TRANSLATIONAL CONTROL OF SYNAPTIC PLASTICITY

by

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ABSTRACT

Activity-dependent and synapse-specific translation of mRNAs is required for long-term changes in synaptic strength (or efficacy). However, many of the components mediating repression, transport and activation of mRNAs are unknown. Translational control in neurons is a highly conserved process and mediated by a ribonuclear particle (RNP). This study shows that RNPs in Drosophila neurons are similar not only to mammalian neuronal RNA granules but also to yeast P-bodies, cytoplasmic foci involved in translational repression and RNA decay. The evolutionarily conserved proteins Me31b and Trailer Hitch localize to RNA granules. Me31b and Trailer Hitch are required for normal dendritic growth. Mutations in Me31b and Trailer Hitch suppress phenotypes resulting from overexpression of Fragile X Mental Retardation protein, suggesting that both proteins may act as translational repressors. In addition, this study reports the identification of novel translational repressors in neurons. Using the overexpression phenotype of Fragile X Mental Retardation protein in a candidate-based genetic screen, I identified dominant suppressor mutations in five genes, including Doubletime/Discs Overgrown, Orb2/CPEB, PolyA Binding Protein, Rm62/Dmp68 and SmD3. Like Me31b and Trailer Hitch, all five proteins localize to neuronal RNPs. Overexpression of each proteins affects dendritic branching of sensory neurons in Drosophila. Identification and further characterization of these novel RNP granule components and dFMR1-interacting proteins may provide further insights into the mechanisms controlling translational in dendrites.
CHAPTER ONE

THE IMPORTANCE OF LOCAL PROTEIN SYNTHESIS IN SYNAPTIC PLASTICITY
Introduction

Perhaps the most investigated question in all of neuroscience is how memory is encoded in the brain. Over the last century remarkable progress has been made in elucidating the engram or mark of memory within the nervous system at all experimental levels (behavioral, cellular, electrophysiological, molecular); however, a coherent amalgam of the process continues to elude us. From a molecular and cellular perspective, the recognition of the requirement for de novo gene transcription and protein translation for long-term changes in synaptic strength and behavioral memory have begun to elucidate how cellular changes occur following biologically significant synaptic events. More specifically and most relevant to this dissertation, that the initial protein synthesis required for these changes occurs “locally”, i.e. at the dendritic side of the synapse, and is using pre-existing, synaptically localized RNAs as templates. This insight has led to an explosion in the field of translational control in neurons. In this chapter, I will review the discoveries that highlighted the importance of new protein synthesis for synaptic plasticity, and review the evidence for “local” or dendritic protein synthesis. I will also briefly discuss the role of local protein synthesis for another phenomenon, called “synaptic capture”.

Protein Synthesis is required for memory and long-lasting changes in synaptic strength

The initial evidence that translation of new proteins may indeed occur in the brain following learning comes from three areas of study in the nervous system: behavioral, electrophysiological and finally cellular studies.
Protein synthesis and consolidation of memory: The early behavioral evidence.

The first behavioral evidence for a requirement of protein synthesis for learning and memory was uncovered in the early 1960s when researchers found that infusion of the protein synthesis inhibitor puromycin into the hippocampus blocked maze learning in mice (Flexner et al., 1963). Later studies then uncovered that long- but not short-memory requires protein synthesis. Specifically, for the transition from short- to long-term memory, termed consolidation (Flexner, 1967). Parallel experiments at the time were being performed in goldfish, which demonstrated a similar effect of puromycin on memory when trained by a shock avoidance task (Agranoff and Klinger, 1964) but did find exceptions to the puromycin block. Follow-up examinations also demonstrated the protein synthesis requirement for memory consolidation in goldfish, which the authors refered to as “fixation” (Agranoff et al., 1965).

Later studies in insects (flies and honeybees) demonstrated that the requirement of protein synthesis for memory consolidation holds true across species (Tully et al., 1994; Wustenberg et al., 1998). Tully and colleagues demonstrated that long-term memory in flies was disrupted upon feeding the protein synthesis inhibitor cyclohexamide immediately after training (Tully et al., 1994). Other forms of one-trial learning such as foot-shock conditioning in rats is also dependent on new protein synthesis, as is behavioral sensitization (Castellucci et al., 1989) and associative learning in Aplysia (Montarolo et al., 1986), passive avoidance learning in birds (Freeman and Young, 1999; Tiunova et al., 1996) and longer term habituation response in crabs (Pedreira et al., 1996).
In mice, several groups have attempted to alter protein translation with various methods and have succeeded in altering behavior as well. For example, mice conditionally expressing a dominant-negative regulator of MAP kinase (MEK1) exhibited an inhibition of protein translation, and showed deficits in late-phase electrophysiological correlates of memory (long-term potentiation, introduced in detail in the next section), spatial learning, and contextual fear conditioning (Kelleher et al., 2004). Removing key molecules involved in regulation of protein translation alters protein synthesis and has a demonstrated effect on behavior (Klann and Dever, 2004). In knockout mice of 4E-BP2 protein (a translational inhibitor that functions through binding with the eIF4E, the cap binding protein) researchers found that the behavior of the animals was altered such that they exhibited enhanced spatial learning and long-term contextual fear conditioning (Banko et al., 2005). The authors hypothesized that an enhanced basal rate of translation in the knockout mice was responsible for these changes (there were also changes in LTP). Sonenberg and colleagues (Costa-Mattioli et al., 2007) found similar effects when they examined plasticity in mice lacking GCN2 (a protein kinase that inhibits translation initiation by phosphorylating eukaryotic initiation factor 2α). Again, like in the previous study, they observed an enhancement of learning in spatial training following “weak” training. Interestingly, the intense training led to poorer results when tested for spatial memory.

Finally, a study of spatial memory in knockout mice of cytoplasmic polyadenylation element binding protein (CPEB) found that these animals exhibited normal spatial learning but impaired extinction of memory (Berger-Sweeney et al., 2006). Extinction means that the normal loss in frequency or intensity of a response when
reinforcement was removed did not occur as in control, in other words the animals retained the memory longer or could not “unlearn”.

These experiments demonstrate that protein synthesis is required for longer forms of behavioral “memory” and that protein synthesis was indeed occurring in the brain in a manner physiologically relevant to longer forms of memory formation. However, the differential requirement of protein synthesis for short versus long-term memory raises the question of what defines and separates these two states on a molecular and cellular level, or how does protein synthesis facilitate memory consolidation from short to long-term? A detailed discussion of this question is beyond the scope of this introduction but a brief answer may be: short-term memory is likely caused by modifications of existing proteins while long-term memory requires permanent structural changes brought on by both translational and transcriptional activation (Dudai, 2004).

**Protein synthesis and consolidation of memory: The physiological underpinnings.**

The discovery of long-term potentiation (LTP) by Bliss and Lomo (Bliss and Lomo, 1973) formed the foundation for a potential cellular and molecular mechanism of learning and memory and provided an appropriate experimental model to dissect learning and memory on a subcellular level. LTP has since been dubbed “the physiological correlate” of learning and memory and it has not only demonstrated how neuronal connections may be modified following experience but it has also provided a rich model system to study the cellular and physiological requirements and constraints of synaptic plasticity.
Bliss and Lomo used the well-known anatomical structure of the rabbit hippocampus to stimulate the perforant pathway (cells in the entorhinal cortex synapse on CA3 pyramidal cells in the hippocampus via the perforant path) and record from postsynaptic CA3 cells (Bliss and Lomo, 1973). Test stimulations of the perforant pathway at low stimulation frequencies yielded a baseline synaptic response, measured by excitatory field potentials. A high frequency stimulation train (100 Hz) was then applied for 3-4 seconds. After this high frequency stimulation, stimulation of the perforant path at low frequency (test condition) showed an increase in excitatory field potentials and a decrease in the latency of output from CA3. The heightened high frequency response was robust for the duration of the experiment (6 hours) and since the change was “long” lasting, the phenomenon was named long-term potentiation (LTP).

This experiment demonstrated that high activity patterns in the perforant pathway can in fact increase or modify the synaptic response of a downstream population of neurons for an enduring amount of time. It also provided the first neurophysiological evidence for an enhanced communication between two populations of neurons upon “learning”, something that had been speculated to occur within the brain during learning and memory for more than a decade prior (Hebb, 1949). Whether this phenomenon is in (Kim and Linden, 2007) but it is widely accepted as an appropriate model for studying learning and memory.

Like memory, LTP also demonstrates two phases, an early-phase (1-3 h) and a late-phase (>3 h), dependent upon the number and timing of the stimulus used for induction. It has been well established that the late phase but not the early phase of LTP is protein synthesis-dependent (Bliss and Collingridge, 1993; Frey and Morris, 1997;
Krug et al., 1984). Of note, one exception of this rule is the “neurotrophic factor induced potentiation of the Schaffer collateral”, a specific type of LTP that requires local protein synthesis immediately (Kang and Schuman, 1996). Additionally, inhibition of protein synthesis has a specific time window to effectively block late LTP (L-LTP). Application of a protein synthesis inhibitor around the time of LTP induction proves the most effective way to block L-LTP formation. In fact, application of an inhibitor prior to stimulation by an L-LTP producing protocol leads to only a potentiation resembling the rapidly decaying early LTP (Otani et al., 1989). Careful experiments have been done to demonstrate that it is in fact translation of existing mRNAs that is required first, rather than translation of new mRNA (although that is required later) (Kelleher et al., 2004).

Since L-LTP results in the formation and stabilization of new synapses it is likely that the newly synthesized proteins are synaptic modifier. However, there is also evidence for the translation of translation factors and other signaling proteins following LTP induction (Abraham and Williams, 2008).

In conclusion, protein synthesis is required for long-term memory and the likely physiological correlate of memory, LTP. Importantly, at least some of the new protein translation required for long-lasting forms of synaptic plasticity is in fact occurring locally within dendrites, close to the synapse. Researchers investigating the cell biology of the neuron demonstrated that dendrites not only contain components of the translational machinery and mRNAs but are also translationally competent. This allows the dendrite to compartmentalize plastic changes within the neuron, and likely the dendrite itself such that only specifically activated synapses are being modified.
Figure 1.1 Representation of the temporal phases of long-term potentiation (LTP).

A, A train of tetanic stimulation produces a potentiation of the synaptic response that slowly decays within approximately 60-90 minutes and is considered early-phase LTP (E-LTP). E-LTP is insensitive to both translational and transcriptional inhibition. B, Late-phase LTP (L-LTP) is produced by 4 repeated tetani and lasts on the order of hours. When a transcriptional inhibitor (Actinomycin D) or translational inhibitor (Anisomycin) is applied the potentiation is unaffected for the first 60-90 minutes and then mirroring E-LTP returns to baseline. Figure excerpted from Kelleher et al. (Kelleher et al., 2004).
**Cellular evidence for dendritic protein synthesis**

In the early 1980s, the dogma was that protein synthesis occurred only in the neuronal cell body (Steward and Schuman, 2003). However, the identification of synapse-associated polyribosomes shattered this assumption (Steward and Levy, 1982). Using electron microscopy, Steward and Levy (1982) observed polyribosomes at the base of spines in granule cells of the dentate gyrus. Serial section reconstructions revealed that the incidence of polyribosomes at the base of spines was approximately 25%. Other studies looking at single ribosomes yield estimates closer to 50% in the spine base and even higher for other locations (Spacek and Hartmann, 1983).

Are dendritic ribosomes translationally competent? Subcellular fractionation has been the main way to address this question. However, this approach is somewhat limited because of contamination from neuronal and glial cell bodies (Chicurel et al., 1993). Using radiolabeled amino acids and subsequent cellular fractionation does in fact demonstrate the presence of radiolabeled proteins almost immediately after stimulation in a time window that is insufficient for protein transport from the cell body (Steward et al., 1991). This data, however, should be taken with an element of skepticism because of the possible contamination of subcellular fractionation. More refined methods leading to a subcellular fractionation with greater purity have demonstrated the translation of CAMKII α subunit in synaptoneuroosomes (a synaptic, subcellular fraction isolated by sucrose gradients) (Scheetz et al., 2000).

Perhaps the most convincing work demonstrating the translational competence of ribosomes in dendrites comes from cultured hippocampal neurons and the use of a GFP-tagged reporter. Aakalu et al. (Aakalu et al., 2001) used the 3' UTR of CAMKII mRNA
attached to a myristolated GFP, which inserts CAMKII-GFP into the membrane at the translation site. Unstimulated cultured neurons showed a basic GFP fluorescence in the dendritic arbor while chemically stimulated neurons (by BDNF application) showed a much larger GFP fluorescence (4-fold increase). Additionally, physical separation of dendrites from the cell body did not abrogate fluorescence in unstimulated or stimulated dendrites, confirming that translation was occurring independent of the cell body (Aakalu et al., 2001). Finally, stimulation known to induce LTP has been shown to also induce the movement of polyribosomes into spines (base to head), suggesting that the transport of the translational machinery is sensitive to neuronal activity (Ostroff et al., 2002).

The first RNA identified in dendrites was the MAP2 RNA using in situ hybridization (Garner et al., 1988). Since then, many more RNAs have been found in dendrites by both transcript specific methods (e.g. in situ hybridization, PCR) and en masse methods (Syto 14, EtBR, microarrays, macroarrays; (Eberwine et al., 2002; Kiebler et al., 1999; Tang et al., 2001). A review by Eberwine et al. (Eberwine et al., 2002) compiled an incomplete list of RNAs found in dendrites listing no less than 125 types of transcripts. Following that, his lab identified 400 mRNAs localized to dendrites of cultured hippocampal neurons (Eberwine et al., 2001) representing approximately 5% of expressed genes (Eberwine et al., 2001). To identify localized RNAs involved in long-term facilitation in Aplysia, the marine sea slug, a cDNA library was made from isolated sensory neurites. Complete sequencing of this library led to the identification of 250 distinct transcripts primarily enriched for cytoskeletal elements and proteins involved in translation (Moccia et al., 2003). At present the exact makeup of RNAs found in dendrites, exclusively or otherwise is up for debate. Additionally, separating those
transcripts required for synaptic maintenance from those that regulate synaptic strength is just beginning to be addressed, if in fact such a distinction can be made.

**Is “local” or dendritic protein synthesis required for synaptic plasticity?**

By now, new protein synthesis has been established as a requirement for long-lasting forms of memory and dendritic/synaptic protein synthesis appears to be the likely mechanism. The first study to actually show that the protein synthesis occurred “locally” or within the dendrite was in 1993 when the authors observed that the majority (90%) of tritiated amino acids were incorporated into the dendritic laminae of the hippocampus following stimulation (Feig and Lipton, 1993). The time-scale was too short for it to be transported from the cell body. However, the stimulation protocol was not known to cause changes in synaptic strength, and therefore, no direct link was made between the dendritic protein synthesis and synaptic potentiation.

The first study to directly link local protein synthesis with synaptic enhancement came from the Schuman lab (Kang et al., 1996). They demonstrated that local protein synthesis in dendrites is required for the rapid enhancement of synaptic transmission upon the exposure to the growth factor BDNF.
Figure 1.2 Pictoral summary of strategies used to determine the role of local protein synthesis within synaptic plasticity. Excerpted from Sutton and Schuman (2006).
Studies that actually directly link behavioral memory to local translation are rare due to the technical difficulties of such experiments. However, two studies have directly addressed this issue. Ashraf et al. (2006) was the first to examine dendritic protein synthesis in the antennal lobe of *Drosophila* during olfactory learning, and has indeed demonstrated that local protein synthesis occurs at the site where learning is assumed to occur. To do this, Ashraf et al. (2006) trained flies expressing a yellow fluorescent protein (YFP) reporter of dendritic protein synthesis (3′UTR from *CAMKIIα* RNA containing the dendritic targeting and a YFP coding sequence) and compared YFP expression levels in different antennal lobe glomeruli following olfactory learning. The authors reported that the two odors presented resulted in stimulation of YFP synthesis in some glomeruli of trained relative to untrained flies. This suggests that local translational activation in the dendritic regions of the antennal lobes does in fact occur when experiences are encoded into long-term memory.

Another more direct study linking the role of dendritic protein synthesis to learning and memory is from Miller et al. (2002). The authors tested spatial and contextual memory in mice that lacked the 3′UTR of the *CAMKII* mRNA, the location of the dendritic targeting element of the *CAMKIIα* mRNA, within the hippocampus. This leads to a near elimination of synaptic *CAMKIIα* within the dendritic laminae of the hippocampus. These mice also exhibit decreases in both spatial and contextual fear conditioning paradigms. This supports the idea that the establishment of long-term hippocampal-dependent memories requires dendritic protein synthesis. However, because this knockout was not temporally controlled, there is the possibility that developmental effects may lead to the memory impairment.
Although large amounts of behavioral data are limited in their interpretation, overwhelming evidence from multiple areas of neuroscience and many model systems has reinforced the requirement of new “local” protein synthesis for longer forms of synaptic plasticity. The importance of studying and understanding the involved regulatory mechanisms, the mRNAs locally targeted and translated, and their relationship to neuronal activity can be further underscored by the role of local protein synthesis in another important property of the nervous system: synapse-specificity of LTP and a mechanism referred to as “synaptic capture”.

**How do synapses communicate within a neuron?**

Two recent studies address the question of how new proteins/mRNAs may be appropriately targeted and how synapses may be appropriately potentiated. Initially, LTP studies uncovered the ability of a neuron to potentiate two synapses equally, even if one is stimulated at a level not normally significant enough to induce potentiation of the synapse (McNaughton et al., 1978). This results implied that some diffusible factor must circulate in the cell after a sufficiently strong stimulus. Other synapses receiving any amount of stimulation can then “capture” the circulating diffusible factor, which somehow jump-starts the process of longer-term synaptic change. Exactly how this occurs is still not known, but the experiments performed by Uwe Frey in Richard Morris’ lab and Kelsey Martin and colleagues in Eric Kandel’s lab have demonstrated that it is dependent on local protein synthesis at the original site of stimulation.

Frey and Morris (Frey and Morris, 1997) showed that local protein synthesis was required for “synaptic capture” to occur by the application of a protein synthesis inhibitor
directly to the strongly stimulated synapse following tetanic stimulation. As expected, based on our knowledge of LTP, this blocks late LTP formation. Additionally and quite surprisingly, this also blocks the potentiation at the second synapse where a suboptimal stimulus for potentiation was given and normally would lead to equal potentiation as the first stimulus. This demonstrates is that local protein synthesis is required for potentiation to occur associatively at two or more synapses (Frey and Morris, 1997).

Consistently, studies asking the same question in a very different system determined very similar requirements of local protein synthesis (Martin et al., 1997). In the late 1990s Kelsey Martin working in Eric Kandel’s lab made the *Aplysia* system ever more reductionist by culturing a bifurcated sensory neuron onto to two motor neurons. Just as in the intact animal, five pulses of 5-HT application increases the post-synaptic EPSP for up to 24 hours and aptly named the phenomenon long-term facilitation (LTF; see Figure 1.3). Mirroring the findings in LTP, both branches of a bifurcated sensory neuron exhibited LTF equally, even if one side was stimulated only with one pulse of 5-HT (see Figure 1.3). Normally, one pulse of 5-HT is sufficient to induce only a transient potentiation, termed short-term facilitation (STF).

Like in LTP and memory, LTF is also dependent on protein synthesis while STF is not. And just as in the Frey and Morris experiment, local application of protein synthesis inhibitor at the site of 5-HT stimulation blocked LTF at this site and the second site receiving one pulse of 5-HT, which normally potentiates equally following this protocol. Despite the fact that this is in a separate organism (*Aplysia*), a separate culture system and that the potentiation occurs pre-synaptically, as opposed to post-synaptically, it again implies that a protein synthesis dependent diffusible element is required to confer
synapse-specificity, and reinforces the important role of local protein synthesis in the nervous system.
Figure 1.3 Long-term Facilitation is Synapse-Specific. Capture is initiated by 5 pulses of 5-HT at one synapse of the bifurcated sensory neuron and then captured by 1 pulse of 5-HT at the other synapse. Excerpted from Kandel (2001).
Summary

Above I have presented evidence that long-lasting forms of synaptic plasticity not only require new protein synthesis but in fact require translation of existing mRNAs within the dendrite or synapse, a type of translation referred to as “local”. Since these remarkable discoveries, research has focused on identifying not only the mRNAs that are locally translated but also on the regulatory factors involved in this process. Remarkably and to the benefit of the field, many of the regulatory factors for translational control are conserved with other systems allowing evolutionary-based approaches to identify new regulatory factors.

I will spend the next chapter discussing the most relevant problems of translational control regulation in neurons, which are: (1) How are mRNAs targeted and transported to synapses? (2) How is their translation repressed during transport and finally (3) How is their translation induced or repressed upon synaptic activity?
CHAPTER TWO

MECHANISMS OF TRANSLATIONAL CONTROL
Introduction

As I have discussed in the first chapter, translational control is required for two important aspects of learning and memory: long-term changes in synaptic strength and the method by which synapses communicate within the neuron, otherwise known as the “synaptic tagging”. These properties are likely conferred to neurons by translation of “on site mRNAs”. For this to occur, mRNAs must be transported and targeted to synapses, and then stored at synapses in a translationally quiescent state. Upon sufficient activation of the synapse, the mRNAs must then be translated. Therefore, I have divided the discussion into three parts:

1) Transport and targeting of existing mRNAs to postsynaptic sites
2) Translational repression of mRNAs at synapses
3) De-repression and translation of mRNAs upon synaptic activation

I will first give a brief overview of translation and introduce the model systems that have provided the most insight into translational control. Because much evidence suggests that the machinery of translational control is conserved among species, discussion of mechanisms occurring in non-neuronal system will be presented, if there is little evidence from neuronal studies.

An overview of translation.

Protein translation, the process of synthesizing protein from mRNA, is a three-step process: initiation, elongation, and termination. I will first describe cap-dependent
translation in three-steps and then provide a brief overview of cap-independent translation.

