *Drosophila* (Akins et al., 2009; Antar et al., 2006; Christie et al., 2009; Hanson and Madison, 2007). These changes in excitatory transmission properties highlight an important element in both murine and *Drosophila* systems: FMRP-dependent phenotypes are often revealed under states of heightened neuronal activation and are likely due to the functioning of FMRP downstream of synaptic activity (Antar et al., 2006; Aschrafi et al., 2005; Bear et al., 2004; Khandjian et al., 2004; Muddashetty et al., 2007; Nosyreva and Huber, 2006; Park et al., 2008; Repicky and Broadie, 2009; Stefani et al., 2004; Tessier and Broadie, 2008; Todd et al., 2003). Identifying the specific activation conditions that drive FMRP function will likely be critical for understanding the molecular nature of the disease.

FMRP functions downstream of both broad neuronal activation and specific neuronal signaling pathways including Gq-coupled receptors, which has been best characterized for the group 1 metabotropic glutamate receptor (mGluR) (Bear et al., 2004; Volk et al., 2007). mGluR-dependent LTD/LTP synaptic plasticity mechanisms depend on FMRP activity, and mGluR antagonists either partially or completely alleviate structural, functional and behavioral FXS phenotypes in both murine and *Drosophila* disease models (Choi et al., 2010a; Choi et al., 2010b; de Vrij et al., 2008; Dolen et al., 2007; McBride et al., 2005; Osterweil et al., 2010; Pan et al., 2008; Yan et al., 2005). Together, these studies have led to the 'mGluR theory of FXS', which hypothesizes that FMRP limits the translation of specific

proteins under the influence of mGluR stimulation (Bear et al., 2004). The *Drosophila* genome encodes only a single mGluR (DmGluRA), compared to the eight separate receptors in mammals (Bogdanik et al., 2004). The simplicity of the *Drosophila* system, coupled to the evolutionary conservation of the activation pathways, has provided an excellent basis to test the mGluR hypothesis in the *Drosophila* FXS model. Molecular analyses reveal an inverse regulative relationship between dFMRP and DmGluRA: DmgluRA is overexpressed in *dfmr1* null animals and dFMRP is overexpressed in *DmGluRA* nulls (Pan et al., 2008). These results are consistent with dFMRP regulating DmGluRA downstream of the receptor signaling, though whether this is directly through mRNA binding or indirectly through an intermediate pathway is unknown. However, compared to the *dfmr1* null, the *DmGluRA* null shows only minor structural defects in NMJ structuring and synaptic bouton number/size (Bogdanik et al., 2004). Thus, at most, DmGluRA-mediated glutamatergic signaling is only one factor influencing the role of dFMRP in shaping NMJ architecture, and there must be additional signaling factors at play. In contrast to mild structural defects, the *DmGluRA* null shows more striking defects in activity-dependent synaptic function, including elevated transmission amplitudes during high frequency stimulation and abnormally strong hyperpotentiation following high frequency stimulation (Bogdanik et al., 2004; Pan et al., 2008; Repicky and Broadie, 2009). The functional defects are more severe in *DmGluRA* mutants compared to the *dfmr1* null, suggesting again at least some differential requirement. The fact that this functional overlap is only partial may be due to a diverse set of pathways that are initiated downstream of DmGluRA signaling, which appear to only partially overlap with mechanisms regulated by dFMRP. Finally, at a molecular level, loss of DmGluRA leads to an overall increase of both GluRII receptor classes, and therefore elevated total ionotropic GluR abundance at the NMJ synapse. This is in contrast to the loss of *dfmr1* which differentially alters the ratio of GluRIIA and GluRIIB receptor classes (Pan and Broadie, 2007). Once again, this relationship indicates a convergence between DmGluRA signaling and dFMRP dependent pathways, but only a partial overlap of function.

The critical analysis of the link between DmGluRA and dFMRP signaling has come from a combinatorial genetic approach in doubly null mutant animals. The double null mutant partially restores the *dfmr1* null synaptic overgrowth defects at the NMJ (Pan et al., 2008). However, while the *dfmr1* null branching over-elaboration is reduced by removal of DmGluRA, the double mutants actually produce an even greater number of synaptic boutons per terminal. Thus, the overlap in regulation of synaptic architecture is partial. At the ultrastructural level, the density of synaptic vesicles clustered around active zone sites is restored to normal in double mutants, rescuing the increase in density in *dfmr1* null terminals (Pan et al., 2008). However, functional readouts of DmGluRA-dFMRP interaction are more complex. Double mutants exhibit enhanced short-term facilitation and long-term augmentation during high frequency stimulation, which are both equally or more severe than defects in the *DmGluRA* null alone (Bogdanik et al., 2004; Repicky and Broadie, 2009). Conversely, after a high frequency stimulus train, the enhanced potentiation characterizing *DmGluRA* null animals is completely rescued in the double mutant. In addition, *dfmr1* null NMJs show a characteristic cycling of transmission amplitudes after high frequency stimulation, and this is only partially restored by removing DmGluRA (Repicky and Broadie, 2009). The structural and functional results together illustrate that dFMRP is

required for only some of the DmGluRA-dependent signaling pathways. At a molecular level, the increase in the GluRIIA receptor class observed in both *dfmr1* and *DmGluRA* nulls alone is additively increased in double mutants (Pan and Broadie, 2007). Likewise, the decrease in the GluRIIB receptor class in *dfmr1* null animals is lessened in double mutants, presumably due to the additive effect of the increase in GluRIIB numbers in the *DmGluRA* mutant. These data indicate that pathways induced by DmGluRA activation converge with pathways regulated by dFMRP, but do not indicate a linear pathway from DmGluRA to dFMRP. Taking these data together, a picture emerges of the translational regulator dFMRP able to compensate partially for loss of the signaling receptor DmGluRA, and the receptor also able to partially compensate for loss of dFMRP. Clearly more work is required to dissect these two converging pathways in order to determine precisely the mechanism by which these genes modulate synaptic transmission and thereby affect the developmental defects in FXS.

In addition to identifying signaling activities that control dFMRP function, a critical question is to determine when dFMRP functions, and therefore whether targeted interventions may need to be performed during specific windows of development as opposed to maturity. The *Drosophila* model is well suited to this dissection as transgenic methods allow gene expression to be temporally controlled (Fischer et al., 1988; McGuire et al., 2004). Using the inducible GeneSwitch system to express dFMRP during a precise window of development, constitutively throughout development or only at maturity, the ability to rescue *dfmr1* null larval NMJ phenotypes has been thoroughly assessed (Gatto and Broadie, 2008; Gatto and Broadie, 2009; Osterwalder et al., 2001). Using constitutive expression, targeted neuronal presynaptic reintroduction of dFMRP fully rescues all NMJ structural defects, though there is no rescue of defects in synaptic vesicle cycling. Reintroduction of dFMRP only during a short window (12 hours) in the early larval stages is equally effective in rescuing NMJ structural defects (Gatto and Broadie, 2008). One important caveat to this finding is that the *in vivo* half-life for dFMRP is approximately 25 hours (at 25°C), which extends the window of targeted reintroduction. Nevertheless, dFMRP clearly has a primary function during early stages of synaptogenesis to enable subsequent synaptic maturation. In contrast to the early temporal rescue, reintroduction of dFMRP in late 3rd instar stages only marginally rescues *dfmr1* null NMJ structural defects (Gatto and Broadie, 2008). These results indicate that structural plasticity is required to remove excess synaptic branches and supernumerary boutons present in the mutant condition. It is clear that the *Drosophila* NMJ is capable of such plasticity, but the time period available in the GeneSwitch analysis may have limited the ability for structural corrections to manifest (Eaton et al., 2002; Heckscher et al., 2007; Rohrbough et al., 2000). This consideration aside, these results indicate an early requirement for dFMRP in synaptogenesis, which can only be weakly compensated for by later reintroduction of dFMRP. Whether early intervention is similarly necessary to alleviate functional defects at the NMJ, or behavioral readouts of the affected circuit, remains to be tested. It will be critical to understand how early versus late intervention strategies affect downstream dFMRP-dependent molecular pathways that directly control synaptic development.