**Initiation**

Initiation of translation is the step during which the mRNA is becoming associated with the 40S ribosomal subunit. This occurs in preparation for elongation and involves the interaction of proteins with a tag bound to the 5'-end of an mRNA molecule, the 5' cap. The eukaryotic Initiation Factor 3 (eIF3) associates with the small ribosomal subunit, and sterically prevents the large ribosomal subunit from prematurely binding before the eIF4F initiation complex is complete (see Figure 2.1). The eIF4F complex consists of three other initiation factors: eIF4A, eIF4E and eIF4G, each with its own role in initiation.

eIF4A is a DEAD box-helicase responsible for removing secondary structure from the RNA. eIF4E is a cap-binding protein, whose binding is often cited as the rate-limiting step of cap-dependent initiation. eIF4G is a scaffolding protein which directly associates with both eIF3 and the other two components of the eIF4F complex (4E and 4A). The final component that binds to the scaffold (4G) is Poly-A Binding Protein (PABP). PABP binds the poly-A tail of most eukaryotic mRNA molecules circularizing the RNA. This circularization leads to an increase in translation efficiency because the translation complex can remain intact and does not need to reassemble after each round of translation (Sachs and Varani, 2000).

Once the “initiation complex” is assembled, ribosomes are able to bind the mRNA, and the accompanying protein factors move along the mRNA chain towards its
3'-end, scanning for the 'start' codon (AUG) on the mRNA. The Met-charged initiator tRNA is brought to the P-site of the small ribosomal subunit by eukaryotic Initiation Factor 2 (eIF2). At this point the original complex dissociates, the 60S RNA binds and the complete ribosome (80S) then commences translation elongation.
Figure 2.1 Schematic of the translational initiation complex. The translation initiation complex begins to assemble when eIF4E binds the cap. The scaffolding protein, eIF4G, binds eIF4A, eIF4E and PABP circularizing the RNA and allowing eIF3 to bring the ribosome to the complex.
**Elongation**

Translational elongation describes the period between which the 'start' and 'stop' codons are read and the corresponding proteins are assembled. The initiator tRNA occupies the P site in the ribosome, and the A site receives an aminoacyl-tRNA and a peptide bond is formed between the two amino acids. During peptide elongation, each additional amino acid is added to the nascent polypeptide chain using its appropriate tRNA in a three-step cycle (Lehninger et al., 2008).

** Termination**

Termination is the stage at which the stop codon is reached causing the release factor to bind and the release of the nascent protein into the cell (Lehninger et al., 2008).

**Cap-independent translation**

Cap independent translation involves the commencement of translation at an Internal ribosomal entry site (IRES). First discovered in a virus as an ingenious way to inhibit host translation while promoting its own viral transcripts (Bienkowska-Szewczyk and Ehrenfeld, 1988) but now known to occur throughout biology. IRES sequences are often considered condition and tissue-specific. For example the N-myc IRES is active in multiple cell types but most active in neuronal cells (Jopling and Willis, 2001). The identification of IRES sequences in dendritically localized mRNAs (RC3/Neurogranin, ARC, CamKIIa, dendrin, MAP2; Pinkstaff et al., 2001) and the switch to IRES-dependent translation following synaptic activity in the *Aplysia* bag cell system (Wayne et al., 2004) has renewed interested in the possibility of learning and memory utilizing
this strategy to switch from maintenance type translation to synaptic activity induced translation. This will be addressed again in more detail in the last section of this chapter.

**Definition of terms**

mRNAs are regulated by two types of factors, *cis* and *trans* factors. *Cis* factors refer to signals within the mRNA transcript while *trans* factors refer to proteins that regulate the mRNA by binding to it. *Cis* factors are most often found in the 3’ UTR of the mRNA and *trans* factors are any RNA-binding proteins.

**Translational regulation across systems**

One cannot go on to describe the details of translational control without paying homage to developmental and yeast biologists. Much of what we know about translational control in neurons is gleaned from these fields. Additionally, the most successful attempts identifying neuronal translational control factors have used an evolutionary approach. Below I highlight the advantages each area presents.

**Drosophila germline**

*Drosophila* embryogenesis and oogenesis has been a valuable system for studying protein localization, due to the spatial-temporal localization of mRNAs during development. Much of the success comes from the ability to temporally and spatially control gene expression along with a huge repository of genomic data and pre-existing mutant strains.
Yet another attribute of *Drosophila* biology is its short generation time, the ease of culturing flies in the lab, and the free sharing of mutant strains and reagents. More specifically, the *Drosophila* oocyte is easily accessible and simple to collect. The complex temporal-spatial targeting of RNAs during development provides an elegant and simple system to study translational control. As an egg develops, it enters the sixteen cell stage where the most posterior cell becomes the oocyte while the remaining cells develop into nurse cells, which produce massive amounts of maternal mRNAs that are trafficked through intracellular pores (called ring canals) into the oocyte. These mRNAs remain initially untranslated. Soon after fertilization, the mRNAs are transported to specific locations such that their distribution in the oocyte becomes highly polarized. This mRNA transport requires not only transport factors but also translational regulators, which keep the mRNAs quiescent and prevent degradation until appropriate cues activate translation.

Three specifically localized mRNAs (*bicoid*, *nanos* and *oskar*) provided much insight into the regulation of RNA translational regulation in oocytes. Binding of RNA binding proteins to consensus sequences in the 3’UTR of mRNAs is the most common form of regulation (Standart and Jackson, 1994). In conjunction with a cap-binding protein (e.g. Cup) and another factor (e.g. Bruno), this mechanism circularizes the mRNA in a form inaccessible by the initiation complex (also referred to as masking, discussed in the translation repression section). Additionally, most mRNAs that are repressed and targeted early in oocyte development encode for translational repressors themselves. For example, *nanos*, whose mRNA is posteriorly localized in the egg and translated upon fertilization, is a translational repressor of the mRNA for *hunchback*, which is required for the posterior patterning of the embryo, the successive step in development of the
embryo. *Nanos* mRNA is translated after it is localized to the posterior pole. Subsequently, the Nanos protein, through Pumilio, binds to the Nanos response element (NRE) in the *hunchback* mRNA 3’UTR, and causes translational silencing, a requirement for appropriate abdomen formation in the embryo (Johnstone and Lasko, 2001).

**Xenopus oocyte**

The *Xenopus* oocyte has taught us much about the process of polyadenylation during development (Radford et al., 2008). Like all immature oocytes, *Xenopus* oocytes arrest in prophase 1 of meiosis (Philpott and Yew, 2008). Oocytes have been provided with large amounts of maternal mRNAs, which remain untranslated until a cue for maturation is signaled. The mRNAs with a specific 3’UTR signal, called a cytoplasmic polyadenylation element or CPE, remain translationally quiescent until maturation when the progesterone signal removes the translational inhibition and polyadenylation occurs. This allows the transcripts to be activated and oocyte maturation occurs. Although this is believed to occur in most organisms, the size and accessibility of these germ cells made them a favored model for studying developmentally regulated repression and activation of mRNAs (Tadros and Lipshitz, 2005).

**Yeast**

Yeast has been a cornerstone of RNA research because it is easy to manipulate genetically. mRNA localization and repression of *Ash1* mRNA is used to create the budding yeast offspring (Beach and Bloom, 2001) and since this discovery the field of yeast translational control has exploded. More recent work discovered of what is
called Processing bodies or P-bodies in yeast (Sheth and Parker, 2003). P-bodies are RNA and protein containing granules initially thought to be the site of RNA decay. The formation of P-bodies is induced by environmental stress (Sheth and Parker, 2003). These conclusions were drawn from the identification of RNA-associated decapping and decay factors that are present in P-bodies (Sheth and Parker, 2003). More recent work has identified additional translational regulators associated with P-bodies (Sheth and Parker, 2006). This discovery has expanded the repertoire and role of translational control for which these factors are likely significant. Since reporter mRNAs sequestered within P-bodies during stress can exit from these particles, it has been suggested that P-bodies may actually be the site of much more complex RNA regulation than simple RNA decay (Brengues et al., 2005).

Further evidence showing that polyadenylated mRNAs and the translation factors PABP, eIF4E and G are found in P-bodies underscores the complexity of their function (Brengues and Parker, 2007). This is primarily because these proteins are usually observed in translating polysomes, and P-bodies were once believed to participate solely in RNA decay and repression. More recent models suggest that P-bodies are likely sites of storage and triage for existing mRNAs, where mRNAs are shuttled to polysomes when environmental cues signal translation, and to the degradative pathway when environmental cues signal degradation (Coller and Parker, 2005). This model may explain the presence of translational factors that may be bound to mRNAs prior to their targeting to P-bodies. Another possibility is that mRNAs with translation factors may be the result of stalled ribosomes, shuttled into P-bodies during cellular stress.
Figure 2.2. The steady-state of translational control in yeast. Environmental cues and/or stress determine whether mRNAs are shuttled into P-bodies for storage and decay or to polysomes for translation. Excerpted from Coller and Parker (Coller and Parker, 2005).
**How mRNAs are transported and targeted to synapses.**

The prevailing view of mRNA delivery to synapses is that they are transported in complexes containing protein and mRNAs, often called neuronal RNA granules, ribonuclein particles (RNPs) or RNA transport granules. mRNA targeting to these granules likely occurs through the binding of RNA-binding proteins to sequences in the 3’UTRs in the targeted mRNA. However, since many of these RNA binding proteins are often translational repressors, it is likely that these mediate translational repression of mRNAs associated with RNA granules and that RNA granule transport, mRNA targeting and mRNA repression are all very closely linked. I will discuss transport and targeting of mRNA to synapses in this section and the details of translational repression in the next.

**Transport**

RNA and protein containing granules are exactly as the name suggests, cytoplasmic particles that look granular when examined with various markers. RNA granules have now been found in every organism examined (Johnstone and Lasko, 2001; Kiebler et al., 1999; Kobayashi et al., 1994; Kohrmann et al., 1999; Sheth and Parker, 2003), and there are as many names as there are examined systems: maternal granules or germ granules most often refer to RNA granules that are provided by the mother to the oocyte during development and are found in *Xenopus*, *Drosophila* and *C. elegans*. RNA granules in germ cells are called P-granules in *C. elegans* and polar cell granules in *Drosophila*. In yeast, granules are referred to as processing bodies (P-bodies).

RNA granules were first identified in neurons by using the double stranded RNA binding protein Staufen as a marker. Staufen containing punctae are associated with Syto
14 marked RNA and microtubules in dendrites of cultured rat hippocampal neurons. Hippocampal neurons transiently transfected with the human homolog of Staufen protein tagged with green fluorescent protein (GFP) also demonstrate the same cellular distribution as the rodent version. In addition, Staufen-GFP-labeled granules are trafficked by active bi-directional transport from the cell body into dendrites and vice versa, suggesting that these granules may function as vehicle for the delivery of RNAs to dendrites and may return to the cell body to be recycled (Kohrmann et al., 1999). Additionally, the small ribosomal subunit and several translation factors colocalized with the Staufen marker. The latter finding hints at the possibility that a translational unit or a pre-arranged packet for translation becomes associated with RNA granules upon appropriate delivery.

The idea that specific mRNAs were actually localized to dendrites by the Staufen protein was confirmed by the Schuman group (Tang et al., 2001). Removal of the microtubule binding domain in Staufen decreased the amount of RNA (stained with EtBr) in dendrites of cultured neurons (Tang et al., 2001). Currently, the Staufen protein is one of the most common markers for RNA transport granules, processing bodies and stress bodies in neurons.

RNA granule assembly was originally hypothesized to occur in the nucleus because of hnRNPs found in granules (Hirokawa, 2006). In fact this turns out to be true in other systems (Kress et al., 2004). Live imaging of the \( \beta\)-actin mRNA with Zipcode binding protein confirmed that they associate in the nucleus of neurons (Oleynikov and Singer, 2003). However, whether the nucleus is the site of assembly of all granule components remains to be seen.
Two proteomic studies found a range of shared components of neuronal RNA granules that hint at the mechanism of RNA transport. Kanai et al. (2004) used the Kinesin Heavy Chain 5 (KIF5) to IP RNA granules from adult brain extracts while Elvira et al. (2006) used subcellular fractions from an embryonic mouse brain. Both studies found an abundance of hnRNPs and other nuclear proteins in RNA granules. hnRNPs regulate specific RNAs (an example is β-actin mRNA regulated by Zipcode binding protein), which is consistent with a regulator role of neuronal RNA granules. Both studies also found DEAD-box proteins, which control RNA transport and may unwind RNA secondary structure (Chu and Herschlag, 2008). Finally, both studies described a novel and highly conserved protein in RNA granules, termed CGI-99, which has no known RNA binding domain, and whose function remains a mystery.

The major difference between both studies was the motor protein that was associated with RNA granules. In the Kanai study, it was Kinesin (KIF5) while in the Elvira study it was dynein. It is possible that the developmental state or methods were responsible for these differences since it is likely that both methods of transport are used within a neuron. Because the well described heterogeneity of RNA granules, it is possible that the two studies examined functionally different granules binding to separate motors for transport (Barbee et al., 2006; Sossin and DesGroseillers, 2006).

The Kanai study also described the interaction between some granule components in more detail. Particularly, the interaction between Pur-α, an RNA-binding protein whose exact function is unknown, and the kinesin heavy chain, KIF5. By using mutational analysis, they determined the minimal binding site of KIF5 for RNA-containing granules. It is a 59 amino acid sequence found in the C-terminal of KIF5 that
is conserved among 3 kinesins. Removal of the N-terminal motor domain of KIF5 did not affect the colocalization of KIF5 with the RNA binding protein Pur-α and several mRNAs but inhibited trafficking of RNA granules into dendrites. This suggests that kinesin based transport is indeed a mechanism for this type of RNA granule delivery into dendrites (Kanai et al., 2004).

**Targeting**

Targeting of mRNAs into dendrites or synapses likely requires both *cis* and *trans* factors. Several mRNAs, such as the neuronal splice variant of CAMKII, have dendritic targeting sequences in their 3’ UTRs that are required for dendrite localization (Mori et al., 2000). For example, beta-actin has a Zipcode binding sequence in its 3’UTR. This sequence binds the Zipcode binding protein (ZBP), an hnRNP required for RNA transport into the dendrite (Farina et al., 2003). Additionally, it has been hypothesized that RNA secondary and tertiary structure may influence the binding of targeting and transport proteins (Hirokawa, 2006). However, this has not been tested so far.

The presence of many evolutionarily conserved factors in RNA granules across species underscores the likelihood that similar regulatory mechanisms are used. However, many of the details remain to be worked out in neurons. In germ cell development, 3 strategies of RNA targeting have been identified (Zhou and King, 2004): 1) Motor driven transport; 2) diffusion entrapment; and 3) localized stabilization.

Motor driven transport has been best studied in the oocyte by examining the localization of the *oskar* mRNA transcript. Studies on the transport of *oskar* mRNA have provided strong evidence that the transcript is transported on microtubules with a kinesin
driven motor in association with the RNA binding protein Staufen (St Johnston et al., 1991). As suggested by the Kanai study described in the previous section, kinesin-driven microtubule-based transport of mRNA occurs in neurons as well.

Diffusion entrapment targets nanos mRNA (in *Xenopus* called *Xcat2*) to the posterior pole of the germ cell. In *Drosophila*, microtubules are not required for nanos mRNA’s localization. The prediction is that mRNAs are exported from the nucleus and randomly diffuse throughout the cytoplasm. mRNAs that reach the target destination are then “trapped” by an unknown “stabilization factor”. Not surprisingly, this process is not very efficient, as only 4% of nanos mRNA reaches the posterior pole (Forrest and Gavis, 2003). Consistently, the targeting of the nanos homolog of *Xenopus*, Xcat, appears to localize in the *Xenopus* oocyte in the same way, and is also not dependent on microtubules (Zhou and King, 2004). Notably, in *Drosophila* nanos positive particles appear much smaller and may be different from the large mRNPs (Forrest and Gavis, 2003), perhaps alluding to targeting mechanisms being specific to granule subtypes.

The third method of mRNA targeting described in oocytes is called “localized stabilization”, which protects mRNAs from degradation at specific target sites but not anywhere else. The best-studied example is hsp83 mRNA (Ding et al., 1993). In this case, the mRNA localized to the pole of the oocyte is protected by various factors from RNA degradation. A proper integrity of the polar RNA granule is required for this protection to occur since mutations that disrupt RNA granule integrity interfere with the protection from degradation. Not surprisingly, researchers hypothesize that elements in the 3’ UTR of the mRNA are responsible (Ding et al., 1993).
How RNAs are repressed

In the following section, I present the major mechanisms of translational control and discuss what is known about each type of regulation in dendrites of neurons. Although this section is organized by type of regulation, it is important to note that many of these mechanisms are overlapping with each other, and that for any given transcript several types of regulation can occur.

Much of translational repression occurs at the level of translational initiation. Repression appears to occur both in a general sense, meaning blocking the process of translation using factors common to many or most transcripts, or in a transcript-specific way, which occurs when *trans* factors bind to *cis* elements often found in the 3’ UTR. Examples of both mechanisms are described below.

**Repression of RNAs by masking**

Masking refers to the “hiding” of mRNA transcripts such that neither translational nor degradative factors can access them (Johnstone and Lasko, 2001). Masking likely occurs through binding of mRNAs to masking proteins, which alter the mRNA’s conformation and sterically inhibit binding of the initiation complex. For example, Y-box proteins, like FRGY2, bind to the Y-box sequence in the 5’ UTR of mRNAs and to PABP protein bound to the 3’ end of the mRNA (Matsumoto and Wolffe, 1998). This circularizes the mRNA and inhibits translation by blocking the sites at which the translational initiation factors bind.

A second example of masking is the steric inhibition of cap-binding, which also leads to circularization of the mRNA (Tarun and Sachs, 1996). X-ray crystallography
studies of eIF4E identified two binding pockets, one for the actual cap binding and the other speculated to be the site of RNA-binding proteins (Sachs and Varani, 2000). Since the identification of this second site, many proteins have been demonstrated to interact and bind eIF4E (Richter and Sonenberg, 2005). Like with Y-box proteins, the mRNA is circularized such that translation initiation factors cannot bind.

A third example of masking is mediated by the cytoplasmic polyadenylation element binding protein (CPEB), which binds the cytoplasmic polyadenylation element (CPE) in the 3’UTR of mRNAs. CPEB then recruits the protein Maskin and eIF4E to the mRNA. eIF4E bound to Maskin prevents cap binding, and thus, it may titrate out the available cap-binding protein eIF4E (Barkoff et al., 2000). With Maskin binding to the 5’ end of the mRNA, and CPEB bound to the 3’ UTR, the circularized mRNA is in a complex that is neither accessible to translation nor degradation. Other 3’UTR binding factors that have been hypothesized to create similar complexes have been identified in *Drosophila* as well. For example, Bruno binds a very specific sequence in *oskar* mRNA to silence the RNA (Nakamura et al., 2004). Bruno, like CPEB, forms a complex with Cup, a eIF4E-binding protein, which likely leads to a masking of the mRNA by circularizing the 3’ and 5’ ends (Nakamura et al., 2004).

In neurons, masking of mRNA has not been demonstrated definitively. However, many of the components involved in masking have been identified in neuronal RNA granules, suggesting that this form of regulation may exist (Barbee et al., 2006; Keleman et al., 2007). More recently, a neuron-specific, eIF4E-binding protein, called Neuroguidin has been found in the mammalian nervous system. Neuroguidin also binds to CPEB *in vitro* (Jung et al., 2006). The homologous protein in *Xenopus* is required for
normal neural tube development, suggesting a role of RNA masking-like mechanisms in nervous system development (Jung et al., 2006). Another specific example that may involve masking in neurons and may play a role in synaptic plasticity is the Fragile X Mental Retardation Protein (FMRP, see Chapter 3).

Phosphorylation of eIF4E binding protein by the mTOR pathway negatively regulates translation.

Another mechanism regulating initiation of translation is defined by the mammalian target of rapamycin (mTOR) pathway (Jeffries et al. 1994). The mTOR pathway is named for its inhibition by the secreted bacterial protein rapamycin. mTOR signaling is regulated by numerous upstream pathways. These upstream pathways function as sensors for the subcellular nutrient and energy levels of the cell, including the levels of insulin, growth factors (such as IGF-1 and IGF-2), and mitogens (Cully and Downward 2009).

mTor regulation occurs at the 5’ end of the mRNA by phosphorylation of eIF4E binding proteins (4EBP, an example is Cup or Maskin). This was hypothesized when subsets of RNAs were shown to have their rates of initiation regulated by the phosphorylation state of various initiation related proteins (S6Kines, 4EBP) and especially by the addition of the antibiotic rapamycin (Jeffries et al., 1994). Phosphorylation of 4EBP, for example, is believed to create a conformational change in the protein reducing its binding affinity to eIF4E, and relieving both the repression and masking activity (Imai et al., 2008). However, the details of this regulation are far from
understood and why some proteins are more sensitive to mTOR pathway regulation is not well understood.

Essential proteins in the mTOR pathway are found in dendrites (Raymond et al., 2002). In addition, LTP is sensitive to rapamycin, indicating that the mTOR pathway is involved in regulating synaptic plasticity (Herbert et al., 2002; Tang et al., 2002).

**Adenylation and Deadenylation of mRNAs regulates translation**

A major mechanism of translational repression or activation is the regulation of the presence and length of the polyA tail. The polyA tail at the 3’ end of the mRNA is added by the Poly A Polymerase enzyme and allows binding of Poly A Binding Protein (PABP). PABP binds the scaffolding protein eIF4G circularizing the RNA (Derry et al., 2006). The larger the polyA tail, the more PABP molecules can bind to the PolyA tail. In general, it is believed that extending the length of the poly A tail leads to translational activation and a decrease in the chance of repression or degradation (Richter, 1999).

Deadenylation of a mRNA is most often signaled by a *cis*-element in the 3’ UTR, like the AU-rich element (ARE) or the embryonic deadenylation element (EDEN) of *Xenopus*, which are both bound by specific *trans* factors (Paillard et al., 1998). An example of deadenylation controlling translation is the regulation of *hunchback* mRNA in the *Drosophila* oocyte by the proteins Nanos and Pumilio (Chagnovich and Lehmann, 2001). *Hunchback* mRNA contains a Nanos Response Element (NRE), which when bound by Pumilio and Nanos leads to deadenylation, a process closely tied to degradation (Chagnovich and Lehmann, 2001).
In neurons, there is little doubt that the factors involved with polyadenylation of mRNA exist (Richter and Klann, 2009). What is unknown is the degree to which adenylation and deadenylation are used to control translation of mRNAs at synapses. Perhaps the best example so far of polyadenylation occurring in neurons is from the Richter lab, which demonstrates that polyA tail length increases in response to synaptic activity (Wu et al., 1998).