To test the *in vivo* significance of interactions between dFMRP and its mRNA targets, the great power of the *Drosophila* system is the ability to make combinatorial mutations in a

single animal (Table 1). This methodology has been critical in identifying genes that genetically interact with dFMRP at the larval NMJ (Zhang et al., 2001). In particular, *Drosophila* Futsch, homolog to mammalian microtubule associated binding protein 1B (MAP1B), was the first protein shown to genetically interact with FMRP *in vivo* (Zhang et al., 2001). Mutations in *futsch* alone produce NMJ structural and functional phenotypes that are inverse to those produced by mutations in *dfmr1*. Moreover, *futsch* hypomorphic alleles, which reduce Futsch expression by approximately 50%, completely restore the *dfmr1* null NMJ axonal branching, supernumerary synaptic bouton and neurotransmission defects of the *Drosophila* FXS model (Zhang et al., 2001). dFMRP binds *futsch* mRNA and represses Futsch translation. Null *dfmr1* animals express approximately 2 fold more Futsch than controls, and *dfmr1* overexpression reduces Futsch expression, showing that dFMRP acts as a negatively regulator of *futsch* translation *in vivo* (Zhang et al., 2001). Importantly, this FMRP-MAP1B interaction was subsequently confirmed in mammalian systems, confirming evolutionary conservation of the molecular mechanism (Lu et al., 2004; Wei et al., 2007). Consistent with defects in neuronal microtubules regulation, *dfmr1* null testes spermatid axonemes fail to properly form the microtubule array needed for sperm mobility and male fecundity (Coffee et al., 2010; Zhang et al., 2004). In the mutant condition, microtubule stability is impaired and the characteristic “9 + 2” microtubule arrangement is lost, often resulting in an aberrant “9 + 0” array lacking the central pair of microtubules. Together, studies in the NMJ and testes both suggest that FMRP plays a prominent role in the regulation of the microtubule cytoskeleton.

Using a similar approach to that used to identify a Futsch-dFMRP interaction, other genes have been demonstrated to function with dFMRP at the larval NMJ. For example, loss of function mutations of the cytoskeletal-binding protein Lethal Giant Larvae (Lgl), originally identified as a tumor suppressor gene in cellular proliferation and cell polarity development (Bilder et al., 2000; Strand et al., 1995), dominantly suppress *dfmr1* overexpression phenotypes (Zarnescu et al., 2005). At the NMJ, testing for genetic interaction in the double heterozygote *lgl*⁺; *dfmr1*⁺ condition revealed structural defects similar to the *dfmr1* null; specifically, a >2 fold increase in synaptic bouton number. Neither single heterozygotic mutation alone causes a phenotype, but rather the double mutants act convergently to produce synaptic hyperplasia. dFMRP and Lgl co-localize in cells, can be co-fractionated on density gradients and co-immunoprecipitate from similar complexes (Zarnescu et al., 2005). Although the two proteins likely do not interact directly, the complexes they inhabit share an overlapping set of mRNAs constituents, which each protein presumably regulates in common during transcript transport and/or translation regulative control. It is possible that Lgl interacts with the cytoskeleton to localize or stabilize a subset of mRNAs regulated by dFMRP, although the mechanism of this putative cooperation is uncertain. This cooperative interaction may involve the PAR protein complex and atypical protein kinase C (aPKC), which are necessary for the distribution of proteins defining cell polarity (Ohno, 2001). Interestingly, all of these proteins are necessary for proper NMJ development. Loss of aPKC results in a reduction in the number of synaptic boutons caused by improper organization of the microtubule cytoskeleton in both pre- and postsynaptic compartments (Ruiz-Canada et al., 2004). *aPKC* nulls exhibit increased GluRIIA and concomitantly increased transmission amplitudes in NMJ synaptic terminals. The Par complex colocalizes with aPKC at NMJs,

and mutations in Par complex components mimic aPKC mutants in reduced bouton numbers, increased GluRIIA expression and increased evoked synaptic transmission amplitudes (Ramachandran et al., 2009; Ruiz-Canada et al., 2004). Loss of the complex component Baz/Par3 disrupts postsynaptic actin organization similarly to loss of aPKC. aPKC phosphorylates Lgl thereby releasing it from the actin cytoskeleton and altering the subcellular distribution of Lgl bound targets (Betschinger et al., 2003; Tian and Deng, 2008). Heterozygotic removal of aPKC should hyperactivate Lgl and, interestingly, restores *dfmr1* null NMJ synaptic bouton numbers back to wildtype levels (Zarnescu et al., 2005). Therefore, as with the Futsch interaction, it appears that modulation of cytoskeletal properties is a central aspect of dFMRP function.

More recent evidence for the importance of the regulation of the microtubule cytoskeleton in *dfmr1* null animals has come from the identification of a genetic interaction of dFMRP with *Drosophila* Spastin (Yao et al., 2010). Spastin is a microtubule severing protein whose mutation is one prominent cause of Hereditary Spastic Paraplegia (HSP) (Salinas et al., 2007). RNAi knockdown of Spastin suppresses the rough eye phenotype caused by overexpression of dFMRP, and importantly, double mutant combinations of *dfmr1* and *spastin* show a combinatorial increase in NMJ bouton number over either individual null allele alone (Yao et al., 2010). In direct imaging of the microtubule networks around nuclei in muscle cells, *dfmr1* null cells show a dramatic increase in the ratio of microtubules around the perinuclear region compared to the middle nuclear region. The phenotype caused by dFMRP overexpression in the muscle is the inverse; less microtubule complexity in the perinuclear region. Interestingly, though no differences in Spastin expression are seen in *dfmr1* nulls, muscle overexpression of dFMRP leads to a nearly 3 fold increase in Spastin expression, suggesting that dFMRP positively regulates Spastin. However, it is not known whether this change in protein level is due to direct regulation of *spastin* mRNA by dFMRP. The functional relevance of the alteration in microtubule complexity may be to impact mitochondria transport processes (Chen et al., 2009). Null *dfmr1* axons contain more mitochondria, and these mitochondria appear more motile than in controls. The interpretation is that dFMRP-dependent misregulation of Spastin leads to an inability to properly regulate the microtubule network, thereby altering the dynamic localization of mitochondria. Such a defect may have important consequences for activity-regulated mechanisms of dFMRP function.

In addition to regulation of microtubules, dFMRP also regulates the actin cytoskeleton. This function has been best characterized in dendritic arborization (DA) mechanosensory neurons that extend sensory dendritic processes underlying the larval epidermis (Lee et al., 2003; Schenck et al., 2003). As at the NMJ, loss of dFMRP results in an increase in neuronal branch arborization in the sensory dendrites. Moreover, dFMRP overexpression results in a more simplified dendritic architecture with fewer branches. The small RhoGTPase Rac1 plays a critical role in mediating branching by modulating actin cytoskeleton dynamics to control this growth (Luo et al., 1994; Ng et al., 2002). In multiple neural circuits, loss of Rac1 leads to a reduced structural complexity similar to the dFMRP overexpression phenotype. Indeed, simultaneous overexpression of both dFMRP and Rac1 in DA neurons leads to a modest restoration of dendritic branch structure towards the wildtype architecture (Lee et al., 2003). dFMRP directly binds to *rac1* mRNA, although the effect of this

interaction on Rac1 protein levels has not been assessed. However, it is attractive to speculate that dFMRP represses Rac1 translation, perhaps locally in dendritic processes, to control actin-mediated branching. An additional component of this mechanism is the Cytoplasmic Fragile X Interacting Protein (CyFIP), which binds both Rac1 and dFMRP, although no complexes containing all three proteins have been identified (Schenck et al., 2003). CyFIP is an eukaryotic initiation factor 4E-binding protein (eIF4E-BP) that is capable of regulating the initiation of translation at synapses, and specifically in response to neuronal activity (Napoli et al., 2008). Thus, it is possible that CyFIP acts as a bridge between the translational repressor activity of dFMRP and the Rac1-dependent induction of actin remodeling. Since dFMRP regulates *rac1* mRNA directly, it is likely that a feedback mechanism ensures that the actin cytoskeleton is precisely modulated, particularly during times of neuronal activity (Lee et al., 2003). Yet another component of this pathway in *Drosophila* is the actin-binding protein Profilin/Chickadee (Reeve et al., 2005). Profilin/Chickadee mediates the dynamic turnover of actin by destabilizing F-actin. The role of Profilin/Chickadee in the Rac1-FMRP pathway has been best characterized in the adult circadian activity circuit (Reeve et al., 2005), and will therefore be discussed in below. The discovery of elements of this cytoskeletal regulatory mechanism in multiple *Drosophila* neural circuits once again highlights the importance of misregulation of cytoskeletal dynamics in the FXS disease state.

1.4. dFMRP roles in adult brain neural circuit development

To correlate dFMRP-dependent pathways to higher order behaviors, there is an obvious need to push the *Drosophila* FXS model assays into adult brain neural circuits. Two particularly attractive circuits are 1) the mushroom body (MB) circuit required for olfactory learning and memory consolidation, and 2) the circadian clock circuit involving small and large pigment dispersing factor (PDF) lateral neurons that coordinate daily activity cycles (Dahdal et al., 2010; Dubnau and Tully, 2001; Helfrich-Forster et al., 2007; Sheeba et al., 2008; Zars, 2000). Both circuits are excellent locations for *Drosophila* FXS model study for several reasons. First, both are very well characterized with respect to the neuronal subtypes comprising the circuit. In the MB circuit, a great deal is known about connectivity in the upstream olfactory lobe (OL), the projection neurons (PNs) innervating the MB, and the three classes of MB Kenyon Cells (KCs) required for memory storage (Dubnau et al., 2001; Krashes et al., 2007; Tully and Quinn, 1985). In the circadian clock circuit, the cellular components are similarly well defined at the level of individually-identifiable neurons (Fernandez et al., 2007; Nitabach and Taghert, 2008). Second, in both circuits, a great deal is known about how each neuron class contributes to the associated behavior. Third, a broad array of genetic tools is available for studying both MB and clock circuitry at the level of small groups of neurons, or even the single cell level. This includes an array of specific GAL4 driver lines for controlled gene expression and the targeted delivery of genetic manipulations and reporter constructs, as well as GAL4-based clonal techniques for similar manipulation of single neurons. Single cell analysis has been particularly critical for understanding activity-dependent mechanisms of architectural and functional refinement associated with the cell-autonomous loss of dFMRP (Tessier and Broadie, 2008; Tessier and Broadie, 2011).

The first CNS circuit investigated in the *Drosophila* FXS model was the circadian circuit controlled by the dorsal cluster (DC) photoreception responsive cells and the lateral PDF clock neurons (Morales et al., 2002). Interestingly, dFMRP may function in opposing roles in these two components of the same circuit. In DC neurons, the number of axons that project from the lobula to the medulla is reduced in *dfmr1* nulls compared to controls. Concomitantly, however, the connecting large lateral PDF neurons, which normally project via a fasciculated axon bundle towards the dorsal medial lateral horn where synaptic connections are formed, are overgrown in *dfmr1* null brains (Morales et al., 2002). Axonal pathfinding in this part of the circuit appears somewhat inaccurate, with extra projections prematurely branching off to the medial part of the brain. However, the most robust phenotype is that the synaptic puncta in the lateral horn are more numerous and more broadly dispersed in the *dfmr1* null mutant (Coffee et al., 2010; Gatto and Broadie, 2009; Morales et al., 2002). Overexpression of dFMRP results in the opposite consequence, with synaptic arbors collapsed to occupy a much smaller area containing fewer definable synaptic boutons. The combination of these gain and loss of function *dfmr1* phenotypes in the lateral PDF clock neurons show that dFMRP normally functions to limit neuronal growth and limit synaptic development, comparable to the role at the NMJ synapse. Importantly, the timing of this dFMRP requirement appears limited to a precise stage of circuit development (Gatto and Broadie, 2009). Restoring dFMRP activity to this circuit using the GeneSwitch conditional paradigm is only effective at rescuing *dfmr1* null phenotypes when dFMRP is reintroduced during a late pupal stage of brain development, a period of synaptogenesis, remodeling and synaptic refinement (Gatto and Broadie, 2009). Reintroduction of dFMRP either during the earlier stages of neurogenesis and axonal outgrowth, or during mature stages in adult animals, completely fails to rescue the *dfmr1* null defects in clock circuit architecture. Thus, as in the larval motor circuit, dFMRP functions in a precise developmental window to sculpt synaptic connectivity. Identifying the molecular players interacting with dFMRP at this stage of brain maturation will be critical to understanding the molecular mechanism of dFMRP function within the developing clock circuit.

From embryonic and larval dFMRP analyses there are many strong indications that dFMRP plays a prominent role in regulating the dynamic properties of the cytoskeleton. Similarly in the adult clock circuit, genetic interaction between dFMRP and Rac1 has been identified in the regulation of the downstream actin-destabilizing protein Profilin/Chickadee (Reeve et al., 2005). Double loss of function mutation of *rac1* and *dfmr1* significantly exacerbates the branching defects of the lateral PDF neurons compared to the *dfmr1* null alone. Similarly, *chickadee* mutants genetically interact with *dfmr1* to control clock circuit architecture. Overexpression of *chickadee* alone results in a phenocopy of the *dfmr1* null synaptic overgrowth, and co-overexpression of *chickadee* and *dfmr1* rescues the synaptic arborization of the lateral PDF neurons (Reeve et al., 2005). The mechanism of this interaction is likely direct, as dFMRP binds to and represses the translation of *chickadee* mRNA. This regulation may also involve either an effect on the transcription or stability of *chickadee* mRNA, as there is an elevated level of *chickadee* mRNA in *dfmr1* null brains compared to controls (Tessier and Broadie, 2008). Interestingly, the mRNA level increase is transient, peaking during late stages of pupal brain maturation, immediately prior to eclosion. This window is the same developmental window identified for the transient rescue requirement of dFMRP,

and reinforces the conclusion that dFMRP has a primary function during neural circuit refinement (Gatto and Broadie, 2009). These findings, combined with the evidence of Rac1 and CyFIP genetic interactions in the larval peripheral circuits, strongly indicate that dFMRP mediates regulation of the actin cytoskeleton via translational control of Chickadee/Profilin, in concert with a feedback mechanism to upstream Rac1 signaling molecules.