**Translational repression at the 60S ribosome binding step**

Most mechanisms of translational regulation affect the pre-initiation complex while only a handful of mechanisms are known to affect the ribosome binding step (Ostareck et al., 2001). However, a complex of heteronuclear ribonuclear proteins (hnRNPs) has been demonstrated to attach to the 3’UTR of mammalian 15-lipoxygenase mRNA and sterically inhibit binding of the 60S ribosomal subunit to the pre-initiation complex, likely through attachment to eIF5 (Ostareck et al., 2001). In *Drosophila*, the eIF5 protein homolog interacts with the DEAD-box helicase Vasa, which itself has been implicated in translational regulation of mRNA during oogenesis. Although there is no direct evidence, this suggests yet another mechanism in which translation can be repressed at the 60S ribosomal binding step (Carrera et al., 2000).

Another potential regulator similar to Vasa is Me31b, a DEAD-box helicase protein, and a main topic of this dissertation discussed in Chapter 3. Me31b has been identified as the regulator of *oskar* mRNA during oogenesis (Nakamura et al., 2001), and is a putative neuronal translational repressor. Although highly circumstantial, this is...
perhaps the best evidence so far that translational repression at the 60S ribosome binding step occurs in neurons.

**RNA decay as a mechanism for translational control**

Another potential mechanism to regulate translation of mRNAs is simply removing them from the “pool of translatable mRNAs” by degradation. There are many pathways to degrade mRNAs. Degradation can be initiated by the removal of the 5’ cap, through ARE-mediated decay and “non-sense mediated decay” (NMD) pathways (Silva and Romao, 2009). 5’ Cap removal exposes the mRNA to degradation by exonucleases at the 5’ end. ARE-mediated decay is believed to occur primarily through deadenylation which decreases mRNA stability. ARE refers to an adenine/uridine rich consensus sequence in the 3’UTR. More recent data, however, suggests that ARE-mediated decay may occur through the miRNA pathway (presented later in this section). However, the details are as of yet unknown (von Roretz and Gallouzi, 2008). NMD-mediated degradation is a RNA splicing-dependent pathway that degrades mRNAs with nonsense mutations or premature termination signals.

Much of the details of RNA decay and their relationship to RNPs and repression has been worked out in yeast (Steiger and Parker, 2002). Recently, decapping proteins and 5’ and 3’ exonucleases have been found to colocalize with neuronal RNA granules in mammals and *Drosophila* (Barbee et al., 2006; Vessey et al., 2006), suggesting that RNA decay is a likely mechanism of translational control at synapses in neurons.

**Translational repression occurs at the elongation step**
Only a few examples of translational regulation at the elongation step are known. For example, phosphorylation of elongation factor 2 (EF2) leads to a decrease in translational efficiency, presumably by decreasing the speed in which the ribosome can add amino acids. However, it is interesting to note that phosphorylation of EF2 can also lead to an increase in the translation efficiency of the specific oxytocin mRNA, which is expressed in the rat hypothalamus (Fujisawa et al., 2007). In contrast, a correlative decrease in translational efficiency was found during glutamate-dependent ischemia in the brain (Marin et al., 1997). Although this mechanism evidently occurs in the nervous system, little is known about how widespread this regulation is as far as transcript number or whether it is involved in synaptic plasticity.

**Translational repression occurs via the non-coding RNA pathway**

More and more data suggest that the non-coding RNA pathway is involved in human disease pathology, including cancer, cardiovascular disease, and immune disorders (Li et al., 2009). In this section, I will focus on three groups of non-coding RNAs: long non-coding RNAs and two of the short regulatory RNAs, small interfering RNA (RNAi) and micro RNA (miRNA).

Long non-coding RNAs may affect many aspects of mRNA processing and may provide a substrate to become a small regulatory RNA. Long non-coding RNAs are typically around 200 nucleotides, and often part of a complementary sequence of a transcript known to encode a protein (Ponting et al., 2009). One of the best examples of how a long non-coding RNA can affect RNA processing is Zeb2 mRNA whose long 5’UTR contains an internal ribosome entry site (IRES) required for translation of the
transcript (Beltran et al., 2008). Formation of the appropriate 5’ UTR requires RNA splicing, which will not occur without the presence of a Zeb2 antisense transcript that complements the RNA. In this case, the Zeb2 antisense transcript (e.g., the long non-coding RNA) protects the Zeb2 mRNA against aberrant splicing that would eliminate its ability to be translated efficiently.

An example of non-coding RNAs affecting translational control in the nervous system is brain cytoplasmic transcript (BC1), which contains an anti-sense sequences to many dendritically expressed mRNAs (Lin et al., 2008). BC1 RNA is expressed almost exclusively in the nervous system of mice and humans and colocalizes with polysomes and other mRNAs (Tiedge et al., 1991; Wang and Tiedge, 2004). BC1 RNA inhibits recruitment of the 43S pre-initiation complexes to mRNA. BC1 RNA particularly inhibits the translation of mRNAs with structured 5’ UTRs that require eIF4A-mediated unwinding (Lin et al., 2008). Interestingly, synaptic activity increases BC1 RNA expression and localization to dendrites and synapses (Muslimov et al., 1998). More recently, BC1 has been implicated in Parkinson’s disease; BC1 may regulate dopamine release through GABA expression in the ventral striatum (Centonze et al., 2007). BC1 also functions in cooperation with FMRP in many contexts (see Chapter 3). Finally, long non-coding RNAs have been recently demonstrated to provide substrates for the short interfering RNA pathway during the annealing process of antisense and sense transcripts (Golden et al., 2008).

The two small regulatory RNAs that will be discussed in this dissertation are RNAi and miRNA. The RNAi and miRNA regulatory pathways control translation by either repressing the expression of transcripts (miRNA), or degrading mRNAs (RNAi).
(Tijsterman and Plasterk, 2004). The route taken depends on the perfect or imperfect annealing of the two strands of the small double stranded RNA (Tijsterman and Plasterk, 2004). For example, if the substrate anneals perfectly, it will target transcripts for degradation by the RISC complex. If the annealing is imperfect, then the targeted transcript is translationally repressed by a slightly modified RISC complex.

miRNA substrates originate from gene coding sequences with high degrees of secondary structure. Most often, these sequences form a “G-quartet”, which is a cloverleaf-like organization of double-stranded RNA. Small non-coding RNA substrates form from perfectly matched double stranded RNA, which occurs when a single-stranded RNA sequence can fold back on itself due to an inverted repeat. The perfectly matched double-stranded RNA is then recognized by the double stranded RNAase Dicer and R2D2, while an imperfectly matched RNA associates with Dicer alone. After proteolytic cleavage into short double-stranded fragments (Tijsterman and Plasterk, 2004), the RNAs then dissociate into single-stranded RNAs, which are the bound by the Argonaute protein to form the RNA-induced silencing complex (RISC). Depending on the association of R2D2 (Tijsterman and Plasterk 2004), the RNA is then targeted for immediate degradation or translational repression (Figure 2.3). Translational inhibition is believed to be due to steric inhibition of the initiation factor binding caused by the RISC complex. However, this has not been directly tested yet.
Figure 2.3. Schematic of the micro-RNA and RNA interference pathways. Double stranded substrates with imperfect annealing bind the antisense containing transcripts and cause RISC-mediated translation repression. Double stranded substrates with perfect annealing bind the antisense containing transcripts leading to RISC mediated degradation. Excerpted from Tijsterman and Plasterk (2004).
There is ample evidence of small RNA regulation in the nervous system and miRNAs that are involved in several forms of synaptic plasticity (Corbin et al., 2009). The developing zebrafish nervous system is perhaps the best studied system addressing neuronal mi-RNA expression and localization. Many groups have shown a cell- and development-specific expression of more than a dozen miRNAs (Fiore et al., 2008). Furthermore, the removal of Dicer expression from the developing zebrafish nervous system results in severe developmental defects, implicating small regulatory RNAs as crucial regulators of normal nervous system development (Giraldez et al., 2005).

In Drosophila, the RISC-complex component Armitage colocalizes with a CAMKII mRNA reporter at synapses. In Drosophila, CAMKII mRNA has a homologous site for miR-130 binding in its 3’ UTR (Ashraf et al., 2006). Synaptic activity (in this case pharmacological activation of muscarinic receptors) leads to a proteasome-dependent degradation of Armitage and an upregulation of CAMKII reporter expression (Ashraf et al., 2006). This example provides some insight into how regulatory RNAs may be involved in more than just development and may function in synaptic plasticity, potentially regulated by various forms of synaptic activity.

Another neuronal example for microRNA-mediated regulation of protein synthesis is miRNA-134, a negative translational regulator of the actin regulator “lim-domain containing protein kinase 1” (limk1) protein (Schratt et al., 2006). The attenuation of Limk1 translation limits dendritic spine size (Schratt et al., 2006). Both, miR-134 and Limk1, colocalize to the dendritic shaft and local translational regulation has been beautifully demonstrated by adding a dendritic anchoring coding sequence to Limk1 mRNA. miR-134 dependent miRNA regulation appears to be sensitive to synaptic
activity in cultured mammalian neurons (Schratt et al., 2006). For example, BDNF activation of TrkB receptors releases the microRNA-134 repression of Limk1 mRNA via an unknown mechanism (Schratt et al., 2006).

Other evidence to support small ncRNA function in the brain is that FMRP, a known neuronal translational regulator, genetically interacts with Argonaute (Jin et al., 2004). Argonaute is a component of the RISC complex and colocalizes with other RISC components in the somatodendritic compartment (Caudy et al., 2002). FMRP has been suggested as a possible recruiter of RNAs into the RISC complex. However, FMRP’s requirement in miRNA and RNAi function is a topic of contentious debate, and will be addressed later in both Chapter 3 and 5.

**Translation and Synaptic Activity**

Because protein synthesis inhibition blocks long-term formation of memory, cellular physiological correlates of memory require new protein synthesis. Much indirect evidence has been presented to support this idea. Various forms of synaptic activity have shown both transcript-specific and general increases in translational activities.

Considering the biological role of protein translation for synaptic plasticity, it is apparent that not all types of mRNAs should be translated upon synaptic activity. For example, if a neuron receives a stimulus that is intended to strengthen a synapse, a mRNA encoding a protein that opposes this purpose should not be translated but repressed. Some evidence that synaptic activity can lead to a decrease in translation does exist (Costa-Mattioli et al., 2009). Additionally, a switch in translation from cap-dependent to cap-independent translation (Dyer et al., 2003) has also been demonstrated.
to occur upon stimulations leading to changes in synaptic strength. Next, I will present what is known about the effect of synaptic activity on translation. What will become apparent is that the signal transduction pathways that connect synaptic activation with translational control are not well understood.

**Various forms of synaptic activity lead to transcript specific increases in translation**

Several mRNAs have been shown to be translated in response to various forms of synaptic plasticity. Below several examples are briefly presented.

Tetanic stimulation of the Schaffer pathway in cultured slices of the rodent brain leads to a rapid increase (by 20%) in the translation of the CAMKIIα subunit, which likely occurs in dendrites (Ouyang et al., 1999). Because the increase occurs rapidly, and local application of the protein synthesis inhibitor anisomycin abrogates the increase, protein transport from the cell body is not responsible. Additionally, the 3’UTR of CAMKII (which contains the dendritic localization signal) fused to a GFP coding sequence and a myristilation sequence for immediate membrane insertion, showed a 4-fold increase in translation following BDNF application, as measured by GFP fluorescence (Aakalu et al., 2001).

The auto-catalytic zeta subunit of PKMζ undergoes increased translation following LTP inducing stimulation (Osten et al., 1996). This study also demonstrated that both late LTP maintenance and PKMζ subunit translation are blocked by protein synthesis inhibitors. Unfortunately, whether this translation occurred locally was not addressed.
Arc is one of the best-characterized LTP-induced proteins. Arc mRNA is synthesized within minutes after stimulation paradigms that lead to learning or LTP. Newly transcribed Arc mRNA is transported into dendrites and becomes localized to active synapses (Steward et al., 1998). This was demonstrated using both behavioral and tetanic stimulation.

An example of new translation caused by stimulation protocols leading to LTD is MAP1b, a microtubule associated protein. Stimulation of metabotrophic glutamate receptors (mGluR) with the agonist DHPG leads to mGluR1-dependent LTD and triggers a protein synthesis-dependent increase in MAP1b protein (Hou et al., 2006).

Interestingly, MAP1b mRNA is translationally repressed by FMRP, and FMRP knockout animals show altered mGluR1-dependent LTD and exhibit increased basal levels of MAP1b protein (Lu et al., 2004).

**How translational activation may occur through synaptic activity**

Although the exact mechanisms are not well-understood, insights into how translational activation occurs upon synaptic stimulation are emerging. One way is simply to remove the trans factors that are bound to the mRNA and repressing translation. Another one is to localize the mRNA to a compartment that is translationally active, such as a polysome. Examples of both are described below.

The Kosik group has demonstrated that RNA from Staufen-containing granules moves to polysomes upon synaptic stimulation (Krichevsky and Kosik, 2001). The granules that they describe are rich in ribosomal subunits, Staufen protein, and mRNAs but are not believed to be translationally competent due the absence of translation
initiation factors. Depolarization of cultured cells by application of KCl leads to a rearrangement of these granules into smaller subunits, which then may allow ribosomal assembly and subsequent translation.

Another example is provided by FMRP. FMRP binds with to CYFIP, which contains a non-canonical eIF4E binding site, making it a 4EBP (Napoli et al., 2008). The hypothesis is that CYFIP and FMRP bind up eIF4E on one side, the mRNA on the other side, and “mask” the RNA from translation. The CYFIP-FMRP complex is found in a putatively translationally dormant mRNP complex, from which a repressor can be removed by application of BDNF to cultured cells (Napoli et al., 2008).

Removal of translation repression by removal of the repressor RNG105 following synaptic stimulation has been previously demonstrated (Shiina et al., 2005), but in contrast to the Kosik study, it appears the repressor leaves the complex. RNG105 disassociates from granules following BDNF stimulation, and the dissociation is coincident with local translation occurring near the granules (Shiina et al., 2005).

As proposed above, dissociation of the repressor complex is yet another method that could allow translational activation. Indeed, the number of P-body like structures in mammalian cultured neurons is reduced by ~60% after chemically induced stimulation simulating synaptic activity (Zeitelhofer et al., 2008).

**Activation of components mediating translation**

Another strategy to increase translation following synaptic stimulation is to components of the translational initiation complex more readily available. Surprisingly, this appears to occur by translational activation itself.
For example, the translational control protein FMRP appears to be locally translated. *FMRP* mRNAs were identified in a cDNA library made from subcellular fractions, termed synaptoneurosomes, which mostly contains sheared of synapses (Weiler et al., 1997). *FMRP* mRNA apparently associates with translational complexes in synaptoneurosomes within 1–2 min after mGluR1 stimulation. In addition, mGluR1 stimulation also increases the expression of FMRP protein (Weiler et al., 1997). This data is reminiscent of regulating the regulatory molecules of translation in developmental pathways.

A more direct and likely positive strategy affecting translation upon synaptic stimulation, is by increasing locally the amount of available ribosomal subunits and translation factors. This may occur primarily through activation of 5′TOP mRNAs, which are encoding nearly 90 proteins that have been assigned to the translational apparatus. Some examples are ribosomal proteins, elongation factors EF1α and EF2 and poly(A)-binding protein. These mRNAs are likely repressed upon growth arrest caused by environmental stress. This response is mediated by a cis-regulatory element (TLRE) in the 5′UTR (Costa-Mattioli et al., 2009).

Interestingly, LTP, mGluR-dependent LTD in rodents, and long-term facilitation in *Aplysia* increase the translation of 5′ TOP mRNAs (Costa-Mattioli et al., 2009). Using reporters, stimulation increases translation of 5′TOP proteins locally in dendrites (Gobert et al., 1998; 2008). Consistently, 5′ TOP RNAs are enriched in dendrites of hippocampal neurons (Poon et al., 2006) and in neurites of *Aplysia* neurons (Moccia et al., 2003). However, there is no evidence yet that translation of 5′TOP proteins is a requirement for synaptic plasticity.
Summary

The data presented in this chapter address the complex and various mechanisms to target, transport, repress and activate mRNA in neurons. Much to our experimental advantage, these mechanisms are highly conserved with one exception: the translational activation upon synaptic activity, which may mirror the translational response to cellular or environmental stress. In the next chapter, I will discuss the translational regulation mediated by Fragile X Mental Retardation Protein (FMRP). FMRP’s relevance to human health is apparent from its role in a devastating human neurological disease, and studies addressing its role have provided much insight into mechanisms of regulating translation in neurons.
CHAPTER THREE

FRAGILE X MENTAL RETARDATION PROTEIN IS AN RNA-BINDING PROTEIN

THAT IS ASSOCIATED WITH INHERITABLE MENTAL RETARDATION IN

HUMANS
Introduction

In this chapter I, will discuss the canonical translational regulator, Fragile X Mental Retardation Protein (FMRP). This discussion will serve two purposes: 1) to introduce the mechanisms by which FMRP protein controls translation; and 2) to illustrate the enormous difficulty in assigning precise roles to translational regulators in the nervous system.

Etiology of Fragile X Mental Retardation Syndrome

Fragile X Mental Retardation Syndrome is the most frequent cause of heritable mental retardation, and among the most common single gene disorders (Hagerman et al., 1996). The disease often appears by age 3 when parents notice numerous developmental delays, including motor, speech and language comprehension (Hagerman et al., 1996). Many of the children suffering from this disorder exhibit hyperactivity, anxiety and autistic-like behaviors. Fragile X Mental Retardation is also a known genetic cause for autism spectrum disorders.

Fragile X Mental Retardation Syndrome was cytogenetically mapped to an area of constriction on the X chromosome, which created a “fragile” looking X chromosome (O'Donnell and Warren, 2002). More detailed mapping identified a CGG repeat expansion just upstream of the FMRP gene promoter (O'Donnell and Warren, 2002). While the number of CGG repeats in normal individuals is around 30, individuals with Fragile X Syndrome can have over 200 repeats, which impairs expression of the FMRP gene (O'Donnell and Warren, 2002). It is speculated but not confirmed that an abnormal
chromatin structure (likely caused by DNA methylation) leads to the aberrant gene transcription of FMRP and the disease (O'Donnell and Warren, 2002).

Fragile X syndrome causes abnormalities in dendritic spine shape and number, possibly due to incomplete spine maturation (Irwin et al., 2002; Irwin et al., 2001). Knock out models in both mice and flies mirror this cellular phenotype (Lee et al., 2003; Thomas et al., 2008). However, the behavioral effects in mice are mild (Kooy, 2003). Gross structural defects in the brain have also been demonstrated in the fly model (Michel et al., 2004).

**Fragile X Mental Retardation Protein is an RNA binding protein with unknown cellular function.**

Some of the first functional predictions of FMRP came from the Warren lab, which demonstrated that human FMRP bound to approximately 4% of human fetal brain RNAs (Brown et al., 2001). Sequence analysis identified two KH-domains in the middle of the human FMRP protein (domains shared by other known-RNA binding proteins) that were identical in fly and mouse FMRP (Brown et al., 2001). Additionally, FMRP contains an RGG box, which is an RNA binding domain shared with hnRNP proteins (Ashley et al., 1993a). The functional importance of the RNA-binding ability of FMRP was supported by the observation that a patient with a severe form of the Fragile X syndrome had a mutation in the KH domain while the FMRP promoter did not contain excessive repeats (Siomi et al., 1994).

FMRP may act as a repressor of translation for several dendritic RNAs, including CAMKII, Arc, Map2 and beta-actin (Zalfa et al., 2003; Zhang et al., 2001). However, its
role as a repressor may not be so straightforward since FMRP also is present in the cellular polysomal fraction and associates with mRNPs. Hence, it could mediate transport or repression of translation. It has been suggested that FMRP in the polysomal gradient may be associated with large, stalled and aggregated pre-initiation complexes that are not necessarily translationally active (Zalfa et al., 2006).

Further insight into the FMRP association with the polysomes came from a patient who contained a missense mutation within the FMRP coding region and a particularly severe neurological phenotype (Feng et al., 1997). Copying the mutation in vitro abolished the association of FMRP with polysomes without affecting its ability to bind polyadenylated mRNAs (Feng et al., 1997). An interesting inference from this study is that the most important function of FMRP may be to associate mRNAs with translation rather than directly control translation. However, conflicting evidence from both in vitro and in vivo work suggests that the neurological phenotypes can be attributed to an excess of translation caused by the absence of FMRP (Li et al., 2001). The abrogation of translation was demonstrated by in vitro translation assays, which showed a dose-dependent decrease in translation as FMRP protein levels increased. Using quantitative PCR from mRNAs enriched in the polysomal fraction from mice containing a loss of function mutation in FMRP, translational dysregulation of many mRNAs was seen. Of the 251 mRNAs present in dFMRP-containing complexes, 136 transcripts were increased while 115 were decreased in copy number compared to wild type cells (Brown et al., 2001). Again, this suggests that there may be a dual effect on translation by FMRP protein.
In *Drosophila*, loss of FMRP impairs associative olfactory conditioning. This deficit was rescued by the application of protein synthesis inhibitors (Bolduc et al., 2008), demonstrating a role for FMRP during protein synthesis for memory formation.

Two other indirect regulatory roles have also been hinted at, one in RNA processing and another for the nuclear export of mRNAs (Eberhart et al., 1996). The identification of a functional nuclear localization signal and nuclear export signal suggests that FMRP may also have a role in mRNA transport shuttling mRNAs from the nucleus to the cell soma (Eberhart et al., 1996). Another group observed an enhancement of *FMRP* mRNA splicing in a mouse model, which may occur as an auto-regulatory loop of translational repression. Finally, to add more complexity, the ARE consensus sequences found in the 3’ UTR may be in fact a cue for translational activation through the FMRP. This casts doubt not on the role of the ARE site as a *cis*-factor leading to RNA decay and on the role of FMRPs as a repressor of protein translation (Vasudevan et al., 2007).