A particular benefit to studying dFMRP function in the circadian clock circuit is the ability to easily correlate molecular and cellular changes with circadian behavior. Consistent with gross changes in circuit architecture, the *Drosophila* FXS model exhibits profound circadian activity defects (Banerjee et al., 2007; Dockendorff et al., 2002; Inoue et al., 2002; Morales et al., 2002; Sekine et al., 2008). In locomotor analysis during alternating light and dark cycles, a large portion of *dfmr1* null animals are totally arrhythmic; they do not cycle between active and inactive periods, but rather exhibit heightened activity without a clear sleep period. Confoundingly, when sleep periods are measured directly, *dfmr1* null animals show prolonged sleep, while animals over-expressing dFMRP show reduced bouts of sleep (Bushey et al., 2009). This seeming contradiction certainly requires further investigation. Under conditions of constant darkness, *dfmr1* nulls exhibit an even more pronounced arrhythmicity, indicating a defect in circadian clock output (Dockendorff et al., 2002; Inoue et al., 2002; Sekine et al., 2008; Sofola et al., 2008). Importantly, the severity of the arrhythmicity of each individual *dfmr1* null animal positively correlates with the degree of overexpansion of the lateral PDF neuron synaptic contacts in the dorsal horn (Sekine et al., 2008). The striking behavioral defect helped to identify a genetic interaction between dFMRP and Lark (Sofola et al., 2008), an RNA-binding protein which globally regulates genes involved in circadian behavior (Newby and Jackson, 1996; Zhang et al., 2000). Lark and dFMRP can be coimmunoprecipitated, suggesting that some mRNA targets may overlap between the two proteins (Sofola et al., 2008). Interestingly, loss of function *lark* mutants exhibit reduced levels of dFMRP, although *dfmr1* null animals express wildtype levels of Lark, thus perhaps indicating that dFMRP is directly regulated by Lark but not vice versa (Sofola et al., 2008). Moreover, overexpressing Lark in the lateral clock neurons leads to a modest behavioral arrhythmicity, which can at least be partially rescued by introduction of the heterozygous *dfmr1* null mutation. In addition, genetic knockdown of *lark* and concomitant overexpression of dFMRP leads to a dramatic loss of rhythmicity, reminiscent of *dfmr1* null animals (Dockendorff et al., 2002; Inoue et al., 2002). These strong behavioral readouts certainly demand further investigation of the downstream targets of these two interacting RNA-binding proteins to elucidate the precise mechanisms of circadian control.

The second CNS focus of the *Drosophila* FXS model has been the MB learning and memory center. MB neurons come in 3 Kenyon Cell (KC) classes (alpha, beta and gamma neurons), which are required for different aspects of learning, short- and amnesia-resistant memory formation, and long-term memory consolidation (Akalal et al., 2010; Blum et al., 2009; Isabel et al., 2004; Lee et al., 1999; Wang et al., 2008b; Yu et al., 2006). This circuitry has particular relevance due to the cognitive impairments of FXS patients. The characteristic MB lobe structure can be analyzed either for gross anatomical alteration or for fine architectural changes at the single neuron level using the genetic clonal technique of Mosaic Analysis with Repressible Cell Marker (MARCM) (Lee and Luo, 2001). The powerful MARCM technique allows GFP labeling of a single *dfmr1* mutant neuron in an otherwise

wildtype brain, permitting characterization of cell autonomous effects of *dfmr1* loss or gain of function. At a gross anatomical level, the *dfmr1* null MB exhibits defects in axonal lobe formation, of variable penetrance and dependent on genetic background (Chang et al., 2008; McBride et al., 2005; Michel et al., 2004; Pan et al., 2004). Phenotypes range from dramatic loss or rearrangement of alpha/beta lobes, to improper beta lobe axonal projections across the brain midline. Single cell MARCM analysis consistently reveals over-branched and over-extended axons with supernumerary synaptic varicosities, similar to excessive neuronal growth and bouton formation in the clock and larval motor circuits (Pan et al., 2004; Tessier and Broadie, 2008). Overgrowth is also apparent in dendritic arbors, where PNs connect to their postsynaptic KC inputs of the MB circuit, indicating that dFMRP plays cell-autonomous roles in the structural maturation of both sides of the synapse. Overexpression of dFMRP in single MB neurons again caused the opposite consequence of *dfmr1* loss of function: reduced and simplified synaptic connections in both dendritic inputs and axonal outputs (Pan et al., 2004). Importantly, *dfmr1* synaptic overgrowth phenotypes are both developmentally-regulated and activity-dependent (Tessier and Broadie, 2008). During the late stages of pupal brain development, *dfmr1* null axonal branches are overgrown, with excessive synaptic contacts. Subsequently, at late stages of development and during early adult use, there is a failure to properly eliminate synaptic contacts via an activity-dependent pruning mechanism. Using light to hyperexcite gamma neurons expressing a light-activated cation channel, wildtype neurons prematurely undergo axonal pruning and synapse elimination, but no refinement was induced in *dfmr1* null neurons (Tessier and Broadie, 2008). Thus, pruning is dependent on both dFMRP and activity. This dependence is at least somewhat bidirectional, as hyperexcitation leads to reduced dFMRP expression and loss of activity leads to an upregulation of dFMRP (Tessier and Broadie, 2008). Consistently, mammalian FMRP associates with polyribosomes in an activity-dependent manner to regulate the translation of its own mRNA (Khandjian et al., 2004; Lagerbauer et al., 2001; Stefani et al., 2004). Thus, activity induced translation is tightly controlled at many levels to ensure that the timing of production of growth factors is well coordinated with both intrinsic and extrinsic signals.

One critical consequence of neuronal activity is the mobilization of calcium in response to membrane depolarization. Many types of calcium signaling pathways function in neurons, dependent on the source of calcium, the magnitude and duration of the calcium changes, the frequency of calcium influx and the precise developmental stage of the neuron (Berridge et al., 2000; Lnenicka et al., 2006; Lohmann, 2009). Calcium signaling is known to play key roles in axonal outgrowth, synapse formation and the refinement of synaptic connections. *dfmr1* null MB neurons exhibit a developmentally regulated defect in processing both internal and external calcium signals after depolarizing stimulation (Tessier and Broadie, 2011). MB calcium dynamics were investigated in *dfmr1* null animals using the genetically-encoded calcium sensor gCAMP, which changes fluorescence depending on calcium concentration (Akerboom et al., 2009; Nakai et al., 2001). In *dfmr1* null neurons in intact brain MB circuits, there is a defect in 1) calcium release from internal organelles as well as 2) calcium influx across the plasma membrane (Tessier and Broadie, 2011). These defects are cell intrinsic as evidenced by consistent findings at the single cell level in a dissociated MB cell culture system. One possible joint mechanism for both phenotypes is misregulation

of calcium buffering proteins. Both Calmodulin and Calbindin sequester cytosolic calcium, and expression of their mRNAs is severely reduced in *dfmr1* null brains (Tessier and Broadie, 2011). The reduced expression of calcium buffers could have profound impacts on neuronal development and physiology. In addition to developmental roles, the regulation of dFMRP on calcium dynamics could play key functions in the activity-dependent mechanisms described above. Interestingly, the role in regulating activity could involve cooperative interactions between dFMRP and the CPEB Orb protein. During oogenesis, translation is positively regulated by Orb, but this regulation is maintained within reasonable limits by the repressor dFMRP, just as dFMRP is thought to mitigate local translation after neuronal firing. Indeed, a nervous system specific CPEB, Orb2, is present in *Drosophila* and genetically interacts with *dfmr1* in the eye (Cziko et al., 2009). CPEBs play critical roles in synaptic plasticity and may act as “memory markers” to help identify synapses during memory formation (Keleman et al., 2007). It will therefore be critical to determine if dFMRP functions in a similar feedback control mechanism for nervous system translation via mitigation of neuronal CPEB/Orb2 expression.

A great advantage of MB circuit analysis is the ability to assay well-characterized MB-dependent learning/memory behaviors. Consistent with MB circuit defects, *dfmr1* null animals exhibit a significant defect in learning and striking loss of long-term memory formation, a protein synthesis dependent process (Bolduc et al., 2008; Tully et al., 1994). A memory defect also occurs when dFMRP is overexpressed, indicating that precise levels of translational control are necessary for memory storage. This behavioral readout has been exploited to identify multiple dFMRP interactors. For example, the RNA-binding translational repressor Staufen colocalizes in similar cytoplasmic granules as dFMRP and functions in long-term memory formation, suggesting an overlapping function with dFMRP (Barbee et al., 2006; Brendel et al., 2004; Dubnau et al., 2003). Consistently, double heterozygotic mutation of *staufen* and *dfmr1* causes a dramatic reduction in the ability to form long-term memory, although each single heterozygous mutation shows no memory defects (Bolduc et al., 2008). Long-term memory is also dependent on dFMRP interaction with the microRNA pathway. As with *staufen*, double heterozygous mutations of *dfmr1* and the RNA Induced Silencing Complex (RISC) core component *argonaut-1* produce a dramatic reduction in long-term memory storage. Blocking protein synthesis with chemical inhibitors rescues this genetic interaction, further confirming that deregulated protein synthesis destroys memory capacity (Bolduc et al., 2008). The specific mRNAs translated in response to memory-inducing cues, however, remain to be identified. One possible convergent mRNA target of Staufen and dFMRP is *cheerio/filamin A*, which is required for reorganization of the actin cytoskeleton (Flanagan et al., 2001; Li et al., 1999). The *cheerio* and *dfmr1* mutations interact to disrupt protein synthesis dependent memory (Bolduc et al., 2010). dFMRP positively regulates Cheerio expression in response to memory-inducing stimuli, although it is not clear if this is a direct or indirect mechanism. Also, whether the dFMRP positive regulation of Cheerio integrates with the microRNA-mediated protein synthesis mechanism of memory storage remains to be determined. Nonetheless, as with larval circuits and adult clock circuit, these results further suggest that dFMRP-dependent regulation of the cytoskeleton is a critical pathway underlying FXS disease-relevant behavioral defects.

1.5. dFMRP and the microRNA pathway

Despite a great deal of effort, the molecular mechanism by which FMRP regulates mRNAs remains quite uncertain. A number of possibilities have been proposed including control of mRNA stability and/or trafficking, repression of translation mediated by direct mRNA binding via specific secondary structure (G-quartet) recognition, and translation regulation through microRNA pathways (Ashley et al., 1993; Caudy et al., 2002; Darnell et al., 2005a; De Diego Otero et al., 2002; Feng et al., 1997; Lagerbauer et al., 2001; Schaeffer et al., 2001; Weiler et al., 2004; Xu et al., 2004; Zalfa et al., 2007; Zalfa et al., 2003; Zhang et al., 2007). In *Drosophila*, dFMRP was initially discovered in unbiased biochemical screens to associate with multiple components of the cellular machinery responsible for the maturation and function of microRNAs (Caudy et al., 2002; Ishizuka et al., 2002; Jin et al., 2004). dFMRP can cofractionate and coimmunoprecipitate with multiple ribosomal proteins, the double stranded RNA nuclease Dicer and Argonaute-1/2 components of the RNA induced silencing complex (RISC), which functions to degrade mRNAs or repress translation (Caudy et al., 2002; Ishizuka et al., 2002; Megosh et al., 2006). The dFMRP-Argonaute association is critical for regulating the expression of a specific dFMRP target mRNA encoding the degenerin/epithelial sodium channel Pickpocket (Xu et al., 2004). dFMRP binds directly to *pickpocket* mRNA, and *dfmr1* null animals exhibit an increase in *pickpocket* mRNA levels (Xu et al., 2004). Conversely, overexpression of dFMRP results in a decrease in *pickpocket* mRNA levels, indicating a bidirectional mode of regulation. Loss of function *argonaut-2* mutants similarly exhibit an elevation of *pickpocket* mRNA, but this effect is not dependent on dFMRP. However, the reduced *pickpocket* mRNA levels caused by dFMRP overexpression can be rescued by *argonaut-2* mutation, suggesting a cooperative effect by which dFMRP may act as a target locator for RISC components (Xu et al., 2004).

In addition to these molecular interactions, *dfmr1* mutants genetically interact with both *argonaut-1* and *argonaut-2* mutants (Jin et al., 2004; Pepper et al., 2009). In the *Drosophila* eye, a loss of function *argonaut-1* mutant rescues the rough eye phenotype caused by dFMRP overexpression, which confirms the overlapping pathways of regulation between these two proteins (Jin et al., 2004). At the larval NMJ, double trans-heterozygous mutations in *dfmr1* and *argonaut-1*, or *dfmr1* and *argonaut-2*, cause synergistic over-production of synaptic boutons; in the case of the *dfmr1/+; argonaut-1/+* combination, more numerous boutons than in the homozygous *dfmr1* mutant alone (Jin et al., 2004; Pepper et al., 2009). However, it is not clear yet whether specific mRNAs may be differentially regulated by Argonaute-1/dFMRP and Argonaute-2/dFMRP complexes, or how those complexes may regulate synapse development. In the absence of dFMRP there is a reduced association of Argonaute-1 with Dicer, which may be responsible for the reduced expression of miRNA-124 in *dfmr1* null animals (Xu et al., 2008). Interestingly, miRNA-124 overexpression limits dendritic development in DA neurons and thus, the dendritic overgrowth defects associated with FXS may be due to a failure of RISC to silence targets of miRNA-124 (Xu et al., 2008). Indeed, excessive protein synthesis of miRNA targets may be at least partially responsible for FXS memory defects (Bolduc et al., 2008). Future efforts to identify these targets and determine their role in synapse development will certainly be required to test this hypothesis. In support of these efforts, target identification is increasingly feasible as more refined

miRNA target parameters become defined. Multiple databases such as mirBase (<http://www.mirbase.org>) and microCosm (<http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/>) are valuable tools in performing candidate gene targeted approaches to determine the full extent by which this pathway is affected in the *Drosophila* FXS model.