It appears that we are still fairly distant from understanding how FMRP controls translation, either through activation, repression, nuclear export or transport. Perhaps the most enlightening data so far come from FMRP’s localization with both polysomes and the smaller mRNP elution fraction (Zalfa et al., 2006). This may help to explain the conflicting effects on translation of specific transcripts. This further suggests that sometimes FMRP appears as a repressor and other times as an activator, dependent upon the state of FMRP phosphorylation. Biochemical data demonstrate that most of FMRP in RNP particles is phosphorylated while FMRP in the polysome fraction is dephosphorylated (Ceman et al., 2003), hinting at a mechanism to switch from an
activator to repressor state. Additionally, mGluR activation leads to rapid
dephosphorylation of FMRP by Protein Phosphatase 2 (Narayanan et al., 2007). This
demonstrates a link between synaptic activity and the phosphorylation state of FMRP,
which may relieve translational repression and explain FMRP’s role in mGlu2-dependent
LTD.

Another recent insight into the possible dual role of FMRP as repressor and
activator of translation is based on the idea of transcript masking, which is simply defined
as the “hiding” of the transcripts with various trans-factors, preventing both translation
and degradation (discussed above). The best example comes from Drosophila oocytes
where CPEB, Cup, and eIF4E binding protein form a complex that leads to repression of
a mRNA transcript by circularization. When Cup is not present, CPEB promotes
adenylation and translation (Nakamura et al., 2004). A similar phenomenon has been
implicated in neurons with the protein Maskin instead of Cup (Huang et al., 2003).

There are several parallels with FMRP and the FMRP-interacting protein, CYFIP.
CYFIP contains a non-canonical eIF4E binding site, which attaches to the 5’ cap of a
mRNA. The CYFIP-FMRP complex is found in the putatively translationally dormant
mRNP complex, from which a repressor complex can be removed by synaptic
stimulation (Napoli et al., 2008). An interesting side note is that PABP is also present in
this complex, apparently through the binding of CYFIP, which may constitute yet another
example of how a mRNA can be switched from a repressed to an activated state through
the rearrangement of an attached complex (Zalfa et al., 2006). Hence, it is possible that
the phosphorylation state of FMRP could also determine whether FMRP interacts with
CYFIP in repressed mRNPs or in translating polysomes.
Despite two potential mechanisms of how FMRP switches from a state repressing translation to promoting it, the models cannot explain FMRP’s various other effects on mRNA. Other known and suggested mechanisms of regulation by the non-coding pathway involving FMRP (BC1 RNA and other more controversial involvement in RNAi/miRNA discussed in following section) do not clearly fit into this mechanism. As has become apparent with many other translational regulatory proteins, the complexities are numerous and indecipherable at this point.

**FMRP has a role in synaptic plasticity**

Although cognitive deficits are very apparent in Fragile X Mental Retardation patients, unraveling the molecules underlying the cognitive deficits is difficult. Although conditional knockouts of FMRP in mice would be the most appropriate way to attack the problem, it has not been done yet. However, as discussed in the next paragraph, it does appear that Fragile X loss of function animal models exhibit an altered neurophysiology.

Activation of mGluR-dependent LTD is enhanced in the hippocampus of FMRP knockout mice (Huber et al., 2002) but LTP is unaffected. Appropriate synaptic function in this system, depends on a constant endocytosis of AMPA receptors. In FMRP knockout mice, AMPA receptors are internalized at an increased rate, which is likely the main cause for the enhanced LTD (Garber et al., 2008). How this defect connects to protein synthesis is unclear. The best explanation so far is that FMRP acts as a negative regulator of a factor that acts downstream of mGluR; Reduced amounts of this factor may then increase the amount of AMPA receptor internalization (Bear et al., 2004). The mGluR theory has been confirmed pharmacologically in several animal models.
Treatment with either a mGluR antagonist (MPEP) or genetic reduction of mGluR rescued the morphological, behavioral and physiological phenotypes of FMRP KO animals (Tucker et al., 2006; Yan et al., 2005). Further support came from in vitro data showing that elongated spines can be induced by excessive mGluR activation, which appear similar to the abnormal spine structure seen in Fragile X patients (de Vrij et al., 2008). In addition, FMRP represses MAP1b and Arc mRNA expression at the synapse (Caudy et al., 2002; Zalfa et al., 2003). Both proteins are required for AMPA receptor recycling. MAP1b is required for AMPA receptor endocytosis since knockdown of MAP1b arrests endocytosis (Davidkova and Carroll, 2007). Additionally, pharmacological induction of AMPA receptor endocytosis led to a translation-dependent increase in MAP1b protein, suggesting a regulatory feedback loop (Davidkova and Carroll, 2007).

mGluR dependent LTD depends on rapid translation of Arc, another known target of FMRP repression (Waung et al., 2008). However, mGluR induction also leads to rapid Arc synthesis. Of mention, Arc is also a well-known regulator of AMPA receptor recycling (Waung et al., 2008).

It is still not clear if the effects induced by GluRs upon loss of FMRP result from a dampening of the translational control system or by another less direct mechanism. Determining the specific target that is directly responsible for the increased amount of AMPA receptor turnover could help to confirm that translational control is indeed the major cause of Fragile X Mental Retardation Syndrome. In addition, this would foster designing a focused pharmacological or genetic approach to mitigate the neurological effects of the disease.
FMRP and the non-coding RNA pathway

FMRP’s interaction with BC1 RNA, a long non-coding RNA, was discovered by screening for mRNAs that may be regulated by FMRP. The brain specific non-coding RNA, BC1, is a translational repressor and interacts with several RNA binding proteins, including FMRP (Zalfa et al., 2006; Wang et al., 2005). Investigations into whether FMRP may function in the miRNA or RNAi pathway are controversial (Kim et al., 2009; Jin et al., 2004). Colocalization data show FMRP protein associated with RISC components in mammals and in Drosophila (Ishizuka et al., 2002). In Drosophila, neuronal RNA granules and transport RNPs have shown to contain both FMRP and several RISC components (Barbee et al., 2006; Li et al., 2008). However, the genetic/functional data come only from Drosophila labs (Ishizuka et al., 2002; Jin et al., 2004), which at the very least could be interpreted as a species-specific effect of Fragile X protein involvement in the RISC complex; there are some known differences in this complex between invertebrates and vertebrates.

The Warren lab in collaboration with the Moses group demonstrated that removal of one copy of the Argonaute1 gene (Ago1), a core component of the RISC pathway (see Figure 2.3), can suppress the phenotype of FMRP overexpression in the Drosophila eye (Jin et al., 2004). Additionally, the heterozygous loss of FMRP function phenotype in synaptogenesis is suppressed by loss of one copy of Ago1, suggesting that FMRP signaling may require the RISC pathway (Jin et al., 2004). However, since FMRP mRNA itself is translationally regulated (Schaeffer et al., 2003), it is possible that FMRP itself is regulated by the RISC pathway. However, this possibility was not addressed.
Another possibility is that FMRP has a transcript-specific RNAi function. If so, FMRP may associate specific RNAs with the RISC complex without being a core member itself.

Immunoprecipitations (IPs) using GST-tagged FMRP in *Drosophila* S2 cells copurified several RISC components, including the DEAD-box helicase (RM62/DMP68) and several small ribosomal subunits (Ishizuka et al., 2002). Furthermore, RNAi-induced knockdown of FMRP significantly reduced RNAi activity, as assayed by the fluorescence intensity of a GFP-tagged RNAi reporter, suggesting that FMRP is required for efficient RNAi. However, this being S2 cells and a reporter transcript, this assay may not reflect FMRP function under *in situ* conditions. Much more in-depth work is required to assess whether FMRP does indeed function as one of the core components of the RNAi pathway, or acts by bringing specific RNAs to the RISC components.

**Summary**

In conclusion, it appears that FMRP is required for appropriate nervous system development and synaptic plasticity, more specifically, LTD. FMRP protein can potentially regulate translation, possibly as both a translational activator and repressor. In addition, FMRP may have other roles in nuclear mRNA export, cytoplasmic mRNA transport and possibly a mRNA processing role. FMRP is perhaps the most well-studied translational regulator in the nervous system, and is an effective genetic tool for identifying new translational regulators. In the next two Chapters (4 and 5), I will present evidence of FMRPs usefulness as a tool for studying translational control in the nervous system.
CHAPTER FOUR

STAUFEN AND FMRP CONTAINING NEURONAL RNPS ARE STRUCTURALLY AND FUNCTIONALLY RELATED TO SOMATIC P-BODIES
**Introduction**

Localized translation of mRNAs has emerged as a major mechanism for regulating dynamic intracellular processes such as those involved in early embryonic development and synapse plasticity (Johnstone and Lasko, 2001; Martin, 2004). In the specific cases of growth cone guidance and synapse plasticity, temporally and spatially restricted repression of mRNA translation allows subcellular locations within a single neuron to transiently achieve different molecular and functional properties. This allows growth-cone turning in specific directions or, potentially, synapse-specific alterations required during learning and memory (Martin, 2004; Richter and Lorenz, 2002).

Similarly, in dendrites, translationally repressed RNAs mobilized by synaptic stimulation are translated through control mechanisms that may include polyadenylation of mRNAs at stimulated synapses (Richter and Lorenz, 2002). It is likely that such locally translated mRNAs influence dendritic growth as well as maintain protein synthesis-dependent forms of synaptic plasticity (Martin, 2004; Ye et al., 2004).

Translational repression often occurs in cytoplasmic, ribonucleoprotein (RNP) particles. In the mammalian nervous system, Staufen-containing RNP are thought to mediate translational repression and/or mRNA transport of dendritically localized mRNAs (Kiebler and Bassell, 2006). These granules often contain the Fragile-X Mental Retardation Protein (FMRP), a translational repressor that negatively regulates dendritic growth (Nimchinsky et al., 2001) as well as mRNAs translationally regulated at synapses (Kanai et al., 2004; Knowles et al., 1996; Kohrmann et al., 1999; Krichevsky and Kosik, 2001; Mallardo et al., 2003). However, the compositional diversity, cellular functions, and underlying mechanisms of Staufen-containing RNPs remain largely unknown.

The shared presence of Staufen (Stau) and an associated protein, Barentsz (Btz),
on maternal and neuronal RNPs suggests a compositional similarity between at least two
classes of RNA storage/transport granules (Kiebler et al., 1999; Macchi et al., 2003;
Mallardo et al., 2003). This hypothesis is further supported by roles for Stau in both
maternal and neuronal mRNA transport (St Johnston et al., 1991; Tang et al., 2001), and
for FMRP (dFMR1 in Drosophila) in translational repression during Drosophila oocyte
development (Costa et al., 2005). While additional shared components may soon be
identified using biochemistry combined with proteomics (Elvira et al., 2006; Kanai et al.,
2004), there is currently limited information on how far biochemical and functional
similarities between neuronal and maternal RNPs extend.

A third class of conserved somatic cytoplasmic RNPs, termed cytoplasmic RNA
processing bodies (“P-bodies”; also termed GW182 or DCP bodies), have been described
in yeast, C. elegans, and mammalian cells. P-bodies contain non-translating mRNAs and
multiple proteins involved in mRNA degradation and translational control (Kiebler and
Bassell, 2006). While first described as sites of mRNA decapping and 5’ to 3’
exonucleolytic degradation (Cougot et al., 2004; Sheth and Parker, 2003), P-bodies have
recently been shown to function in conventional and miRNA-mediated translational
control as well as mRNA storage (Brengues et al., 2005; Coller and Parker, 2005; Liu et
al., 2005; Pillai, 2005). Indeed, shared features of yeast mRNA turnover and translational
control pathways are indicated by the observation that two proteins that accumulate with
mRNA in P-bodies, Dhh1p and Pat1p, promote both mRNA decapping and translational
repression (Coller and Parker, 2005). Similarities between P-bodies and maternal RNPs
are further suggested by the known functions of Dhh1p-orthologous, DEAD-box RNA
helicases (Me31B, CGH-1, and Xp54) in maternal RNA granules of Drosophila, C.
*elegans*, and *Xenopus* oocytes respectively (Ladomery et al., 1997; Nakamura et al., 2001).

More recent experiments have demonstrated not only that mRNAs can move from P-bodies to polysomes (Brengues et al., 2005) but also that specific polyadenylated mRNAs can move into a P-body, remain polyadenylated and re-enter the translational pool again later (Brengues and Parker, 2007). These RNAs are accompanied by the proteins PABP, eIF4E, eIF4G, components that have been associated with another granule type, stress granules (Sossin and DesGroseillers, 2006). Not only does this suggests that P-bodies are triage sites for mRNAs entering and exiting translation this also suggests conservation with yet another type of granule, the stress granule. These observations have led us to hypothesize that many RNA granules, within and between organisms, have many functions in translational control and will share a core composition and function.

In this work we provide experimental support for a model in which *Drosophila* neuronal Staufen-containing RNPs (also referred to here as "staufen RNPs" or "staufen granules") share fundamental organization with maternal RNA granules and somatic P-bodies. Staufen RNPs visualized in *Drosophila* are shown to contain not only maternal translational control and RNA-transport molecules, but also components of miRNA, nonsense-mediated decay (NMD), and RNA-turnover pathways present on somatic P-bodies. Additionally, we present functional data showing that Me31B/Dhh1p, a protein present in neuronal staufen granules, P-bodies, and maternal RNA granules, functions: a) together with another dFMR1-associated, P-body protein (Trailer Hitch/Scd6p) in dFMR1-driven, Argonaute-dependent translational repression in the developing eye disc;
b) dendritic elaboration in larval sensory neurons, a process previously shown to be regulated by translational repressor proteins Pumilio (Pum), Nanos (Nos), and dFMR1; and c) in bantam miRNA-mediated translational repression in the developing wing imaginal disc. Thus, in addition to documenting broadly conserved composition and function of RNA granules in neuronal, germline, and somatic cells, we identify Me31B as novel component of the dFMR1 pathway, which acts as critical regulator of dendritic morphogenesis and microRNA function in vivo.

**MATERIALS AND METHODS**

*Drosophila stocks*

Fly stocks were raised at 25°C on standard cornmeal and agar media. Wild-type (Oregon-R and w^{1118}) were from Ramaswami lab stocks. Other strains were obtained from *D42-Gal4-chaGal80* (constructed by S. Sanyal with components from T. Kitamoto and G. Boulianne); *C380* (V. Budnik); *C155* (C. Goodman); *UAS-dFMR1* (T. Jongens); *sev-dFMR1* (P. Jin); *UAS-Stau-GFP* (A. Brand); *UAS-Me31B^{hpn}* (R. Ueda); and *elav, UAS-GFP:MCP:nls and UAS-CamKII^{3' UTR}−ms2* (S. Ashraf and S. Kunes). *Gal4^{477} ; UASmCD8-GFP, UAS-Flip Act<CD2<Gal4* was constructed using strains from W. Grueber and S. Sanyal; *hsFLP-1; FRT 40A , armadillo-lacZ* (Bloomington); “hid-reporter” and “bantam reporter” lines are described in Brennecke et al. (Brennecke et al., 2003). *Me31B* alleles were as previously described (Nakamura et al., 2001).

*Drosophila neuron primary cell culture*

Cells for culture were obtained from the thoracic-abdominal (ventral) region of the CNS of late third-instar larvae. Tissues were dissected and placed into a Liberase
enzyme (combination of collagenase and dispase) solution and incubated at room
temperature for one hour. Tissues were then rinsed in culture medium (Schneider’s-or
IL15-based medium) and subjected to two mechanical trituration steps. Cells were plated
onto coverslips coated with Concanavalin A and laminin in tissue culture dishes and
allowed to grow at 25 °C for 3-4 days prior to immunostaining. We used a composite
Gal4/Gal80 system (D42-Gal4; chaGal80) to drive expression of a functional Stau:GFP
fusion protein (UAS-Stau:GFP) or dFMR1 (UASdFMR1) in a subset of motor neurons.
Cells were identified using confocal microscopy by the presence of Stau:GFP (or
dFMR1-positive) punctae allowing for the identification of a discrete population of
neurons in an otherwise heterogeneous neuronal culture.

**Immunohistochemistry**

Primary antibodies used for neuronal granule staining are listed in Table 4.1.
Additional primary antibodies used were mouse anti-β Galactosidase (Molecular Probes),
rabbit anti-GFP (Molecular Probes), mouse anti-DLG 4F3 (Developmental Studies
Hybridoma Bank), and goat anti-HRP-TRITC (Sigma). Secondary antibodies used were
FITC (Sigma), Alexa 488-, Alexa 555-, Alexa 568-, and Alexa 647-conjugated anti-rat,
mouse and -rabbit IgG (Molecular Probes). Cultured cells were fixed and stained using
as follows. Briefly, 3-day old ventral ganglion cell cultures were rinsed in prewarmed
PBS buffer [pH = 7.2] and fixed for 10 min in 3.5% paraformaldehyde in PBS. Cells
were blocked for 30 min in Block solution (PBS containing 0.1% Triton X-100, 2% BSA,
and 5% normal goat serum). Primary and secondary antibodies were diluted in Block
solution and incubated with cells for 2 hr and 1 hr respectively at RT. After rinsing,
preparations were mounted in Vectashield Mounting Medium (Vector Labs) and imaged on a Nikon PCM2000 laser confocal microscope using Simple PCI software. Further discussion of methods used to examine colocalization of neuronal granule components can be found in the Supplementary Material.

For larval CNS preparations, wandering third instar larvae were processed according to the method of Sanyal et al., 2003 with the following modification. To permeabilize the sheath surrounding the ventral ganglion, CNS preps were treated with 50 µg/ml collagenase diluted in HL-3 saline (+Ca\(^{2+}\)) for 3 min prior to fixation. Immunostaining of *Drosophila* oocytes was done essentially as described in Wilhelm et al. (Wilhelm et al., 2003) with the following alterations. Ovaries were dissected in room temperature PBS + 0.1% Triton X-100 and fixed for 10 min in one part 3.7% paraformaldehyde in PBS to six parts heptane.

**Immunoprecipitation of Me31B**

Immunoprecipitation from head extracts with rat anti-Me31B was carried out essentially as described (Nakamura et al., 2001). Samples were separated by SDS-PAGE, transferred to PVDF membrane (Millipore) and analyzed by Western blotting. Proteins were detected by ECL (Amersham). 

**Analysis of *Drosophila* rough eye phenotypes**

*Drosophila* genotypes used for SEM analysis and tangential eye sectioning where as follows: dFMR1 overexpression: +/SevdFMR1. Me31B suppression:

Me31B\(^{\Delta1}\) FRT40A/SevdFMR1 and Me31B\(^{\Delta2}\) FRT40A/SevdFMR1. Me31B “rescue”:

Me31B\(^{\Delta2}\) FRT40A/SevdFMR1; +/- P[w MeAflIII]. Tral suppression: +/SevdFMR1; +/- Tral
\( FRT2A \) and \( +/- SevdFMR1; +/- Tral^{Δ4} FRT2A \). Tral “rescue”: \( P[Tral-10]/SevdFMR1; +/- Tral^{Δ4} FRT2A \). All indicated stocks (above) were crossed to \( w^{1118} \) to generate heterozygotes for subsequent analysis.

Further analysis of the rough-eye phenotype using scanning electron microscopy (SEM) and in tangential eye sections is described below.

**Generation and characterization of me31B-mitotic clones**

Mitotic recombination clones were induced 48 ± 2hr after egg laying (AEL) in staged larvae by heat shock at 37\(^\circ\)C for 90 min. Larval genotypes used were \( hs-FLP1; FRT40A, arm-lacZ/Me31B Δ1, FRT 40A; bantam-reporter (or hid-reporter) \). Discs were dissected at 120 ± 2hr AEL, fixed with 4% formaldehyde and stained with different antibodies. The discs were mounted in Vectashield (Vector Labs) and analyzed by confocal microscopy (Zeiss LSM 510) with a 20x objective. Clone areas were measured and analyzed using Adobe Photoshop.

**Generation of tral null alleles**

Null alleles for \( tral \) were generated by imprecise mobilization of transposon \( P[w^{GS12288} GSV6] \) in a homozygous viable P-element insertion line, in which GSV6 element (Toba et al., 1999) is inserted 395 bases distal to the 5’ side of the initiation codon of the \( tral \) coding region. We generated excision lines by crossing virgin \( GS12288 \) females to males possessing \( Δ2-3 \) transposase. From 200 independent excision lines established, four lines were found to contain a deletion within the \( tral \)-coding region. Breakpoints of the deletion lines were determined by a direct sequencing of the PCR products amplified
from genomic DNA.

**Construction of tral transgenes**

A genomic “rescue” transgene for *tral* was constructed as follows. A ~6.2 kb PstI/StuI genomic fragment, which contains entire *tral* locus and ~1.2 kb upstream promoter region, was isolated from a *Drosophila* genomic clone in λFixII (the *Drosophila* genomic library was provided by B. Suter) was subcloned into pCaSpeR3 to yield the P[w\(^+\) Tral \(^+\)] plasmid. To make Gal4 responsive *tral* transgenes, the plasmid pTGW-Tral was generated by PCR amplifying the *tral* open reading frame into pENTR-TOPO (Invitrogen) using standard techniques. The open reading frame was then transferred to pTGW (which contains the pUASt promoter and an amino terminal eGFP tag followed by a Gateway cassette and is available from the DGRC) via the Gateway cloning system (Invitrogen). This transgene was renamed pUASt-GFP-Tral. Both P[w\(^+\) Tral \(^+\)] and pUASt-GFP-Tral plasmids were used as substrates for P-element-mediated germline transformation.

**Construction of me31B transgenes**

Targeted overexpression of *Me31B* was achieved by cloning the wild-type *Me31B* open reading frame into the pUAST vector (Brand and Perrimon, 1993) and generating flies through germline transformation (Genetic Services, Inc.). Transformed stocks with UAS-*Me31B* alleles were crossed to various GAL4 driver stocks.

**FRAP Analysis**
Cultured motor neurons from larval neuronal tissue of *C380, cha-Gal80* Stau:GFP animals were used for FRAP experiments at 3-days of age. FRAP data collection was obtained using a Zeiss 510 Meta confocal system with an inverted microscope and the LSM software package. Stau:GFP cells exhibiting multiple numbers of granules were chosen for testing. Granules in neurites (but near the cell body) were tested with an average of 2 granules per cell being subjected to FRAP.