The reduced Dicer/RISC complex association in the absence of dFMRP likely has global effects on miRNAs. In the *Drosophila* ovary, dFMRP associates with at least 30 miRNAs, including the well characterized *bantam* miRNA, suggesting that downstream targets of many miRNAs may contribute, at least in part, to *dfmr1* null phenotypes (Yang et al., 2009). Bantam miRNA functions in germ cell development, where it genetically interacts with dFMRP to control the maintenance of germ line stem cells. Interestingly, *bantam* miRNA is also required in epithelial cells, but not the neurons themselves, to regulate the arborization of adjoining sensory DA neuron dendrites in *Drosophila* larvae (Parrish et al., 2009). It will be important to determine if this role is specific to *bantam* miRNA, or if other developmentally regulated miRNAs, such as miRNA-124, are also able to regulate synapse development in a similar trans-tissue mechanism. This work suggests a potential new mechanism by which dFMRP regulates neuronal morphogenesis from neighboring cell types, rather than neurons themselves. This hypothesis can be easily tested in the *Drosophila* FXS model using the powerful array of tissue-specific gene expression systems combined with genetic mutations in each gene. In the future, it will be important to expand dFMRP/miRNA interaction analyses from larval peripheral circuits into the central brain circuits to determine whether this mechanism allows dFMRP to regulate the development of central synapses responsible for FXS behavioral defects.

1.6.1. Developmental molecular analysis of dFMRP function

FMRP has been proposed to bind hundreds of transcripts and may act as a general mRNA regulator (Brown et al., 2001; Darnell et al., 2005b; Miyashiro et al., 2003). However, attempts to validate these mRNAs as *bone fide* targets of FMRP regulation *in vivo* have largely been unsuccessful. Therefore, the scope of FMRP function still remains a mystery. The majority of validated FMRP/dFMRP mRNA targets have been identified based on a candidate gene approach, rather than systematic analyses. The candidate approach is subject to serendipity, proceeds in a piecemeal fashion, and does not provide a broad overall picture of cellular functions controlled by FMRP. To circumvent these imitations, several attempts have been made to perform global analysis of FMRP mRNA-binding activity, and mRNA expression in null mutant animals, using microarray technologies (Bauer et al., 2008; Brown et al., 2001; D'Agata et al., 2002; Gantois et al., 2006; Zarnescu et al., 2005). For example, to identify dFMRP targets, immunoprecipitation was used to purify directly bound mRNAs that were then probed on microarrays (Zarnescu et al., 2005). As in mammals, the list of putative targets is long and, confoundingly, there is little overlap between mRNA sets identified by different approaches or different laboratories. Nevertheless, the use of combined microarray analysis on genes that genetically interact with dFMRP has proven valuable in identifying key dFMRP regulatory networks. For example, microtubule regulation was again highlighted in microarray analyses of dFMRP and its genetic interactor Sticky, a citron kinase that regulates microtubule organization (Bauer et al., 2008). Heterozygous loss of function *sticky* mutants can rescue rough eye phenotypes caused by

dFMRP overexpression, and similarly loss of dFMRP exacerbates *sticky* mutant eye phenotypes. Indeed, there is considerable overlap between the identities of mRNAs which show varied expression in *dfmr1* and *sticky* loss of function mutants, as assessed by microarray and quantitative reverse transcription PCR (Bauer et al., 2008). The high degree of mRNA regulation overlap strongly indicates that these diverse proteins regulate convergent pathways that alter the cytoskeleton, once again implicating the cytoskeleton as a key target of dFMRP regulation.

In addition to the still unknown range of FMRP mRNA targets, the nature of FMRP regulatory functions on those targets is similarly unclear. Both mammalian and *Drosophila* FMRP has been implicated in regulating mRNA transport, mRNA stability and mRNA translation (Ashley et al., 1993; De Diego Otero et al., 2002; Feng et al., 1997; Lagerbauer et al., 2001; Weiler et al., 2004; Xu et al., 2004; Zalfa et al., 2007; Zalfa et al., 2003; Zhang et al., 2007). For example, total brain mRNA levels are elevated in a development stage dependent manner in *dfmr1* null animals (Tessier and Broadie, 2008). In addition, many single candidate target transcripts have altered levels in *dfmr1* loss of function mutants (Table 1) (Epstein et al., 2009; Monzo et al., 2010; Monzo et al., 2006; Tessier and Broadie, 2008; Tessier and Broadie, 2011; Xu et al., 2004; Zalfa et al., 2007; Zhang et al., 2007). Clearly, from the developmental work in the *Drosophila* FXS model, RNA binding studies will need to be performed on controlled developmental time points. Indeed, many targets of dFMRP are likely transient in nature, identifiable only during specific windows of dFMRP function (Gatto and Broadie, 2008; Papoulas et al., 2010; Tessier and Broadie, 2008; Tessier and Broadie, 2011). Moreover, the sensitivity of *dfmr1* phenotypes to neuronal activity levels indicates that coupled activity-dependent assays are also needed. The *Drosophila* FXS model is particularly attractive for these studies because the entire tiled array of the *Drosophila* genome can be spotted on a single chip, compared to the multichip platform needed for mammalian genomes (Bertone et al., 2004; Oliver, 2006; Stolc et al., 2004). Tiled arrays would identify a wealth of additional information over classical arrays, including specific mRNA isoforms, noncoding mRNA segments and other classes of RNAs such as microRNAs (Matsumoto et al., 2007). Also promising is the rapidly advancing technology of RNA-Seq which can provide direct sequence analysis of dFMRP-bound transcripts (Marioni et al., 2008; Wang et al., 2009). Indeed, this powerful approach will likely soon overtake standard array technologies.

Of course, the *Drosophila* model is most valuable as a truly unique vehicle for genetic screens. Comparable screens cannot practically be done in the mouse FXS model. Unfortunately, this vital research avenue has yet to be fully explored, and the few limited *Drosophila* screens to date have focused on phenotypes arising from dFMRP overexpression (Reeve et al., 2008; Yao et al., 2010; Zarnescu et al., 2005). In the eye, excess dFMRP produces a degenerative rough eye phenotype, and an enhancer/suppressor screen identified both classes of genetic interactors. The majority of these genes have yet to be characterized, although multiple *lgl* mutations were defined as *dfmr1* suppressors, as discussed above (Zarnescu et al., 2005). Additionally, ubiquitous overexpression of dFMRP causes lethality, allowing a simple search for viable suppressors. Perhaps not surprisingly, the majority of suppressors were mapped to point mutations in *dfmr1* itself, and this has provided the basis for a detailed structure-function analysis of the dFMRP N-terminus, whose function was

previously unknown (Reeve et al., 2008). Also, a non-genetic, small chemical screen has been used to identify pharmacological suppressors of *dfmr1* (Chang et al., 2008). For unknown reasons, null *dfmr1* animals die when raised on high levels of dietary glutamate, and drugs that interfere with either GABA synthesis or transport are able to prevent this glutamate-mediated lethality. This work strongly suggests that GABAergic pathways are a critical component of dFMRP regulation (Chang et al., 2008). Consistently, an independent study showed that GABA receptor subunit expression is significantly decreased in *dfmr1* null brains (D'Hulst et al., 2006). Thus, drugs targeting GABAergic pathways may be a new starting point for therapeutic intervention in the human disease state. These results also inform about a possible FXS mechanism; specifically that the FXS disease state may represent an imbalance between excitatory and inhibitory synaptic connections (Gatto and Broadie, 2010). Further investigations of this hypothesis will be necessary to identify dFMRP mRNA targets involved in each type of circuit, and to determine how misregulation of those targets may be causally linked to the FXS disease state.

1.6.2. Proteomic analysis of dFMRP function

FMRP is best characterized as a translational regulator. Therefore, it is of the highest importance to understand changes in the proteome that occur in the absence of FMRP. A number of different proteomic approaches have been used in different tissues to assess which proteins may be aberrantly expressed in FXS disease models (Liao et al., 2008; Monzo et al., 2010; Papoulas et al., 2010; Zhang et al., 2005; Zhang et al., 2004). Surprisingly, while FMRP is reported to bind to hundreds of different mRNAs, the total number of proteins that detectably change by proteomic analysis is astoundingly small. Only a relative handful of proteins have been identified to either be up- or down-regulated in null mutants. The primary technology employed for these analyses has been 2-dimensional differential gel electrophoresis (2-DIGE) combined with mass spectrometry analysis (Monzo et al., 2010; Papoulas et al., 2010; Zhang et al., 2005; Zhang et al., 2004). This technology has been steadily advancing over the years and is well equipped to characterize either large changes in the complete proteome, or changes to single proteins. Nevertheless, as a standard electrophoretic gel is integral to this technique, there are many limitations, including the limited size of proteins that can be resolved on the gel and the requirement for the protein to be relatively abundant, i.e. low abundance proteins may not be identifiable. In the *Drosophila* FXS model, 2-DIGE has been performed on null *dfmr1* whole heads and isolated brains (Zhang et al., 2005; Zhang et al., 2004). Among over 1400 proteins queried, only 32 proteins were identified to have a significant change in level in the absence of dFMRP; that is, only ~2% of the proteins detectably changed (Zhang et al., 2005). Most of the abundance changes were exceedingly small (<10%), and many consisted of shifts in the ratio of post-translationally modified protein variants. Nevertheless, an important discovery from this initial proteomic work was the finding that brain monamine biosynthesis pathways as regulated by dFMRP. Both dopamine and serotonin levels are significantly elevated in the *dfmr1* null brain, owing to misregulation of rate-limiting biosynthetic enzymes (Zhang et al., 2005). These results are consistent with recent findings suggesting that different neurotransmitters and enzymes involved in neurotransmitter biosynthesis are misregulated in the FXS disease state (Fulks et al., 2010; Wang et al., 2010; Wang et al., 2008a). Thus,

despite limitations, 2-DIGE has proven effective at identifying physiologically relevant changes in protein expression.

2-DIGE proteomics has also been done on *dfmr1* null adult testes and embryos (Monzo et al., 2010; Zhang et al., 2004). In mutants, testes are enlarged and fail to properly form microtubules needed for spermatid axoneme development, and cleavage furrows fail to form properly in embryos. Once again, an exceedingly small number of protein changes were identified in these locations: only 23 and 45 proteins, respectively, were significantly altered in *dfmr1* null animals (Monzo et al., 2010; Zhang et al., 2004). Similar to results obtained from microarray analysis, the identity of proteins characterized in different 2-DIGE studies seldom overlaps. However, a few classes of proteins do seem to be constant. In particular, metabolic genes and chaperone/heat shock proteins are consistently identified in all analyses. In embryos, this later group was found to be directly targeted by dFMRP, as the chaperone complex CCT was misregulated in *dfmr1* mutants and the mRNA for specific subunits can be bound by dFMRP (Monzo et al., 2010). Nonetheless, each of these 2-DIGE analyses carries the same limitations. In no case has it been possible to effectively monitor the complete proteome; only proteins within a restricted size and PI range can be assayed. Moreover, only a single time point has been analyzed and no developmental time lines have been examined. This latter point is a particular concern giving that many mammalian and *Drosophila* phenotypes are only relevant during short periods of neuronal maturation, as discussed above. Thus, it is extremely likely that such limited proteomic analyses have missed significant protein changes that are critical to our understanding of the FXS disease state.

New proteomic approaches are being developed to address these concerns (Brewis and Brennan, 2010; Zhang et al., 2010b; Friedman et al., 2009). We have recently undertaken multiple methodologies to characterize protein changes in brain and synapse development. First, we have revisited advanced 2-DIGE proteomics with mass spec analysis of protein spots to perform the first studies controlled for developmental time. We have focused our work on four developmental time points, which show significant changes in candidate protein expression during late stages of brain maturation and activity dependent neural circuit refinement (Figure 1). Using a multiplexed gel system comprising four replicates for each time point, highly significant results have been obtained comparing not just how proteins change between *dfmr1* null to wildtype brains, but also how proteins change in each genotype across developmental time points. Principle component analysis (PCA) of the complete proteomics data set validates this time point based methodology. The largest component of variation across all samples is developmental stage, accounting for 35% of the variation, while the second largest component is genotype, accounting for 30% of the variation (Figure 2). Consistent with earlier studies, the number of identified protein changes remains relatively small, albeit several times larger than in adult tissues and with more striking changes in protein levels (Monzo et al., 2010; Zhang et al., 2005; Zhang et al., 2004). As before, metabolic proteins are the primary class found to be affected in the *dfmr1* null condition. However, this analysis also reveals significant effects on microtubule and actin cytoskeletal proteins, consistent with the genetic interaction studies described above. This latter finding further suggests that cytoskeletal dynamics may be regulated differently

in *dfmr1* mutants at different developmental time points. Thus, temporal analysis will add tremendous depth to our knowledge of the dynamic nature of dFMRP function.

Another recent advancement in proteomics is the use of isobaric tag labeling for quantitative mass spectrometry (Li et al., 2007; Unwin, 2010). The lack of gel separation in this technique removes any limitations based on protein size or PI range, which are unavoidable with gel-based technologies. The multiplexing of different mass tags allows for a significant number of biological replicates to be analyzed in unison, thus providing tightly quantitative data. We have performed this proteomic approach using proteins from *dfmr1* null and control brain synaptosomal preparations to enrich for proteins at the synapse (Figure 3). This fractionation dramatically eliminated the large number of metabolic and chaperone/heat shock proteins that have been isolated by other techniques. Instead, the majority of proteins found to change in abundance in *dfmr1* null synaptosomes are involved in neurotransmission and cytoskeletal dynamics, as parallel studies have predicted. Thus, selecting not just developmental time points, but specific subcellular locations for proteomic analysis is providing more pinpointed information about dFMRP function than classical global analyses have been able to reveal. Unfortunately, there is currently no one silver bullet methodology that will enable a complete picture of the function of dFMRP. Proteomic analysis, while critical for understanding functional changes, will not provide information as to whether affected proteins are directly or indirectly regulated by dFMRP. Likewise, classical global analysis of mRNA levels will also miss this important distinction. Probing microarrays, or using advanced RNA-Seq techniques with mRNAs that have been purified from dFMRP bound complexes will advance our understanding of direct versus indirect regulations though neither will inform us about the molecular significance of the protein-mRNA association. We will still have to ask, are these mRNAs regulated at the level of translation, stability or localization? Clearly, combinatorial techniques will need to be applied, and harnessing the power of *Drosophila* genetics to include multiple mutations into these global analyses will greatly enrich our understanding of dFMRP spatial and temporal regulation of mRNA targets.