A small region of interest (ROI) was drawn around each granule to be tested. Using the LSM software program, bleach and time series protocols were set up - with parameters that established to allow complete bleaching and optimal imaging of the unbleached particles (50 scans of the ROI at 50% Argon laser power for bleaching, and an average 4 rapid scans at low laser power taken every 30 seconds for a duration of 15 minutes to monitor recovery). An unbleached granule was used as a control. Granule fluorescence data was determined using LSM software after appropriate background subtraction.

Data were exported and processed in Excel. Granule fluorescence from bleached granules were "normalized" at each time point to an unbleached control granule tracked in the same neuron. Normalized values were averaged to generate the relative intensity values plotted on the graph (so n=6 neuron; 11 particles). The fluorescence recovery curve was plotted (± SEM) using nonlinear regression. Fitting was carried out using the Marquardt method (Graphpad Software).

**Analysis of CamKII mRNA localization**

For localizing CamKII mRNA, the strain *elav Gal4, UAS-GFP:MCP:nls; UAS-*
CamKII 3' UTR –ms2 was used (Ashraf et al. 2006). Only about 10% of neurons in this culture showed fluorescent particles in neurites (others showed almost exclusively nuclear fluorescence). This diversity may derive in part from the fact that the elav Gal4 driver used here (for technical ease due to the number of transgenes involved) is expressed more widely than the D42-Gal4; chaGal80 driver used in all the other experiments. Cells containing neuritic CaMKII mRNA were selected for analysis.

**Immunological reagents**

Sources of primary antibodies used for immunohistochemistry; immunoprecipitation, and Western analysis are listed in Table 4.1. Those generated as part of this work are described below.

For Rat anti-Me31B, His-tagged Me31B protein (Met2-Gln294) was expressed in E. coli BL21 cells by IPTG induction, and purified with Ni-NTA agarose (Nakamura et al., 2001). The protein was further purified with a preparative disc SDS-PAGE system. Polyclonal rat antibodies were generated by Kitayama Labs (Nagano, Japan). The Pcm antibody was made by expressing the 54 kD C-terminal portion of Pcm as a His-tag fusion protein in E. coli using the expression vector pET28a. The histidine tag was removed by thrombin treatment and the Pcm protein fragment cut from the gel for use in raising antibodies. The antibody was prepared by Eurogentec. The full length DCP1 open reading frame was PCR amplified from EST clone GH04763 and cloned into pENTR. The DCP1 open reading frame was then transferred to pProEX-HTc that contained a Gateway cassette via the Gateway cloning system. His6-tagged DCP1 was expressed and purified under denaturing conditions and used to generate rabbit polyclonal antisera using standard protocols (Covance). The 416 aa of UPF1 were PCR
amplified from EST clone RE52657 and cloned into pENTR. UPF1-416 was then transferred to pProEX-HTc that contained a Gateway cassette via the Gateway cloning system. His6-tagged UPF1-416 was expressed and purified under denaturing conditions and used to generate rabbit polyclonal antisera using standard protocols (Covance).

Analysis of colocalization of neuronal granule components

**Excluding bleed-through:** Image acquisition parameters for double-stained images were rigorously tested to exclude bleed-through of signal from one channel to the other. Thus, single-fluorophore-labeled control specimens were viewed under imaging conditions used in our experiments to ensure no bleed-through into the other channel. In addition, internal controls usually present in each experiment - bright punctae seen in one or other channel, but not in both - also confirmed the absence of bleed-through.

**Thresholding and colocalization analysis:** A background signal, defined as fluorescence from regions of coated cover slips devoid of cells, was subtracted from each image. This was generally low and even. Each background-subtracted image was independently stretched for optimal contrast (min/max corresponding to 0-256). Percent colocalization of neuronal granule components was then determined using the Manual Count function of Metamorph (Universal Imaging Corp.) software. To identify the fraction of Staufen/ dFMR1 labeled particles that contained specific P-body or other RNA granule component, we first identified and counted all Stau, Stau:GFP or dFMR1-positive particles in neurites of cultured motor neurons, We then examined both the merged images as well as in other single-channel to determine the presence of the other proteins of interest.

Most double-labeled particles appeared yellow in the merged image. However,
some double-labeled particles contained disproportionately high or low levels of one or other fluorophore. Because these did not appear quite yellow in the merged image, they were identified more easily by examining the single channels. To avoid ambiguity in assessing the precise spatial overlap of particles seen in each individual channel, circles were drawn manually around Stau:GFP particles in the green channel, and a “region transfer” routine in Metamorph was used to computationally transfer these circles to identical coordinates on the red channel.

Localizing Scd6 to yeast P-bodies

For Fig 4.4E-G, P-bodies were visualized using strain yRP2166 (MATa, ura3Δ, leu2Δ, his3Δ, met15Δ, SCD6-GFP). This strain was transformed with pRP1155, which expresses a DCP2RFP fusion. Cells were grown in 2% dextrose media until OD600=0.3. Cells were harvested in media lacking glucose and visualized by confocal microscopy.

Analysis of rough eye phenotypes by SEM and tangential sectioning

The rough-eye phenotypes were easily scored and, in each case, suppression and “rescue” phenotypes were readily distinguishable as demonstrated by “blind” experiments in which experimenters had to assess relative roughness of the eyes viewed under a dissection microscope. Phenotypes were often even more evident in tissue sections (lower panels in Figure 4.5) that examined organization of individual cells in ommatidia.

To examine eye phenotypes by scanning electron microscopy, whole adult flies of appropriate genotypes were fixed for 2 hours in 1% formaldehyde, 1% glutaraldehyde, 100 mM sodium cacodylate [pH 7.2]. Flies then went through a graded series of ethanol
dehydration (25, 50, 75, and 100% ethanol at least 4 hours per grade) and were dried using a critical point dryer. Flies were coated with gold and examined on a Philips 515 scanning electron microscope. Briefly, adult eyes were dissected, fixed in Trump’s fixative (4% paraformaldehyde, 1% glutaraldehyde, 100mM sodium cacodylate [pH 7.2], 2mM sucrose, 0.5 mM EGTA) post-fixed in 0.01% osmium tetroxide, dehydrated in ethanol, and then embedded in Epon/Araldite resin. Sections (1µM) were cut on an ultramicrotome, stained in toluidine blue, mounted in VectaMount Permanent Medium (Vector Labs) and examined and photographed with a Nikon Eclipse E800 microscope and a SPOT RT Mono2000 (2.1.1) camera using SPOT software.

**Analysis of Dendritic Complexity**

Experiments were done in a Gal4 477, UAS-mCD8-GFP; UAS-flp, Act<CD2<Gal4 background in which flp-recombinase target sequences (“<”) flanking `CD2 stuffer sequence is often excised thorough the activity of Gal4 477-driven Flp recombinase. Thus, in this background, individual Gal4 477 positive da sensory neurons are occasionally very brightly labeled by Actin-Gal4 mCD8:GFP. In genetic backgrounds carrying a Gal4-responsive transgene, the transgene is also strongly expressed.

Wandering third instar larvae of the appropriate genotype were filleted and fixed essentially as described in Grueber et al. (Grueber et al., 2002). Fixed specimens were rinsed with PBS and mounted muscle side up on Superfrost slides (VWR). Imaging was performed on a Nikon PCM2000 laser confocal microscope. Two micron Z sections were taken through the ddaC neurons of segments A2 and A3 with a 20X objective. Z stack projections were assembled with Simple PCI software. Images were sorted and
selected for quantification based on image quality, lack of extensive folding of the underlying cuticle, and absence of damaged or broken branches or tissue artifacts. No more than two neurons per animal were used in the analysis. Dendrites were traced on a digital tablet using Simple PCI software. Only the medial half of the dendrites where counted to avoid confounding results caused by including the axon. The medial half was determined by including all processes exiting from the medial half of the cell body from the primary to terminal branches. The reversed Strahler method (Uylings et al., 1975) was used to count the number of branches per branch order in accordance with the protocol used in Grueber et al. (2002). In addition, data regarding branch lengths and branch point number were also collected.

RESULTS

**Neuronal staufen granules in *Drosophila***

To identify and characterize *Drosophila* RNPs involved in neuronal translation control, we combined a primary cell culture system (Kraft et al., 1998) with microscopic localization of transgenically expressed Stau, a highly conserved protein of maternal RNPs and mammalian neuronal granules (Ferrandon et al., 1994; Kiebler et al., 1999). A Stau:GFP fusion protein expressed in *Drosophila* ventral ganglion neurons is concentrated in puncta within neurites of 3-4 day old primary cultures of dissociated larval ventral ganglia, with large puncta observed in the cell body (Figure 4.1A; Figure 4.2). Of 292 granules analyzed in 9 Stau:GFP expressing cells, 56.5% of granules were within 1 micrometers of branch points and 33.9% away from branch points (Figure 4.1A and inset). This observed localization of staufen granules is consistent with the
previously proposed role for translational regulation in controlling dendritic branching in
*Drosophila* (Ye et al. 2004). *In vivo*, pan-neuronally expressed Stau::GFP revealed
similar particles within peripheral nerves exiting the larval central nervous system as well
as in cell bodies within the ventral ganglion (Figure 4.1B).

To determine whether these Stau::GFP particles were similar to mammalian RNPs
involved in neuronal mRNA regulation, we asked if they contained other established
components of mammalian neuronal RNPs. As shown in Figure 4.1, Stau::GFP-
containing granules were strongly labeled by antibodies against dFMR1 (Figure 4.1C-E)
or Btz (Figure 4.1F-H). Stau::GFP and dFMR1 colocalized extensively but not
completely in wild-type and Stau::GFP- or dFMR1-overexpressing neurons (Table 4.1;
Figure 4.1; Figure 4.3). These results indicate that dFMR1 and Stau exist substantially in
the same granules, but can also be observed in separate yet related particles (see
Discussion).
Figure 4.1. *Drosophila* neurons have ribonucleoprotein particles containing Stau, dFMR1, Btz and a dendritically targeted mRNA. (A) Stau:GFP (green) in cultured *Drosophila* motor neurons counterstained with an anti-HRP antibody (red). The inset shows Stau:GFP puncta at the base of small neurite branches (arrows). These puncta show occasional bidirectional movement within neurites. (B) View of a *Drosophila* larval ventral ganglion and emerging nerve from an animal expressing Stau:GFP in the nervous system. (C-E) Confocal image pair and merged image of a cultured motor neuron labeled for Stau:GFP (C) and endogenous dFMR1 (D). Dashed boxes show regions optimized for displaying faint spots: the yellow arrowheads show that particles appearing red on the merged image E, in fact contain Stau:GFP (green). (F-H) Cell double labeled with Stau:GFP (F) and endogenous Btz (G). (I-K) *Drosophila CaMKII* mRNA (I) visualized by ms2-tagged CamKII mRNA combined with MCP:GFP detection (Ashraf et al., 2006) is present on dFMR1-positive particles (J). (L) In FRAP experiments in live cultured motor neurons expressing Stau:GFP, images of a staufen granule were recorded (“before”), immediately after bleaching (“0 sec”), and once every 30 sec during the course of recovery. (M) For each time point, fluorescence intensity within a small region of interest (ROI) was measured and plotted on the graph after normalization to a paired “unbleached” spot. From the data set (n = 6 cells; 11 spots), a fluorescence recovery curve was calculated using nonlinear regression. Rectangles frame the bleached particle; ROIs, not shown, were smaller and closer to spot dimensions. Scale Bar: 10µm. As seen in Barbee et al. (2006)
Table 4.1. Percent Colocalization of P Body Components with Stau- and dFMR1-Containing Granules\(^a\)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Stau:GFP Expressing</th>
<th>dFMR1 Overexpressing</th>
<th>Wild-Type ((\nu^{1118}))</th>
<th>Wild-Type ((\nu^{1118}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>v. Stau</td>
<td>v. dFMR1</td>
<td>v. dFMR1</td>
<td>v. Stau</td>
</tr>
<tr>
<td>Stau</td>
<td>—</td>
<td>89.0 (11; 402)</td>
<td>77.2 (8; 409)</td>
<td>—</td>
</tr>
<tr>
<td>dFMR1</td>
<td>60.3 (19: 524)</td>
<td>—</td>
<td>—</td>
<td>45.1 (17; 1223)</td>
</tr>
<tr>
<td>Me31B</td>
<td>71.0 (12; 526)</td>
<td>85.0 (4; 207)</td>
<td>60.0 (14; 432)</td>
<td>50.2 (8; 440)</td>
</tr>
<tr>
<td>Traf</td>
<td>100.0 (6; 32)</td>
<td>90.6 (7; 335)</td>
<td>56.8 (6; 491)</td>
<td>ND(^b)</td>
</tr>
<tr>
<td>Fcm</td>
<td>68.0 (7; 17)</td>
<td>72.3 (7; 343)</td>
<td>66.3 (8; 401)</td>
<td>ND(^b)</td>
</tr>
<tr>
<td>DCP1</td>
<td>85.0 (5, 91)</td>
<td>85.6 (11, 346)</td>
<td>75.3 (3, 87)</td>
<td>ND(^b)</td>
</tr>
<tr>
<td>UPF1</td>
<td>90.0 (6; 130)</td>
<td>80.8 (10; 304)</td>
<td>58.3 (3; 96)</td>
<td>ND(^b)</td>
</tr>
<tr>
<td>Ago-2</td>
<td>74.3 (4; 109)</td>
<td>73.0 (6; 293)</td>
<td>ND(^c)</td>
<td>ND(^c)</td>
</tr>
</tbody>
</table>

\(^a\)Values are expressed as percent colocalization (number of cells analyzed; number granules analyzed).

\(^b\)Antibodies both rabbit polyclonal.

\(^c\)Percent colocalization not determined.

Table 4.1. Percent Colocalization of P-body Components with Stau- and dFMR1 Containing Granules.
Figure 4.2. Neuronal staufen granules are distinct particles in the cytoplasm of cultured *Drosophila* neurons. (A and B) show the same neuron under phase contrast microscopy (A) and with green Stau:GFP particles overlaid on a dim phase image (B). The particles clearly exist (indicated by arrows) within the neuronal cytoplasm and in healthy, well-developed neurites. (C-E) Confocal images pairs (C and D) and the merged image (E) show that an enzyme Lk6/MNK is not significantly enriched in Stau:GFP positive particles. Thus, neuronal granule association is a specific property shown by a restricted group of RNA regulatory proteins. Scale Bar: 10μm. As seen in Barbee et al. (2006)
For additional evidence that Staufen granules could be involved in translational repression, we also examined whether a known dendritically transported mRNA was present in these Staufen/dFMR1-positive granules. Recent work has shown that Drosophila CaMKII mRNA is transported along dendrites through a process stimulated by neuronal activity (Ashraf et al., 2006). This phenomenon is analogous to activity-stimulated movement of mammalian CaMKII mRNAs in Staufen-positive neuronal RNPs (Kosik and Krichevsky, 2002). To visualize CaMKII mRNA, we cultured neurons co-expressing a GFP-tagged, nuclearly-targeted RNA virus capsid protein (GFP:MCP) and CaMKII mRNA, multiply tagged with binding sites for MCP (Ashraf et al., 2006). Figure 4.1I-K shows that CaMKII mRNA-containing puncta observed in neurites overlap with protein markers of staufen granules.
Figure 4.3. Endogenous Stau and dFMR1 are extensively colocalized in control \( w^{1118} \) and dFMR1 overexpressing neurons. (A-C) Image pair and merged image showing colocalization of Stau (A) and dFMR1 (B) in \( w^{1118} \) neurons. The percentage colocalization observed in A-C (45.1% of dFMR1-positive puncta are positive for Stau) is increased to 92.1% in dFMR1 overexpressing cells (D-F). The improved colocalization could arise either from clustering or merging of small granules of variable composition into larger granules of more average composition; or from increasing the likelihood of detection of either antigen by increasing the number of protein molecules within each particle. The second explanation certainly contributes, as shown in Figure 4.2 (N-P and insets), where Me31B particles that appear Stau-negative in one set of imaging conditions are clearly seen to have Stau under better optimized imaging.
conditions. Scale Bar: 10µm. As seen in Barbee et al. (2006).
The presence of Stau, dFMR1, Btz, and, in at least some cases, CaMKII mRNA in overlapping puncta indicates that these foci represent Drosophila neuronal RNPs likely to function in the transport and translational regulation of neuronal mRNAs. Consistent with this hypothesis, these Staufen/dFMR1-positive granules also stain with antisera against the RNA-binding protein Ypsilon Schachtel (Yps; Figure 4.4A-C) and the Zipcode Binding Protein (ZBP/Imp; Figure 4.4D-F), both of which function in transport or regulation of localized mRNAs (Mansfield et al., 2002; Munro et al., 2006).

Moreover, these granules also stain positive for: a) the RNA binding proteins and translational repressors Pum and Nos, recently implicated in neuronal translational control and dendrite morphogenesis (Ye et al., 2004); b) the cap-binding translational-initiation factor, eIF4E (Sonenberg and Gingras, 1998); together with c) the eIF4E-inhibitory protein Cup, which represses translation by binding to and blocking eIF4E function (Figure 4.4P-R). The presence of Cup is consistent with translational repression of particle-associated mRNAs (Lasko et al., 2005). Control experiments established that colocalization of various granule proteins described is observed in neurons of multiple genotypes: a) wild-type control; b) UAS-dFMR1; or c) UAS-Stau:GFP, although images are typically shown from the bright, easily-imaged neuronal granules observed in cells expressing transgenically encoded Stau:GFP or dFMR1.
Figure 4.4. *Drosophila* staufen granules contain proteins involved in mRNA transport and translation. Image pairs and merged pairs of cultured dFMR1 expressing larval motor neurons double stained for dFMR1 and various proteins indicated. Confocal image pairs show Yps (A-C) Imp/ZBP (D-F), Pum (G-I), Nos (J-L), eIF4e (M-O) and Cup (P-R) are present on dFMR1-positive neuronal RNPs. Scale Bar: 10µm. As seen in Barbee et al. (2006).
The above results reveal two general properties of these granules in *Drosophila* neurons. First, in all cases a major class of granule exists wherein various proteins colocalize. For example, in wild-type cells 77.2% of dFMR1-containing particles are positive for Staufen and 75.3% for DCP1 (Table 4.1). Second, the observation that there are clearly granules that stain strongly for some, but not all markers, suggests there are subclasses of particles. Two additional observations suggest that these subclasses of particles are related to the major class of granules. First, they share components such as Stau or dFMR1. Second, the increased brightness of staufen granules in Stau:GFP or dFMR1 overexpressing cells (compared to wild-type), and the increase in colocalization is presumably due to growth, or fusion, of related and dynamic endogenous granules when assembly components are present in abundance. Such a model is supported by fluorescence recovery after photobleaching (FRAP) analyses (Figure 4.1L-M). These experiments show that staufen granules are dynamic, allowing relatively rapid exchange of at least a fraction of Stau:GFP with the cytoplasm. Taken together, these results are most consistent with *Drosophila* neurons containing a family of potentially interacting RNPs with related composition and function.

**Neuronal staufen granules are related to somatic P-bodies**

Recent work on yeast and mammalian P-bodies has suggested that they are dynamic RNPs like neuronal staufen granules (Andrei et al., 2005), and can be sites of transient translational repression (Bhattacharyya et al., 2006; Brengues and Parker, 2007; Brengues et al., 2005). Thus, we asked whether *Drosophila* staufen granules are related to P-bodies. We first tested whether neuronal staufen RNPs contained hydrolytic
enzymes that mediate removal of the $m7\text{GDP}$ (7-methyl-GDP) cap and subsequent 5’-3’ degradation. These enzymatic events are respectively mediated by a decapping enzyme that includes the Dcp1p subunit, and by the 5’ to 3’ ribonuclease Xrn1p (Parker and Song, 2004). Both Dcp1p and Xrn1p are integral components of yeast and mammalian P-bodies (Cougot et al., 2004; Sheth and Parker, 2003).

Remarkably, the *Drosophila* homologs of the degradative enzymes Dcp1p and Xrn1p (termed DCP1 and Pacman/Pcm) are clearly concentrated in Staufen- and dFMR1-containing RNPs (Figure 4.5B-D and 4.5F-H; Figure 4.6A-F). The presence of these enzymes suggests that *Drosophila* staufen granules may have additional roles in the control of mRNA turnover.
Figure 4.5. Neuronal staufen granules contain P-body components mediating translation repression and RNA decay. Yeast P-body proteins tagged with GFP in *S. cerevisiae* cells (left column) with their *Drosophila* orthologs localized relative to Stau:GFP in cultured Stau:GFP-expressing motor neurons. (A-D) Dcp1p/DCP1; (E-H) Xrn1p/Pcm; (I-L) Upf1p/UFP1; (M-P) Dhh1p/Me31B; (Q-S) and Ago-2 are also present on neuronal staufen granules. The inset with a magnified view of small Me31B particles in a neurite show that these also contain Stau:GFP. Scale Bar: 10µm for neurons. As
seen in Barbee et al. (2006).
We also examined the localization of other proteins known to concentrate in P-bodies and promote P-body formation. To date, mRNAs are known to be targeted to P-bodies by three pathways: i) the miRNA pathway by miRNAs and Argonaute (Ago) proteins (Liu et al., 2005; Pillai et al., 2005); ii) the NMD pathway, which is primarily driven by Upf1p (Sheth and Parker, 2006); and iii) a general pathway that works on bulk mRNA and is mediated by the Dhh1p and Pat1p proteins in yeast (Coller and Parker, 2005).

Association of Ago-1 and Ago-2 with dFMR1 has been argued by genetic and biochemical tests in Drosophila (Ishizuka et al., 2002; Jin et al., 2004). We therefore tested whether these proteins were present on Staufen- and dFMR1-positive granules. While the generally poor quality of the Ago-1 antibody for immunohistochemistry (data not shown; G. Hannon, personal communication) did not allow us to easily examine its presence in Drosophila neuronal granules, Ago-2 could be visualized within these particles (Figure 4.5Q-S; Figure 4.6J-L). This is consistent with recent analyses suggesting that miRNAs may function in granules such as P-bodies (Chu and Rana, 2006; Jakymiw et al., 2007; Liu et al., 2005; Pillai et al., 2005) although recent data suggested that P-body formation is not necessary for RNAi (Eulalio et al., 2007). Thus, our observation that Ago-2 is present in dFMR1-containing staufen granules is consistent with a possible role for FMRP/dFMR1 in miRNA/RNAi-mediated gene silencing (Kosik and Krivecksky, 2002) not necessarily as a component of the RISC complex.