1.7. Moving Forward

The *Drosophila* FXS disease model has proven to be an extremely powerful system to analyze the molecular players and cellular mechanisms involved in this debilitating disease. While the focus is rightly still on the core player, FMRP itself, genetic analysis is making it increasingly clear that understanding how other proteins cooperate to regulate pathways in common with dFMRP is equally important for our ability to design successful intervention strategies. To this end, exploiting the genetic strengths of the *Drosophila* system will be absolutely critical for advancing future research. Primarily, this means designing and undertaking new forward genetic screens to take advantage of the robust phenotypes in the *Drosophila* FXS model. In addition, we must incorporate new techniques and embrace new understandings of *Drosophila* biology. For example, both the adult brain mushroom body circuit and embryonic motor circuit have been shown to remodel in a developmentally controlled manner in response to neuronal activity (Tessier and Broadie, 2008; Tripodi et al., 2008). This is contrary to the dogma that invertebrate circuits are hardwired. Rather, the plasticity exhibited by *Drosophila* circuits closely resembles the plasticity in mammalian

systems, and the *Drosophila* FXS model work highlights the need to understand dFMRP function in activity-dependent pathways in critical developmental refinement processes. Furthermore, although the *Drosophila* system is rightly valued for genetic prowess, new advances in electrophysiology and *in vivo* imaging are providing critical new tools for cellular and physiological analyses in this disease model. It is now possible to record single neuron synaptic responses within intact brains, and to image dynamic physiological responses to stimuli such as light or odor (Gu and O'Dowd, 2007; Jayaraman and Laurent, 2007; Yu et al., 2006; Zhang et al., 2010a). New generation genetically encoded tools such as advanced calcium reporters (e.g. gCAMP3.0), light-activated ion channels (e.g. channelrhodopsin2) and the newly-described potassium selective glutamate channels (HyLighter) can be specifically targeted to neuron subsets and used to directly monitor or drive synaptic function (Janovjak et al., 2010; Lin, 2010; Schoenenberger et al., 2010; Tian et al., 2009). In addition, new fluorescent probes can be genetically encoded and targeted to virtually any neural circuit. For example, the DenMARK probe specifically localizes to dendritic regions and allows for previously unattainable resolution of these densely packed arborizations (Nicolai et al., 2010). All of these tools are available for *Drosophila* FXS model researchers and can be used to probe the intersections between the developmental programs and the activity-dependent mechanisms in which dFMRP is intertwined. Lastly, it is imperative to correlate molecular and genetic analyses with behavioral outputs of targeted circuits. The *Drosophila* system has a long and distinguished history in the study of learning and memory, circadian activity, aggression, courtship or other behavioral paradigms, and applying these assays to the *Drosophila* FXS model will continue to enhance our understanding of the evolutionarily conserved underpinnings of Fragile X Syndrome.

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Figure 1. dFMRP transiently suppresses protein expression during development

Protein was extracted from control (*w¹¹¹⁸*) and *dfmr1* null (*dfmr1^{50M}*) heads at the indicated hours post-eclosion at 25°C. Two candidate proteins probed by Western Blot analysis indicate transient upregulation during the first 24 hours in the absence of dFMRP. α -Tubulin is a loading control.

Figure 2. 2-DIGE proteomic analysis of *dfmr1* nulls over developmental time

Protein was extracted from wildtype (WT) and *dfmr1* null heads at four developmental times points: 72 hour (P3) and 96 hours (P4) after puparium formation, and 0-3 hours (E0) and 12 hours (12E) after eclosion. **A**) Extracts from 4 biological replicates at each time point were labeled and subjected to 2-DIGE. A representative gel is shown. **B**) Principle component analysis reveals strong statistical correlations for each of four replicates for the two genotypes. **C**) Individual proteins show developmental stage specific changes in expression. An example shown for the four time points shows aberrant persistence of high expression in the mutant at E0 and E12. **D**) A single time point from panel C at eclosion, showing the relative abundance of a protein in the control and null mutant.

Figure 3. Purification of synaptoneuroosomes for proteomic analyses

Head protein extracts were subjected to multiple rounds of high speed centrifugation over sucrose gradients to purify the synaptosomal compartment. Western analysis of each step shows strong enrichment for presynaptic protein synaptotagmin (SYT) and postsynaptic proteins discs large (DLG), but not α -Tubulin. Synaptosomal preparations can be used to increase targeting for synaptic protein changes in proteomic analyses.

Table 1

Genes identified to genetically interact with dFMRP

Gene	Function	Tissue interaction identified	mRNA bound by dFMRP	mRNA levels in <i>dFmr1</i> nulls	protein levels in <i>dFmr1</i> nulls	Reference
trailerhitch	mRNA processing	embryo	yes	upregulated	downregulated	Monzo et al. 2006
cuprin	mRNA translation	embryo	ND	ND	ND	Papoulias et al. 2010
CCT3	chaperone complex	embryo	yes	ND	postranslationally modified	Monzo et al. 2010
CCT4	chaperone complex	embryo	yes	ND	downregulated	Monzo et al. 2010
CCT7	chaperone complex	embryo	yes	ND	upregulated	Monzo et al. 2010
orbl	mRNA translation	ovary	ND	ND	upregulated	Costa et al. 2005
dbl	ubiquitin ligase activity	ovary	ND	upregulated	unaffected	Epstein et al. 2009
bantam mRNA	miRNA	ovary	yes	unaffected	-	Yang et al. 2009
sticky	citron kinase	eye	ND	ND	ND	Bauer et al. 2010
orb2	mRNA binding	eye	ND	ND	ND	Cziko et al. 2009
fusch/MAPIB	microtubule binding	larval NMJ	yes	ND	upregulated	Zhang et al. 2001
dmGlurA	G-protein coupled receptor	larval NMJ	ND	ND	upregulated	Pin et al. 2007, Repicky and Broadie 2009
lg1	cytoskeletal binding	larval NMJ/eye	ND	ND	ND	Zarnescu et al. 2005
aPKC	protein kinase	larval NMJ	ND	ND	ND	Zarnescu et al. 2005
argonaut 1	mRNA processing	larval NMJ/eye	ND	ND	ND	Jin et al. 2004
argonaut 2	mRNA processing	larval NMJ	ND	ND	ND	Pepper et al. 2009
spastin	microtubule severing	larval NMJ/eye	ND	ND	unaffected	Yao et al. 2010
Rac1	GTPase activity, actin organization	DA neurons	yes	ND	ND	Lee et al. 2003
pickpocket	sodium ion channel	sensory neurons	yes	upregulated	ND	Xu et al. 2004
chickadee/profilin	actin binding	lateral clock circuit neurons	yes	upregulated	upregulated	Reeve et al. 2005, Tessier and Broadie 2008
Lark	mRNA binding,actin organization	clock circuit/eye	ND	ND	unaffected	Sofola et al. 2008
staufen	mRNA translation	adult brain	ND	ND	ND	Bolduc et al. 2008
cheerio/flimmin A	actin binding	adult brain	ND	ND	Reduced after spaced training	Bolduc et al. 2010

ND: Not Determined