The critical protein for translation repression in NMD, UPF1, is also present on staufen granules (Figure 4.5J-L; Figure 3.6G-H). Finally, we observed that Me31B, a highly conserved homolog of yeast Dhh1p, is also present on these particles (Figure
4.5N-P; Figure 4.7D-F). Thus, neuronal Staufen- and dFMR1-positive RNPs contain critical components of three different systems of translation repression suggesting that these RNPs, like P-bodies, mediate diverse RNA regulatory events.
Figure 4.6. P-body proteins also colocalize with dFMR1-positive neuronal RNP.

Confocal image pairs and merged images in this gallery show that the P body proteins DCP1 (A-C), Pcm (D-F), UPF1 (G-I), and Ago-2 (J-L) colocalize with dFMR1. These images complement Figure 4.2, which shows that P-body proteins colocalize with Stau:GFP. P-body proteins colocalize with dFMR1 in a large population of *Drosophila*.
dFMR1-containing neuronal granules (Table 4.1). Scale Bar: 10µm. As seen in Barbee et al. (2006).
The presence of similar proteins in staufen granules and P-bodies suggests that these neuronal and somatic RNPs share a similar core biochemical composition. These data also suggest that shared proteins will be common to other types of RNA granules, including maternal RNA granules. Consistent with this view, the decapping enzymes (Dcp1 and Dcp2) have been recently reported to be present on C. elegans P granules (Lall et al., 2005). Moreover, we find both Pcm/Xrn1p and DCP1 colocalize with Me31B in maternal RNA granules in Drosophila nurse cells (Figure 4.8A-F).
Figure 4.7: Me31B, Tral and DCP1 colocalize extensively with dFMR1 in cultured wild-type neurons. (A-C) Immunostaining of larval ventral ganglia shows that Me31B (A), Tral, (B) and dFMR1 (C) are found in the neuronal cytoplasm. (D-L) Individual and merged images of cultured w¹¹¹⁸ larval motor neurons double stained for dFMR1 and various proteins indicated. Confocal image pairs show that Me31B (D-F), Tral (G-I), and DCP1 (J-L) are present on dFMR1-positive neuronal RNPs. Again Me31B and Tral colocalize to a large fraction of Drosophila neuronal dFMR1-positive
granules (Table 4.1). Scale Bar: 10µm. As seen in Barbee et al. (2006).
Figure 4.8. RNA decapping and degradative enzymes are present on maternal RNP granules. (A-C) DCP1 and (D-F) Pcm colocalize with Me31B in cytoplasmic foci in nurse cells (stage 8 is shown). (C and F) Merged images. Scale Bar: 10µm. As seen in Barbee et al. (2006).
Trailer Hitch, a Me31B-associated maternal protein, is present on P-bodies and neuronal staufen granules. Me31B functions during oogenesis as a translational repressor of oskar mRNA in a well-studied eIF4E-Cup-Bru translational control complex (Lasko et al. 2005). This complex also contains a conserved Sm- and FDF-domain RNA-binding protein, Trailer Hitch (Tral). In Drosophila ovaries, Tral coimmunoprecipitates with Me31B and colocalizes with Me31B-containing maternal RNA granules (Boag et al., 2005).
Figure 4.9: Tral is an Me31B/dFMR1 associated protein present on staufen RNPs with a conserved homolog, Scd6p, in yeast P-bodies. (A) Western blot of Me31B coimmunoprecipitates probed with antibodies against Me31B, Tral, dFMR1, and dynamin. (B-D) Me31B (B) and Tral (C) colocalize in neuronal granules of dFMR1 expressing cultured motor neurons (Similar results in w1118 cells are shown in Figure 4.7D-I). (E-G) Yeast cells expressing Scd6p:GFP (E) and Dcp2p:RFP (F) showing colocalization of Scd6p:GFP to P-bodies. Scale Bar: 10µm. As seen in Barbee et al. (2006).
Figure 4.9A shows that a Me31B/Tral/dFMR1 complex coimmunoprecipitates from *Drosophila* adult head extracts, consistent with a model in which the three proteins function together in neuronal translation control. Me31B, Tral, and dFMR1 all have a similar, ubiquitous expression pattern in the central nervous system, showing a predominantly cytoplasmic, steady-state localization (Figure 4.7A-C). In cultured *Drosophila* neurons, Tral also localizes to Staufen- and dFMR1-containing granules (Figure 4.9B-D and Figure 4.7G-I). Moreover, a GFP fusion to Scd6p, the *S. cerevisiae* homolog of Tral, colocalizes with Dcp2p:RFP under high cell density or nutrient starvation, conditions which enlarge yeast P-bodies (Teixeira et al., 2005). Together, these data indicate that: (1) Tral is present on *Drosophila* neuronal RNPs in a biochemical complex that contains Me31B and dFMR1; and (2) Scd6p, the yeast homolog of Tral, is a component of P-bodies. The latter observation further extends similarities between P-bodies and staufen granules.
Me31B and Tral are required for dFMR1 mediated translational repression

The compositional similarity of P-bodies and staufen RNPs suggests that neuronal translational control is regulated through proteins and mechanisms associated with somatic P-bodies. To test this prediction, we focused on the highly conserved DEAD-box RNA helicase, Me31B, which functions in translational repression of maternal mRNAs and in the targeting of mRNAs to P-bodies (Coller and Parker, 2005; Nakamura et al., 2001). The presence of Me31B and Tral with Ago-1 on dFMR1-containing complexes suggests that these proteins may function in neuronal translation control, potentially with dFMR1 in miRNA-mediated processes.

To test whether Me31B and Tral function in dFMR1-mediated translational repression, we asked if defects caused by dFMR1 overexpression in developing eyes were modified in genetic backgrounds deficient for Me31B or Tral. Ectopic overexpression of dFMR1 in the compound eye driven by the sevenless enhancer (sev-dFMR1) results in a “rough-eye” phenotype through a pathway that requires dFMR1 domains essential for translational repression as well as Ago-1 function (Jin et al., 2004; Wan et al., 2000).
Figure 4.10. Me31B and Tral are required for dFMR1-induced defects in the Drosophila eye. (A-E) SEMs of adult compound eyes with paired retinal sections (F-J). Magnification of SEMs is 150X. Tangential sections of each genotype are at approximately the same depth. As seen in Barbee et al. (2006).
As shown in Figure 4.10B loss of a single copy of $me31B$ suppressed $sev-dFMRI$ induced rough eye phenotypes. Unambiguous suppression was observed with either $me31B^{\Delta 1}$ or $me31B^{\Delta 2}$ allele (Figure 4.10B; data not shown). Internally, the disruption of ommatidia caused by $dFMRI$ overexpression was also suppressed as observed in tangential sections (Figure 4.10F and 4.10G). This suppression is a direct result of $me31B$ deficiency, because a genomic $me31B^{+}$ transgene, $P[me31BAflII]$, which is capable of rescuing lethality of $me31B^{\Delta}$ mutants (Nakamura et al., 2001) rescues suppression of the $sev-dFMRI$ rough eye phenotype (Figure 4.10C and 4.10H).

Results with $tral$ mutations were similar. We isolated deletion alleles for $tral$ and found them to result in larval lethality. Both $tral$ deletions dominantly suppressed $sev-dFMRI$ induced rough eyes (Figure 4.10D and 4.10I; data not shown). A $tral^{+}$ genomic transgene ($P[tral-10]$) containing the entire $tral$ locus was sufficient to rescue the lethality of $tral^{-}$ mutants. This genomic transgene also “rescued” dominant suppression of the rough-eye phenotype, thereby demonstrating that phenotypic suppression of $sev-dFMRI$ occurs specifically due to loss of $tral$ (Figure 4.10E and 4.10J).

Given that Me31B, Tral, and dFMRI form a physical complex, the above results suggest that Me31B and Tral act, together with dFMRI, as translational regulators in neuronal cells. An alternative interpretation is that single copy deletions of $tral$ or $me31B$ block apoptosis or other developmental errors induced by $Sev-dFMRI$. However, this is unlikely for three reasons: First, coimmunoprecipitation and colocalization of Me31B, Tral, and dFMRI are more consistent with a direct mechanism. Second, all three proteins have RNA-binding domains that predict roles in translational control. Finally, ectopic expression of Me31B in the eye causes rough eyes via a mechanism requiring amino acid
residues necessary for translational repression (Figure 4.11A-C; see below).
Figure 4.11. Respective effects of Me31B and Tral overexpression in eye and sensory neuron development. (A-C) SEMs of the *Drosophila* compound eye showing that overexpression of Me31B in photoreceptors causes a “rough eye” phenotype (A-B) through a pathway that requires DEAD-box residues essential for translational repression (C). Magnification of SEMs is 150X. (D-F) Class IV ddaC neurons overexpressing *Tral* and *UAS-mCD8:GFP* showing an alteration in higher-order dendrite branching. (E and F) 40X zoom of Class IV ddaC neurons overexpressing *Tral* and *UAS-mCD8:GFP* (E) or and *UAS-mCD8:GFP* alone (F). Note that overexpression of Tral induces fine “tendrils”
at terminal dendritic branches. Scale Bar: 20µm. As seen in Barbee et al. (2006).
Me31B and Tral regulate dendrite morphogenesis in sensory neurons

The observed effect of Me31B (and Tral) induction on dendritic development of sensory neurons (Figure 4.11D-F and Figure 4.12) provides further evidence for function in neuronal translation regulation. Previous studies have established that translational control of gene expression regulates dendrite morphogenesis in vivo. For example, neurons of human Fragile X patients and Drosophila dFMR1 mutants show an increase in dendritic spine number and length (Lee et al., 2003; Nimchinsky et al., 2001). Conversely, induction of dFMR1, Pum, or Nos in Class IV Drosophila da sensory neurons greatly perturbs, and can dramatically reduce, higher-order dendritic branching (Lee et al., 2003; Ye et al., 2004). If Me31B and Tral act in dendritic translational control, we anticipated that their induction would also have specific effects on higher-order dendritic branching.

Overexpression of Me31B in Class IV neurons substantially reduced high-order dendritic complexity (Figure 4.12A-B). In neurons overexpressing Me31B, the number of higher-order dendrites was significantly reduced compared with the control, in which only the reporter gene UAS-mCD8:GFP was overexpressed (p≤0.001; Figure 4.12A-B and 4.12E). To determine if this effect of Me31B induction reflected increased translational repression activity of Me31B, we asked whether similar effects would be shown by induction of an Me31B mutant protein (D207A, E208A) homologous to a yeast Dhh1p mutant incapable of translational repression (Coller and Parker, 2005). Expressed at comparable levels (data not shown), the mutant transgene had no effect on dendritic complexity (Figure 4.12C and 4.12E) consistent with the observed effect being dependent on Me31B induced translational repression.
Overexpression of Tral in Class IV neurons also substantially changed dendrite morphology compared to the control (Figure 4.11D-F). Interestingly, closer examination revealed a significant increase in the number of finer dendritic “tendrils” at terminal dendritic branches compared to control neurons. Differences between effects of Tral and Me31B induction on dendritic arborization are consistent with a relatively specific role for CAR-1, the *C. elegans* ortholog of Tral, in translational control compared to CGH-1 (the Me31B ortholog), suggested by phenotypic differences following RNAi-mediated inhibition of respective proteins in the *C. elegans* germline (Audhya et al., 2005; Navarro et al., 2001).
Figure 4.12. Me31B regulates dendritic growth in sensory neurons. (A) Control Class IV ddaC neuron expressing UAS-mCD8:GFP alone. (B) Class IV ddaC neurons overexpressing Me31B and UAS-mCD8:GFP showing a reduction in higher-order dendrite arborization. (C) The same neurons overexpressing a mutant Me31B incapable
of translational repression (Me31B_{\text{D207A, E208A}}) show normal dendritic branching. (D) Transgenic RNAi dramatically reduces Me31B protein levels. Anti-Me31B staining of third-instar imaginal discs shows that \textit{UAS-Me31B}^{hpn} expressed in the patched domain of wing imaginal discs reduces Me31B levels along the anterior-posterior border (top panel) compared to control wing imaginal discs (lower panel). (E) Class IV ddaC neurons overexpressing a \textit{Me31B} RNA hairpin (\textit{UAS-Me31B}^{hpn}) exhibit abnormal dendrite morphology and increased high-order branching. (F) Numbers of dendritic branches in each order, as revealed by reversed Strahler analysis. Number of neurons analyzed for each genotype are: \textit{UAS-mCD8:GFP} control (n = 15), \textit{UAS-Me31B} (n = 10), \textit{UAS-Me31B}^{D207A, E208A} (n = 11), and \textit{UAS-Me31B}^{hpn} (n = 13). Values are mean ± standard error. A star (*) indicates a significant reduction in 5th-order dendrite branching following Me31B overexpression compared to the control (p<0.001) and a significant increase in 5th-order dendrite branching following \textit{Me31B} RNAi (p<0.001). Scale Bar: 20µm. As seen in Barbee et al. (2006).
In Class IV sensory neurons, loss of nanos or pumilio causes abnormal dendritic growth (Ye et al., 2004). This aberrant growth, visible in about 20% of mutant neurons is most easily apparent as a loss of “tiling”, a term that refers to the complete, non-overlapping coverage of the epidermis by dendrites of wild-type sensory neurons (Grueber et al., 2002; Grueber and Jan, 2004; Ye et al., 2004). We therefore asked whether loss of me31B, achieved by expressing a transgenic RNAi construct that generates a hairpin Me31B RNA (UAS-Me31B\textsuperscript{hpn}) would cause similar defects. As shown in Figure 4.12D, UAS-Me31B\textsuperscript{hpn} sensory neurons showed frequent defects in terminal dendrite morphology and dendritic tiling highly reminiscent of nanos and pum phenotypes. Incomplete coverage of the epidermis was observed in at least 33%, (n = 15 neurons) analyzed. Additionally, Me31B\textsuperscript{hpn} neurons show a modest increase (37%) in high-order dendritic complexity similar to that observed in dFmr1 mutants (Lee et al., 2003). Parallel analyses of a hairpin construct for Lk6, which encodes the Drosophila homolog of the eiF4e-kinase MNK, showed no effect on dendritic branching of class IV sensory neurons (data not shown).

From these data, we conclude that Me31B (and Tral) regulate dendritic arborization of Class IV da neurons. This observation, consistent with observations of other translational repressors such as dFMR1, Pum, and Nos provides a second line of evidence suggesting that Me31B and Tral function as neuronal translational regulators.

**Me31B functions in microRNA mediated translational repression**

Two previous findings led us to the hypothesis that the dFMR1-associated, Me31B protein may be required for miRNA/RNAi function. First, FMRP/dFMR1,
showing strong biochemical or genetic interactions with Ago-1 and Ago-2, is strongly implicated in microRNA mediated translational repression (Kosik and Krichevsky, 2002). Second, miRNA-mediated repression has been proposed to occur in P-bodies of somatic cells (Liu et al., 2005; Pillai et al., 2005) although P-bodies may not be required for microRNA pathway function (Eulalio et al., 2007). Thus, we tested whether Me31B is required in vivo for the function of bantam, an endogenous miRNA that represses hid mRNA translation in wing imaginal discs (Brennecke et al., 2003).

We used two transgenically encoded GFP reporters to assay bantam-mediated translational repression (Brennecke et al., 2003). The "hid reporter", which carries the 3'UTR of hid fused to the 3' end of EGFP-coding sequence, closely reports bantam repression of a native target mRNA. This 3' UTR contains four repeats complementary to bantam target recognition sequences, with several mismatches typically associated with miRNA mediated translational repression. The "bantam reporter", in which 4 synthetic repeats 100% complementary to the bantam target recognition element is fused 3' to EGFP coding module, also reports bantam function.

We used the heat-shock FLP/FRT system to generate me31b Δ/ Δ clones in the wing disc, identified these clones by loss of beta-galactosidase or Me31B staining with respective antibodies (Figure 4.13A-C). We then asked how a control protein (Dlg), hid reporter or bantam reporter expression was affected by loss of Me31B (Figure 4.13F-G). While cells lacking me31B showed no detectable increase in a control protein (Dlg) expression (Figure 4.13G), they showed clear increases in both hid reporter (Figure 4.13C-E) and bantam reporter (Figure 4.13H-J) expression indicating that bantam-mediated silencing does not function in the absence of me31B. These data, from in vivo
analyses of an endogenous miRNA in cells carrying a null mutation for me31B, support a recent study showing a role for RCK (the human homolog of Me31B) in Let-7 miRNA-mediated translational repression in cultured mammalian cells (Chu and Rana, 2006). In addition, our observations extend this study by demonstrating a role for Me31B in repression mediated by perfectly base-paired miRNAs. It should be noted that the requirement for Me31B for efficient repression of the bantam reporter does not necessarily mean that Me31B is required for miRNA mediated endonucleolytic cleavage since it is likely that repression by perfectly base-paired miRNAs can be a combination of translation repression, decapping, and/or endonucleolytic cleavage of the mRNA (Valencia-Sanchez et al., 2006). Importantly, these data demonstrate that Me31B is required for repression mediated by an endogenous Drosophila miRNA. An obvious corollary of our analysis in wing imaginal discs is that Me31B plays a similar role in mediating functions of neuronal miRNAs, although assays to directly test this issue are not immediately available in Drosophila.
Figure 4.13. Me31B is required for the *in vivo* function of an endogenous microRNA.

Heat shock induced clones in wing imaginal discs of *hs-FLP1; FRT40A, arm-lacZ/FRT40A, me31BΔ1*; reporter flies show dark me31B/me31B clones revealed either by staining for LacZ (B) or Me31B (A and C). Anti-GFP staining shows that the hid reporter (D and E) is upregulated in cell clones lacking Me31B (C). Similar analyses (F and G) show that a control protein Dlg (G) is not upregulated in me31B/me31B clones (F). However, consistent with a Me31B requirement in miRNA/RNAi, the bantam reporter expression is upregulated in me31B/me31B lacking cells (H-J). As seen in Barbee et al. (2006).
DISCUSSION

Neuronal staufen RNPs are related to somatic P-bodies

Several observations now indicate that P-bodies, maternal granules, and a major subclass of neuronal RNP are similar in underlying composition and represent a conserved system for the regulation of cytoplasmic mRNAs. As summarized in Table 4.2, known RNA transport and translational repressors shared between maternal and neuronal staufen granules now include, Stau, Btz, dFMR1, Pum, Nos, Yps, Me31B, Tral, Cup, eIF4e, Ago-2, and Imp. Strikingly, in human cells, the Me31B homolog RCK/p54, the Tral homolog RAP55, the four human Argonaute proteins, eIF4E, and a eIF4E binding protein analogous to Cup, 4E-T, are all found in P-bodies (Andrei et al., 2005; Cougot et al., 2004; Kedersha and Anderson, 2007; Liu et al., 2005; Pillai et al., 2005). In yeast, homologs of Me31B (Dhh1p) and Tral (Scd6p) are also known to be in P-bodies (Sheth and Parker 2003), and Dhh1p in particular plays a role in recruiting RNA decapping proteins and exonucleases to these RNPs (Coller and Parker, 2005). Consistent with the above observations in yeast, the enzymes involved in mRNA hydrolysis including the 5’ to 3’ RNA exonuclease Xrn1p/Pcm and the RNA decapping enzyme DCP1 are present on *Drosophila* neuronal staufen RNPs (Figure 4.5) and maternal RNA granules (Figure 4.8). The data unequivocally demonstrate tight spatial proximity of components mediating diverse RNA regulatory processes in *Drosophila* neurons.
### Table 4.2 Conserved Components of P-Bodies, Maternal Granules, and Neuronal Granules

<table>
<thead>
<tr>
<th>Protein Class</th>
<th>Mammalian and Yeast P Bodies&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Drosophila Maternal Granules&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Drosophila Neuronal Granules&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Mammalian Neuronal Granules&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
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<tr>
<td>RNA transport</td>
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<td>Stau, Btz</td>
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<tr>
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<td>dFMR1</td>
<td>dFMR1</td>
<td>FMRP, FXR1, FXR2</td>
</tr>
<tr>
<td>Zip-code binding</td>
<td>?</td>
<td>?</td>
<td>Imp</td>
<td>ZBP1</td>
</tr>
<tr>
<td>Pum domain</td>
<td>?</td>
<td>Pum?</td>
<td>Pum</td>
<td>Pum&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>GTP/GDP-Zn-finger domain</td>
<td>?</td>
<td>Nemo</td>
<td>Nemo&lt;sup&gt;slow&lt;/sup&gt;</td>
<td>?</td>
</tr>
<tr>
<td>DEAD-box RNA helicase</td>
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<td>Me31B</td>
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<tr>
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<td>Traf</td>
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<tr>
<td>Cap-binding</td>
<td>eIF4E&lt;sup&gt;n&lt;/sup&gt;</td>
<td>eIF4E</td>
<td>eIF4E&lt;sup&gt;slow&lt;/sup&gt;</td>
<td>under some conditions</td>
</tr>
<tr>
<td>Enhancers of decapping</td>
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<td>?</td>
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<td>?</td>
<td>PABP&lt;sup&gt;2d&lt;/sup&gt;</td>
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<td>?</td>
<td>Yps</td>
<td>Yps</td>
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<td>5' to 3' RNA decay machinery</td>
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<td>DCP1, Pacman</td>
<td>DCP1, Pacman (others not examined)</td>
<td>?</td>
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<td>Upf-1</td>
<td>Upf-1</td>
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<tr>
<td>miRNA, siRNA machinery</td>
<td>mAGO1, mAGO2</td>
<td>?</td>
<td>Ago-2</td>
<td>?</td>
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</table>

<sup>a</sup> Ortholog present but association with RNA granules has not been described.

<sup>b</sup> Sheth and Parker, 2003; Andrei et al., 2005; Liu et al., 2005a; Pillai et al., 2005.

<sup>c</sup> St Johnston et al., 1991; van Besen et al., 2001; Nakamura et al., 2004; Boag et al., 2005; Nakamura et al., 2004; Wilheim et al., 2000; this work; Wang et al., 1994; Forbes and Lehmann, 1998.

<sup>d</sup> This work (except PABP).

<sup>i</sup> Kieber et al., 1999; Macchi et al., 2003; Kanai et al., 2004; Antar et al., 2005; Zhang et al., 2001.

<sup>j</sup> eIF-4E and PABP can associate with P bodies in yeast under conditions of translational arrest induced by glucose deprivation (Teixeira et al., 2005).

<sup>k</sup> Ye et al., 2004 (describes particles likely to be, but not clearly established as, neuronal RNA granules).

<sup>l</sup> Skytist et al., 2000 (describes particles likely to be, but not clearly established as, neuronal RNA granules).

<sup>m</sup> Upf1 has also been shown to interact with Stau in a Stau-mediated NMD pathway (Kim et al., 2005).

<sup>n</sup> Pat1p acts as a general repressor of translation in yeast (Coller and Parker, 2005).

<sup>o</sup> Yang et al., 2006; this work.

<sup>p</sup> Andrei et al., 2005.

<sup>q</sup> Vessey et al., 2006.

As seen in Barbee et al. (2006).
The large collection of proteins and processes common to P-bodies, staufen granules, and likely to maternal RNA granules suggests that they share an underlying core biochemical composition and function, which would then be elaborated in different biological contexts. For example, one anticipates that proteins involved in mRNA transport will be more prevalent in maternal and neuronal RNPs, which need to be transported for their biological function.

An interesting aspect of neuronal staufen RNPs described here is the diversity of translational repression systems that are present within them. First, in Me31B they contain a protein that works in general translation repression of a wide variety of mRNAs, and can also affect miRNA based repression (Chu and Rana, 2006; Coller and Parker, 2005). Second, in Ago-2, they contain a component specific to miRNA/RNAi dependent repression. Third, neuronal staufen granules also contain UPF1, which was originally thought to be solely involved in mRNA degradation. However, because UPF1 can act as a translation repressor (Muhlrad and Parker, 1999; Sheth and Parker, 2006) and physically interacts with Stau (Kim and Kim, 2006), a reasonable hypothesis is that UPF1 might work in neuronal granules, in conjunction with Stau, to repress the translation of a subset of mRNAs. The presence of multiple mechanisms for translation repression colocalizing in granules in *Drosophila* neurons may allow for differential translation control of sub-classes of mRNA in response to different stimuli.

**Neuronal Granule Diversity and Function**

Evidence accumulating in the literature suggests that there is a potential diversity of RNA granule types in neurons and there have been many attempts to classify them (Kosik and Krichevsky, 2002; Sossin and DesGroseillers, 2006). Our observations in
*Drosophila* neurons are most consistent with a model in which a major subclass of neuronal RNP, in which various translational repressor and mRNA turnover proteins colocalize, is related to other compositionally distinct, diverse RNPs and likely performs many aspects of translational control. Because in most studies the large numbers of proteins have not been assessed in consecutive experiments such as ours, comparisons are difficult to make. However, a major subclass of Staufen-containing RNP is indicated by our data showing substantial colocalization among various proteins we have analyzed (Table 4.1; data not shown). Diversity is indicated by the lack of 100% colocalization: for instance, 55% of Staufen-positive particles in wild-type neurons do not contain detectable dFMR1.

Two types of observations suggest that the apparent subclasses of particles containing Stau or dFMR1, but not both, are related to the particles in which they colocalize. First, these two types of RNPs are clearly compositionally related to particles that contain both proteins. Second, this is supported by the observation that colocalization can be substantially increased under some conditions. Overexpression of either dFMR1 or Stau:GFP increases colocalization between Stau and dFMR1 from 45% in wild-type neurons to more than 80%. Concurrent with increased frequency of colocalization, Stau:GFP, or dFMR1 induction increases apparent particle size (or brightness) and reduces the total number of particles. The increase in colocalization and brightness, as well as reduction in particle number is most easily explained by growth and/or fusion of related RNPs. Significantly, similar effects on mammalian neuronal granule size and number have been reported following overexpression of Stau or another granule protein RNG105 (Kiebler et al., 1999; Shiina et al., 2005). Thus, the underlying regulatory
processes appear conserved between Drosophila and mammalian neurons.

While it remains unclear how FMRP, Stau, or RNG105 enhance granule growth or fusion, it is conceivable that individual mRNAs first form small RNPs whose compositions reflect specific requirements for translational repression of the mRNAs they contain. These small RNPs exist in dynamic equilibrium with larger RNPs in which multiple, diverse translational repression complexes are sequestered. Induction of factors that promote granule assembly by physiological factors or overexpression could push the equilibrium towards mRNP sequestration within large granules. A requirement of this dynamic model, which postulates interactions among different types of RNP, is that the RNPs themselves could change in composition during transport to synaptic domains. This is supported by FRAP analyses showing rapid exchange of Stau:GFP between cytosol and granule (Figure 4.1L-M). However, the limits of our imaging resolution should not be understated. The possibility that smaller granules made up of a handful of molecules are much more physiologically relevant but are unable to be seen using current imaging technology and all granule colocalization studies at this point suffer from this potential confound.

Additional types of RNPs have also been described in neurons. For example, polysomes apparently arrested in translation have been observed near dendritic spines and these RNPs show no obvious similarity to large, ribosome-containing particles termed neuronal RNA granules (Greenough et al., 2001; Knowles et al., 1996; Ostroff et al., 2002). In addition, a potentially distinct RNP containing Stau, kinesin, and translationally repressed RNAs, but not ribosomes, has been purified from the mammalian brain (Mallardo et al., 2003). More recently, it has been shown that RNPs
containing stress-granule markers TIA-1 and TIA-R as well as Pumilio2 are induced by arsenate-treatment of mammalian cultured neurons (Vessey et al., 2006) additionally stress granule components have been seen in P-bodies (Brengues and Parker, 2007). Interestingly, as previously shown for somatic cells, these large stress granules appear tightly apposed to domains containing DCP1 and Lsm1, markers of P-bodies (Vessey et al., 2006). Determining the temporal and compositional relatedness of such varied RNPs, their pathways of assembly as well as their functions, is a broad area of future research not only in neuroscience but also in cell biology.

**Two functionally important translational repressors in dFMR1-containing neuronal RNPs.**

Despite the complexity revealed by the diversity of neuronal RNPs, the importance and significance of the observed colocalization of Me31B, Tral, Argonaute, and dFMR1 in Staufen-positive neuronal RNPs is most clearly demonstrated by functional analyses revealing biological pathways in which these proteins function together.

Several independent lines of evidence are consistent with a function for Me31B in neuronal translational repression as part of a biochemical complex that includes dFMR1. First, sub-cellular localization studies indicate that Me31B and Tral localize to dFMR1-containing RNPs especially prominent at neurite branch points in cultured *Drosophila* neurons (Figure 4.7D-I; data not shown). Second, Me31B, Tral, and dFMR1 coimmunoprecipitate from *Drosophila* head extract, thus confirming the physical association of three proteins (Figure 4.9A). Third, loss-of-function alleles of either Me31B or Tral suppress the rough-eye phenotype seen when dFMR1 is over-expressed in
the sev-positive photoreceptors (Figure 4.10). Fourth, over-expression of Me31B in sensory neurons leads to altered branching of terminal dendrites, a phenotype also seen with over-expression analyses of Nos, Pum, and dFMR1 (Lee et al., 2003; Ye et al., 2004). Finally, reduction of Me31B expression in sensory neurons by RNAi results in abnormal dendrite morphogenesis and tiling defects, phenotypes similar to that observed following loss of *nanos*, *pum*, or *dFmr1* function (Lee et al., 2003; Ye et al., 2004). Significantly, the effect of Me31B on dendritic growth is correlated with its ability to function in translational repression (Figure 4.12C and 4.12E). These five independent lines of evidence provide considerable support for Me31B (and Tral) function in neuronal translation control processes. While the site of functional interaction between dFMR1, Me31B, and Tral (soma or neuronal processes) is not identified here, the importance of the physical interactions is clearly demonstrated.

Several observations also argue that Me31B acts, at least in part, within neurons to promote translation repression and/or mRNA degradation in response to miRNAs. This possibility was first suggested by the physical and genetic interactions of Me31B with dFMR1 (discussed above; Figure 4.7D-F and Figure 4.10), a protein that has been previously been implicated in the miRNA mediated repression (Ishizuka et al., 2002; Jin et al., 2004). Using direct assays for miRNA-mediated function *in vivo* (Brennecke et al., 2003), we show that Me31B is required for efficient repression by the *bantam* miRNA in developing wing imaginal discs (Figure 4.13). This identifies Me31B as a new protein required for efficient miRNA-based repression.

Recently, miRNA-based regulation has been shown to be important for the control of spine growth in hippocampal neurons (Schratt et al., 2006), and to be a target
of protein degradative pathways involved in long-term memory formation in *Drosophila* (Ashraf et al., 2006). Thus, our data predict that Me31B will be important in modulating miRNA function pertinent to developmental of functional neuronal plasticity

**Implications for translational control in neurons**

Our conclusion that Staufen- and dFMR1-containing neuronal RNPs are similar in organization and function to P-bodies has several implications for neuronal translational control. First, the presence of diverse translational repression systems on these RNPs suggests that, like in P-bodies, different classes of mRNAs will be repressed by different mechanisms. This may allow specific RNA classes to be released for new translation in response to different stimuli. Such diversity of control may allow synapses to remodel themselves differently depending on the frequency and strength of stimulation (e.g. LTD or LTP). Second, FRAP experiments indicate that both P-bodies and staufen granules are dynamic structures (Andrei et al., 2005; Kedersha and Anderson, 2007). This argues that like P-bodies, staufen granules are in a state of dynamic flux, perhaps in activity-regulated equilibrium with the surrounding translational pool. Third, the presence of mRNA degradative enzymes on staufen granules suggests regulation of mRNA turnover may play an important role in local synaptic events. For example, if synaptic signaling were to induce turnover of specific mRNAs at a synapse, then stimulated synapses could acquire properties different from unstimulated ones that retain a “naive” pool of stored synaptic mRNAs. Finally, these observations imply that the proteins known to function in translation repression within P-bodies will play important roles in modulating translation in neurons. Thus, we anticipate that proteins of
mammalian or yeast P-bodies such as Edc3p, Pat1p, the Lsm1-7p complex, GW182, and FAST will be present on and influence assembly and function of neuronal granules (Cougot et al., 2004; Eystathioy et al., 2003; Kedersha and Anderson, 2007; Sheth and Parker, 2003).
CHAPTER FIVE

GENETIC MODIFIERS OF \textit{DFMR1} ENCODE RNA-GRANULE COMPONENTS IN \textit{DROSOPHILA}
Introduction

The past decade of research in neuroscience has established that local protein synthesis is required for long-lasting forms of synaptic plasticity and memory. Local protein synthesis is also speculated to confer synapse-specific, activity-dependent changes (Frey and Morris, 1997; Martin et al., 1997; Nader et al., 2000). To accomplish this cellular feat, mRNA is localized and translated in situ within the dendrite (Lin and Holt, 2007; Sutton and Schuman, 2006). The mechanism by which this is orchestrated, however, is still largely a mystery. The identification of ribonucleoprotein particles (RNPs) in neurons has led to some insights and suggests strong functional conservation with translational control processes and with proteins in other systems (Knowles et al., 1996; Kohrmann et al., 1999). The current dogma of translational control in neurons is that mRNAs are transported in RNP complexes along microtubules from the cell body to the post-synaptic sites in dendrites where local synaptic activity can induce their translation (Aakalu et al., 2001; Bramham and Wells, 2007; Kosik and Krichevsky, 2002). Well-studied examples of locally regulated mRNAs include β-actin and CAMKII mRNA. β-actin mRNA is targeted to both synapses in dendrites and the leading edge of a neuronal growth cone. The primary protein responsible for it’s silencing during transport, and incorporation in a neuronal RNA granule is Zipcode Binding Protein (ZBP), named for it’s affinity for the “Zipcode” sequence in the 3’UTR of the β-actin mRNA (Tiruchinapalli et al., 2003). In mammals, both NMDA receptor activation and BDNF application have shown to cause an increase in dendritic transport (Eom et al., 2003; Tiruchinapalli et al., 2003). CAMKII mRNA is regulated by numerous proteins in both rodents and Drosophila; these include Cytoplasmic Polyadenylation Binding
Element Protein (CPEB), RNA granule protein (RNG105) and Fragile X Mental Retardation Protein (FMRP). \textit{CAMKII} mRNA is targeted to dendrites and has also been shown to lead to increased dendritic protein synthesis upon BDNF activation (Aakalu et al., 2001; Huang et al., 2003; Schratt et al., 2006).

One of the best-studied neuronal translational regulators is FMRP, primarily due to its role in human neurodevelopmental disease (O'Donnell and Warren, 2002). Unlike the transcript-specific ZBP protein described above, FMRP’s role in translational regulation is believed to occur more globally by blocking general translation at the step of translational initiation through the non-coding RNA (Gao, 2008; Zalfa et al., 2006). As expected from a more general repressor, the phenotypic results of loss of function or overexpression of FMRP are quite dramatic. In humans, loss of FMRP leads to mental retardation at the behavioral level and abnormal dendritic spine morphology at the cellular level (Irwin et al., 2001; O'Donnell and Warren, 20021). In flies and rodents, similar cellular phenotypes have been demonstrated upon loss of FMRP function (Irwin et al., 2002; Lee et al., 2003; Zhang et al., 2001). Conversely, gain of function alleles in flies lead to the opposite phenotype, a decrease in dendritic complexity (Lee et al., 2003). Recent advances to identify the mechanism by which FMRP regulates these structural changes have identified regulators of several cytoskeletal and cytoskeletal remodeling/adaptor proteins (Rac1, CYFIP, MAP2, MAP1b) as translational targets but the details of translational regulation remain unknown (Lee et al., 2003; Menon et al., 2008; Schenck et al., 2003; Zhang et al., 2001). In mice, enhancement of mGluR1-dependent LTD has been seen in the hippocampus of FMRP knock-out mouse while an increase in synaptic transmission has been observed at the \textit{Drosophila} neuromuscular
junction (Huber et al., 2002; Zhang et al., 2001). *In vitro* analyses predicts that up to 4% of human fetal brain mRNAs bind to FMRP (Ashley et al., 1993b). Additionally, the presence of two KH domains, one “arginine and lysine rich area” (RGG box) and G-quartet structure suggests that FMRP may bind RNA (Siomi et al., 1994).

Biochemical and colocalization data suggest that FMRP interacts with many different proteins, including those in the ncRNA-processing pathway (Barbee et al., 2006; Ishizuka et al., 2002; Jin et al., 2004). In mammalian synaptoneurosomes, FMRP protein colocalizes with components of the RISC complex. In *Drosophila*, neuronal RNA granules and transported RNPs contain both FMRP and several RISC components (Barbee et al., 2006). More circumstantial data have inferred that FMRP is required for the small regulatory pathway since mRNAs known to be regulated by the miRNA pathway are also regulated by FMRP. However, the genetic/functional data come only from *Drosophila* labs, which, at the very least, could be interpreted as a species-specific effect of Fragile X protein involvement in the RISC complex. One possibility suggests that Fragile X protein is required not as a core component of the RISC complex but being responsible for bringing a target mRNA to the RISC complex to be repressed or degraded (Ishizuka et al., 2002).

To complicate matters further, FMRP may also act as a translational activator for specific mRNAs through the ncRNA pathway (Vasudevan and Steitz, 2007; Vasudevan et al., 2007). The localization of FMRP to both ribosomal particles (translating RNAs) and RNP particleless (repressed RNAs) also hints at many roles in translational regulation (Zalfa et al., 2006). These effects of FMRP and other translational regulatory proteins on mRNA repression, localization and translational activation likely require much
orchestration in various forms, resulting in a complex and heterogenous system of RNA granule formation, transport and regulation. However, all of this links are only partially understood. Despite this gap in knowledge, FMRP has proven itself useful arenas effective tool to identify new components of translational control. Harnessing the severe phenotype of dFMR1 overexpression, both novel RNA granule and regulatory components have been identified in neurons.

FMRP’s role as a translational regulator provides an excellent genetic tool to screen for similar proteins by genetic methods. Due to the severe and distinct phenotypes of FMRP overexpression, it is relatively simple to design genetic screens to identify modifiers of FMRP function (Wan et al., 2000). An example of a genetic modifier of FMR1 function is the protein Me31b, a DEAD-box helicase, which is highly conserved from yeast to humans. Me31b has been identified as a translational repressor in yeast (Dhh1p), flies and humans (Rck54/DDX6; Barbee et al., 2006; Chu and Rana, 2006; Coller and Parker, 2005). The overexpression of dFMR1 in the fly eye causes an apoptosis-induced aberrant and deformed structure of the eye lattice, referred to as a “rough eye” (Wan et al., 2000). Modeled after results from Jin et al. (2004) with Ago1, removal of one copy of Me31b in the dFMR1 overexpression background led to suppression of the dFMR1 rough eye phenotype, resulting in a nearly wild-type eye (Barbee et al., 2006). This suggests that Me31b like dFMR1 functions in a translational repressor capacity in fly neurons. In Drosophila, neuronal RNA granules probed for Me31b show not only that Me31b is present but also colocalizes with dFMR1 and Staufen. Additional biochemical evidence supports a functional interaction between Me31b and dFMR1 as well. Furthermore, manipulation of Me31b levels in dendrites
shows similar changes in complexity as those seen upon manipulation of dFMR1 levels (Barbee et al., 2006). Me31b plays also a role in the miRNA and RNAi pathway (Barbee et al., 2006). These results allowed us to deduce that the genetic interaction with dFMR1 could require novel neuronal RNA granule components. Parallel experiments with Lgl and Ago1 (mentioned above) have also uncovered genetic interactions later confirmed by other methods (Jin et al., 2004; Zarnescu et al., 2005).

To identify Me31b-like translational repressors and neuronal RNA granule components, we capitalized on the dFMR1-induced “rough eye” as a screening tool to identify other translational repressors and RNA granule components. Using a candidate-based gene approach, we selected 43 candidates for genetic screening. Of these, 5 genes showed consistent suppression of the “rough eye” phenotype with several loss of function alleles: Doubletime/Discs Overgrown, a casein kinase I homolog; Orb2, a CPEB binding protein; Poly A Binding Protein (PABP); Rm62/Dmp68, a DEAD-box helicase; and SmD3, a splicing factor. Of these 5 genes, 4 have been confirmed to be associated with neuronal RNA granules, and 3 out of 5 show similar effects on dendritic branch complexity as Me31b and dFMR1, although to a different degree. None of these genes is apparently required for the miRNA pathway.

Materials and Methods

Fly stocks and crosses

Fly stocks were raised at 25°C on standard cornmeal and agar media. Wild-type (Oregon-R) were from Ramaswami lab stocks; All fly strains used for screening came from Harvard, Bloomington, and Szeged Stock Centers: UAS PABP (L) was obtained
from by P. Lasko and is described in Sigrist et al., 2001; the strain UASmCD8-GFP, UAS-flip Act<CD2<Gal4 was constructed by S. Sanyal using strains from Bloomington; the UAS dFMR1YFP strain was obtained from D. Zarnescu; sevdFMR1, the strain CyO/Sco was obtained from G. Dreyfuss and is described in Wan et al. (2000); the strain C380; cha gal 80 was made by S. Sanyal from lines available from the Bloomington stock center; the strain hs Flp; hid is described in Brennecke et al. (2003). Recombinants for clonal analysis were made with FRT lines (42D, 80B, 82B) from Bloomington.

**Screening Methods and Scanning Electron Microscopy**

The suppression phenotypes were determined by looking at a minimum of 4 SEM eye images (up to 12) and compared to 2 “average” sev-dFMR1 lines that were outcrossed to w1118. To examine eye phenotypes by scanning electron microscopy, adult flies were fixed for 2 hours in 1% formaldehyde, 1% glutaraldehyde, 100 mM sodium cacodylate [pH 7.2]. Flies then went through a graded series of ethanol dehydration (25, 50, 75, and 100% ethanol at least 4 hours per grade) and were dried using a critical point dryer. Flies were coated with gold or platinum and examined and imaged on a Hitachi 3400N or Philips XL-30 scanning electron microscope.

**Cell culture, Immunocytochemistry and Granule Counting**

Cells for culture were obtained from the thoracic-abdominal (ventral) region of the CNS of late third-instar larvae. Tissues were dissected and placed into a Liberase enzyme solution (combination of collagenase and dispase), and incubated at room temperature for one hour. Tissues were then rinsed in culture medium (Schneider’s-or
IL15-based medium) and subjected to two mechanical trituration steps. Cells were plated onto coverslips that were coated with Concanavalin and laminin, placed in tissue culture dishes, and allowed to grow at 25°C for 3 days prior to immunostaining. We used a composite Gal4/Gal80 system (C380-Gal4; cha-Gal80) to drive expression of a functional dFMR1 (UAS-dFMR1YFP) in a subset of motor neurons. Using confocal microscopy, cells were identified by the presence of dFMR1-positive punctae. This allowed the identification of a discrete population of motor neurons in an otherwise heterogeneous neuronal culture.

Primary antibodies used for neuronal granule staining were: anti- PABP (1:200, rabbit, gift from P. Lasko) described in Sigrist et al. (2000); anti-Orb2 (1:200, rabbit, gift from K. Si) described in Si et al. (2003); anti-Rm62/Dmp68 (1:100, guinea pig, gift from A. Spradling) described Buszczak and Spradling (2006); and anti-Sm protein (1:50, mouse, Upstate Signalling) described in Gonsalvez et al (2006). Secondary antibodies were anti-mouse or anti-rabbit Ig antibodies conjugated with Alexa 568 (Molecular Probes/Invitrogen).

3-day old ventral ganglion cell cultures were rinsed in pre-warmed PBS buffer [pH = 7.2] and fixed for 10 min in 3.5% paraformaldehyde in PBS. Cells were blocked for 30 min in blocking solution (PBS containing 0.1% Triton X-100, 2% BSA, and 5% normal goat serum). Primary and secondary antibodies were diluted in blocking solution and incubated with cells for 2 hr and 1 hr at RT, respectively. After rinsing, preparations were mounted in Vectashield Mounting Medium (Vector Labs) and imaged on a Nikon PCM2000 laser confocal microscope, using Simple PCI software. Control for channel bleed-through was done according to Barbee et al. (2006).
Granule counting and colocalization were done using NIH ImageJ software and the Cell Counter Plug-in. Statistics were performed with Excel software.

**Dendrite Imaging and Quantification**

Experiments were done in a Gal4$^{477}$, UAS-mCD8-GFP; UAS-flp, Act<CD2<Gal4 genetic background, in which flp-recombinase target sequences ("<") flanking `CD2 stuffer sequences are often excised thorough the activity of Gal4$^{477}$-driven Flp recombinase. Thus, in this background, individual Gal4$^{477}$ positive da sensory neurons are occasionally very brightly labeled by the actin-driven mCD8-GFP reporter. In genetic backgrounds carrying a Gal4-responsive transgene, the transgene is also strongly expressed.

Wandering third instar larvae of the appropriate genotype were filleted and fixed essentially as described in Grueber et al. (2002). Fixed specimens were rinsed with PBS and mounted muscle side up on Superfrost slides (VWR). Imaging was performed on a Nikon PCM2000 laser confocal microscope. Two micron Z sections were taken through the ddaC neurons of segments A2 and A3 with a 20X objective. Z stack projections were assembled with Simple PCI software. Images were sorted and selected for quantification based on image quality, lack of extensive folding of the underlying cuticle, and absence of damaged or broken branches or tissue artifacts. No more than two neurons per animal were used in the analysis.

Dendrites were quantified using Neuronmetric software (Narro et al., 2007), an ImageJ Plugin. Settings deviating from default settings are listed as follows: Rolling ball radius= 1 pixel, Gap distance = 20 pixels, Extend distance= 20 pixels,
Maximum deviation = 10 pixels, Length threshold = 20 pixels. Non-parametric tests, 2-way ANOVA and Dunnett multiple mean comparisons were performed using Prism Statistical Software.

**Clone Induction and Imaging**

Mitotic recombination clones were induced 48 ± 2hr after egg laying (AEL) in staged larvae by heat shock at 37°C for 2hr. Larval genotypes used were:

- \( hsFLP1; hid \) reporter/+; \( FRT82B, \) arm-lacZ/FRT82B \( Dbr^{P[5139602]} \); \( hsFLP1;hid \) reporter/+; \( FRT82B, \) arm-lacZ/FRT82B, \( Rm62^{P[01086]} \), \( hsFLP1;hid \) reporter/+; arm-lacZ, FRT80B/Orb2Δ, FRT80B, \( hsFLP1;FRT42D,arm-lacZ/FRT42D,PABP{K10109}^{K10109};hid \) reporter/+; \( hsFLP1;FRT42D,arm-lacZ/FRT42D,SmD3^{EP(2)2176};hid \) reporter/+.

Discs were dissected at 120 ± 2hr AEL, fixed with 4% formaldehyde and stained with anti-mouse LacZ (Abcam) and anti-goat GFP-FITC (Abcam) or (various antibodies). The discs were mounted in Vectashield (Vector Labs) and analyzed by confocal microscopy (Zeiss LSM 510) with a 20x objective. Clone areas were measured and analyzed using Adobe Photoshop.

**Results**

**Candidate Based Screen for Fragile X Dominant Interactors**

The rationale of the screen is based the idea that amelioration (or suppression) of the dFMR1 overexpression-induced rough eye phenotype can occur when one copy of an essential gene involved in a parallel or similar pathway is removed. Improvement of the
phenotype, rather than worsening, gave a more reliable indication of genes that are essential parts of the dFMR1 regulatory pathway (Carthew, 2007).

I selected mutations in 43 candidate genes based on inclusion in one or more of the four categories: 1) known RNA-binding proteins; 2) presence on neuronal RNA granules in mammals; 3) known association with the miRNA/RNAi pathways; and finally 4) presence on RNPs in other systems and organisms. Due to a concern over non-specific interactions (Daniela Zarnescu, personal communication), the following control experiments were performed. I selected 21 random mutations and tested them for interactions with dFMR1, none of which showed an interaction. Initially, 43 “candidate genes” were screened using 1-4 putative loss of function alleles that were available from stock centers (Table 5.2). Suppression of the eye phenotypes were judged by using light microscopy. Of the 43 genes, totaling screened 75 alleles (Table 5.2), 9 genes showed dominant suppression of the FMRP overexpression phenotype.

To confirm these findings, a second round screen was performed with an expanded number of alleles (Table 4.3). This second screen assessed candidate interactions and addressed potential contributions from second site mutations by testing multiple alleles and using Scanning Electron Microscopy (SEM). Of the initially 9 genes, only 5 were found to reliably suppress the FMRP phenotype with more of two alleles. The 5 FMRP-interacting genes were Dbt/Dco, Orb2, PABP, Rm62/Dmp68 and SmD3 (Table 5.1).
### Table 5.1. Multiple alleles of 5 genes suppress the dFMR1 “rough eye” phenotype.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Suppressing alleles</th>
</tr>
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<tr>
<td>Doubletime/Disc Overgrown</td>
<td>$dco^{3BS}$, $dco^{3}$, $dco^{553813}$, $dco^{5139602}$, $dco^{14609}$</td>
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<td>Orb2</td>
<td>$orb2^{BG02373}$, $orb2^{do1793}$, $orb2^{56096}$, $orb2^{d01556}$, $orb2^{A}$</td>
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<td>Poly A Binding Protein</td>
<td>$PABP^{EP310}$, $PABP^{K10109}$</td>
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<td>Rm62/Dmp68</td>
<td>$Rm62^{01086}$, $Rm62^{EY10915}$, $Rm62^{EY06765}$, $Rm62^{d02918}$</td>
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<td>Small ribonucleoprotein D3</td>
<td>$SmD3^{K09029}$, $SmD3^{EP2176}$, $SmD3^{EP2104}$</td>
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Doubletime/Discs Overgrown (Dbt), Orb2, Poly A Binding Protein (PABP), Rm62/Dmp68 and SmD3 have multiple alleles that show suppression of Fragile X Mental Retardation Protein Overexpression “rough eye” phenotype. Adapted from Cziko et al. (2009).
<table>
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<th>Gene name</th>
<th>Allele</th>
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<tr>
<td>abt</td>
<td>ab‡</td>
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<td>al8</td>
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<tr>
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<tr>
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<td>aub†</td>
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<tr>
<td>Aubergine</td>
<td>aub‡</td>
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<td>aub‡</td>
</tr>
<tr>
<td>Batten</td>
<td>batten†</td>
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Table 5.2: Complete List of alleles that were screened in the first round of the genetic screen. Alleles that suppressed the FMRP phenotype are highlighted in yellow. As seen in Cziko et al. (2009).
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<th>Notes</th>
<th>Type of Allele</th>
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Table 5.3: Complete list of alleles that were screened in the second round of the genetic screen. As seen in Cziko et al. (2009).
Figure 5.1. Mutant alleles of Doubletime/Discs Overgrown (Dbt), Orb2, Poly A Binding Protein (PABP), SmD3 and Rm62/Dmp68 show dominant suppression of the FMRP overexpression phenotype in the Drosophila eye. SEM images of Drosophila eye. Two representative alleles with median phenotypes are shown. Where applicable, one revertant from a representative allele of the group is shown. dFMR1 overexpression was driven by a fused sevenless (sev) promoter. A & B, control with sevdFMR1 outcrossed to w^{1118}, eye phenotype is variable. C, Wild-type strain Canton S. D Dbt^{S139602} allele in trans with dFMR1 overexpression. E, Dbt^{S139602} revertant in trans
**FMR1 dominant suppressors are present on neuronal RNA granules.**

FMRP is a well-known component of RNA granules in neurons, which contain translationally repressed mRNAs (Sossin and DesGroseillers, 2006). Thus, we asked whether the genes identified as dFMRP modifiers encode proteins that are present in FMRP-positive neuronal RNPs. Four out of the five identified proteins colocalize, albeit at different levels, with YFP labeled dFMR1 in primary cultures of *Drosophila* motor neurons (Figure 5.2). Granules were visualized by using overexpression of a YFP-tagged dFMR1 construct from a motor neuron specific driver. No specific antibodies were available to confirm the presence of Dbt in neuronal granules but studies in yeast (Roy Parker, personal communication) demonstrate its presence on P-bodies, which are homologous structures (Barbee et al., 2006). To quantify the co-localization of each protein with dFMR1-YFP, the number of YFP spots was counted first. Then, the two color images were merged (green for YFP, red for antibody stain) and the number of colocalizing spots (yellow) was counted. Colocalization rates were 44% for Orb2, 54% for PABP, 58% for Rm62 and 73% Sm proteins (Figure 5.2M). Reverse colocalization was also done, which is defined as the percentage of red antibody stained spots colocalizing with dFMR1-YFP. These rates were 26% with Orb2, 52% with PABP, 65% with Rm62 and 50% with Sm Proteins. The percentage of dFMR1-YFP colocalization with a specific protein are much lower, mainly because of the larger number of endogenous and antibody-stained granules compared to the number of the artificially marked granules due to dFMR1-YFP overexpression. The latter are clearly distinguishable as they tend to be larger and fewer in number. The overall lower percentage numbers reconfirm the large amount of protein heterogeneity within granules (Barbee et al., 2006;
Kohrmann et al., 1999). Nevertheless, our results demonstrate the presence of the identified FMRP-interacting proteins in granules known to contain RNA (Barbee et al., 2006). Due to their presence in neuronal RNA granules, the identified proteins may play a role as translational regulators.
Figure 5.2. Orb2, Poly-A Binding Protein (PABP), Rm62/Dmp68 and Sm Proteins Colocalize with YFP-tagged *Drosophila* Fragile X Mental Retardation Protein (dFMR1YFP) in primary neuron culture. 54.5% dFMR1YFP granules (A) colocalize with Orb2 granules (B). 26.5% of Orb2 positive granules (B) colocalize with dFMR1YFP granules (A). C, Orb2 and dFMR1YFP signals merged. 44.3% dFMR1YFP granules (D) colocalize with PABP granules (E). 52.4% of PABP granules (E) colocalize with dFMR1YFP granules (D). F, PABP and dFMR1YFP signals merged. 57.5% dFMR1YFP granules (G) colocalize with Rm62/Dmp68 granules (H). 65.9% of Rm62/Dmp68 granules (G) colocalize with dFMR1YFP granules (H). I, Rm62/Dmp68 and dFMR1YFP signals merged. 72.6% dFMR1YFP granules (J) colocalize with Sm Protein granules (K). 50.3% of Sm protein containing granules (J) colocalize with dFMR1YFP granules (K). L, Sm protein and dFMR1YFP signals merged. M, Graphical representation of dFMR1YFP containing granules colocalized with specific antigen of interest. N, Graphical representation of specific antigen containing granules with dFMR1YFP. Adapted from Cziko et al. (2009).
**Overexpression of dFMR1 interactors affects dendritic branching.**

Proteins co-localizing with FMR1 could be involved in translational repression or many other alternative functions (see discussion). Previous studies have suggested that overexpression of repressor proteins may inhibit dendritic growth, many causing particularly severe phenotypes (Lee et al., 2003; Ye et al., 2004). In contrast, loss-of function of dFMR1 in *Drosophila* body wall sensory neurons causes an increase in dendritic complexity or branching (Lee et al., 2003). To test this, we examined the identified FMR1-modifier proteins for effect on dendrite branching. Due to ease of identification, visualization and sensitivity of these cells to translational repression, we used Class IV body wall sensory neurons as an assay system for overexpression effects on dendritic morphology (Grueber et al., 2002). Quantification of dendritic branching phenotypes was done by using Neuronmetric software, which measures dendritic area, overall branch length and branch number (Narro et al., 2007). Dendritic density, a normalized measure of complexity, can then be calculated. For the 3 primary measurements taken (area, overall length and branch number), at least one allele of each gene tested showed a statistically significant effect in each one of these measurements. The normalized secondary measurement of dendritic complexity showed a statistically significant effect with one allele from Dbt, PABP and two from Rm62. The variable results between alleles within genes can be attributed to variability in expression that was not assayed or controlled for in this study. Creating true overexpression transgenes would likely increase the severity of the phenotypes and lead to more consistent results (as proof of this concept, UAS PABP (L), a transgene made by the Lasko lab shows an affect on all 4 measurements) (Sigrist et al., 2000). Unfortunately, effects of Orb2
overexpression could not be assessed because overexpression was lethal to the animals (Keleman et al., 2007). In summary, overexpression of 3 of the identified genes, though variable, caused a statistically significant reduction in dendritic complexity. This included Dbt, PABP and Rm62. The effect on dendritic complexity along with the dFMR1 colocalization data suggest that Dbt, PABP and Rm62 are acting in a way similar to other known neuronal translational repressors.
Figure 5.3. Overexpression of FMRP-interacting proteins alters dendritic morphology in class IV body sensory neurons. Overexpression was driven by the Gal4 477 driver. Expression and visualization was enhanced with an act<CD2<Gal4, UAS Flippase. A, Number of branches/cell was decreased in all Poly A Binding Protein (UAS PABP (L), UAS PABP (B), PABP EP310, PABP EY11561), Doubletime (Dbt EP3280, Dbt EY02910), Rm62 (Rm62 3607, Rm62 01915, Rm62 06975) and one of the SmD3 (SmD3 EP2104) overexpression constructs assayed. B, Total dendrite length was significantly reduced in all Poly A Binding Protein (UAS PABP (L), UAS PABP (B), PABP EP310, PABP EY11561), all Doubletime (Dbt EP3280, Dbt EY02910), two of Rm62 (Rm62 3607, Rm62 01915) and increased in one of the SmD3 (SmD3 EP2104). Total dendrite length was significantly increased in one SmD3 overexpression line (SmD3 EP2176). C, Dendrite density was reduced in Dbt overexpression construct Dbt EP3280, PABP overexpression construct UAS PABP (L), Rm62 overexpression construct Rm62 EY 01915 and SmD3 overexpression construct SmD3 EP 2104. D, Number of branches/cell (density) was reduced in Doubletime overexpression constructs Dbt EY02910, PABP overexpression construct UAS PABP (L), Rm62 overexpression constructs Rm62 EY 01915 and Rm62 3607. E, Representative example from each overexpression line. Adapted from Cziko et al. (2009).
None of the new *dFMR1* modifier genes are required for the miRNA pathway.

Recent biochemical and genetic evidence supports a role for FMRP/dFMR1 in the miRNA pathway. FMRP has been shown to have strong biochemical or genetic interactions with Ago-2 (Jin et al., 2004). Several groups have also proposed that miRNA-mediated repression occurs in P-bodies of somatic cells, although recent evidence from *Drosophila* S2 cells suggests that P-bodies formation does not require, but is a consequence of miRNA-mediated repression (Eulalio et al., 2007). Because of their genetic interaction with dFMR1/FMRP we tested whether the 5 identified interactors of dFMR1 are required for the efficient function of *bantam*, an endogenous miRNA that represses *hid* mRNA translation in wing imaginal discs (Brennecke et al., 2003).

Like in Barbee et al. (2006), we used a transgenically encoded GFP reporter to assay *bantam*-mediated translational repression (Brennecke et al., 2003). The "hid reporter”, which carries the 3'UTR of *hid* fused to the 3' end of EGFP-coding sequence, closely reports the *bantam* repression of a native target mRNA. This 3' UTR contains four repeats complementary to *bantam* target recognition sequences, with several mismatches typically associated with miRNA-mediated translational repression.

To generate specific loss of function clones in the wing disc, we used the heat-shock FLP/FRT system. We identified the induced clones by loss of beta-galactosidase activity (Figure 5.4). Unfortunately, our hypothesis of dFMR1 modifying proteins participating in the miRNA pathway was not supported by our results. None of the identified proteins showed an effect on the output of the “hid” miRNA reporter. To control for experimental errors, the presumed miRNA pathway regulator dFMR1 was also tested. Contrary to what was expected, dFMR1 mutations did not modify the
miRNA pathway. It is also important to note that Rm62/Dmp68, a confirmed member of the Drosha complex in mammals and dFMR1 itself did not show an effect on the miRNA reporter fluorescence either, although the Rm62 result may be explained by redundant processing by helicases in the Drosha processing complex (see discussion; Jalal et al., 2007; Kahlina et al., 2004). Our results demonstrate clear limitations of the used method to confirm or assay miRNA function, as dFMR1 has been confirmed both biochemically and genetically to function in the miRNA pathway.
Figure 5.4. Neither identified proteins nor FMR1 are required for miRNA mediated repression of bantam-GFP reporter. A, E, I, M, Q, U 20X clonal marking of wing discs with corresponding antibody or LacZ. B, F, J, N, R, V, 20 X GFP labeled bantam reporter expression. C, G, K, O, S, W, 60X closeup of area where loss of function clones were induced marked with corresponding antibody or LacZ. D, H, L, P, T, X, Corresponding area of clones (circled) with bantam GFP expression. See methods for larval genotypes. Adapted from Cziko et al. (2009).
Discussion

Identification of new neuronal translational regulators

We suggest, that as for previously identified sev-dfmr1 suppressors Ago1, Lgl and Me31b (Barbee et al., 2006; Jin et al., 2004; Zarnescu et al., 2005), analysis of PABP, Smd3, Rm62, Orb2 and Dco proteins, encoded by the sev-dfmr1 suppressor genes identified here, will help elucidate how dFMR1 works in translational regulation, RNA targeting and localization, and ncRNA pathway function.

FMR1 modifier proteins function in translational control.

Three lines of evidence indicate that the genes we identify encode proteins with translational repressor activity. First, with the exception of Dco, all of these proteins have been previously implicated in some aspect of RNA metabolism (discussed later in this section) and are present on dFMR1-containing neuritic granules in which RNA is repressed and transported (Bassell and Warren, 2008). Second, the rough eye phenotype observed in sev-dfmr1 has been linked to the ability of FMRP to repress mRNA translation (Wan et al., 2000). Thus, we would expect the phenotype to be alleviated by mutations that reduce the efficiency of translational repression. Third, overexpression of Dco, Pabp, Orb2 or Rm62 inhibits the dendritic growth of neurons, a phenotype predicted for neuronal translational repressors (Barbee et al., 2006; Lee et al., 2003; Ye et al., 2004). These observations are consistent with the idea that translation of RNAs in neurites, which promotes dendritic branching, is inhibited by overexpression of Dco, Pabp, Orb2 or Rm62 (Aakalu et al., 2001; Martin, 2004). Thus, genetic interaction data,
molecular localization, and one functional test in dendrites indicate that Dco/Dbt, PABP, Rm62 or SmD3 function as neuronal translational repressors.

The identification of several canonical translational-factor encoding genes as suppressors of *sev-dfmrl* highlights the point that individual translational control molecules work in multi-component complexes, and therefore have several functional interactions. PABP is one example of a protein that is currently believed to perform two opposing aspects of translational control. In addition to its well-studied role as a translational activator, PABP can mediate translational repression, e.g. of Vasopressin mRNA although the exact mechanism remains unclear (Mohr et al., 2001). Dual roles in activation and repression are also suggested by the observation that reduced or elevated levels of PABP have similar effects at the Drosophila neuromuscular junction (NMJ) (Sigrist et al., 2000). Additionally, PABP associates with particles containing BC1, a neuron-specific non-coding RNA with translational repressor function, as well as a CYFIP-FMRP complex that may function as a repressor in some contexts but as an activator in others (Muddashetty et al., 2002; West et al., 2002). Similarly, Orb2 homologs (CPEB’s) though required for translational activation of CPE containing mRNAs via polyA-polymerase (Wu et al., 1998), also allow translational repression in combination with Maskin or Cup proteins (Barkoff et al., 2000; Nakamura et al., 2004).

It was somewhat surprising that we identify SmD3, a splicing factor, in our screen for translational repressors. However, SmD3 has additional non-splicing functions: in *C. elegans*, the Sm proteins are required for germ cell mRNP assembly and RNA localization (Barbee et al., 2002). Such a role in translational regulation and mRNP assembly is more consistent with functions predicted by our genetic experiments.
Rm62/Dmp68 is a member of the DEAD-box helicase family that has been shown to be associated with a dFMR1-containing RNAi silencing complex (Ishizuka et al., 2002). It also has additional roles during transcription and mRNA processing as well as potentially in miRNA processing as part of the Drosha complex (Busczak and Spradling, 2006; Camats et al., 2008; Park et al., 2004). Based on the biochemical evidence for Rm62’s presence in FMRP containing complexes, it is not surprising that rm62 mutations show strong genetic interactions with dfmr1. However, the mechanism of suppression remains unknown.

Finally Dco/Dbt, is by far the most elusive protein in regard to its potential function in the translational regulatory pathway. Dco/Dbt, a casein kinase I (CKI) is best known from circadian biology where it phosphorylates Per and expedites its degradation (Pruess et al., 2004). dFMR1 protein has several phosphorylation sites, one of which in S2 cells has been demonstrated to be phosphorylated by a CKII protein (Siomi et al., 2002). While, the functional requirement for CKI dependent dFMR1 phosphorylation is as of yet, not understood, there is considerable evidence that the phosphorylation state of FMRP may actually determine its role in translation. Biochemical data demonstrate that most FMRP in granules is in the phosphorylated state while FMRP in the polysome fraction is dephosphorylated (Ceman et al., 2003) suggesting a mechanism to switch state from an activator to repressor, and an important regulatory role for kinases that phosphorylate FMRP (Narayanan et al., 2007; Narayanan et al., 2008).

Another interesting potential link between the two proteins is the behavioral observation that patients with Fragile X Mental Retardation often display circadian disturbances (O'Donnell and Warren, 2002). This altered circadian rhythm is also present
in the Drosophila dfmr1 mutants that usefully model fragile-X syndrome (Dockendorff et al., 2002).

The identification of these proteins as sev-dfmr1 modifiers illustrates the many possibly regulatory roles of RNA-associated proteins. In addition, the data associating Dco/Dbt with RNA regulation indicates unexplored and novel mechanisms of RNA regulation in neurons.

dFMR1/FMRP’s role in the non-coding RNA pathway

Given that dFMR1/FMRP is thought to function in miRNA-dependent translational repression, we were particularly interested in asking whether these dFMR1 interactors had any role in this pathway. To address this issue, we employed a sensitive in vivo assay that uses a fluorescent reporter to reveal the strength of translational repression via an endogenous (bantam) miRNA. When combined with genetic mosaic analysis, this assay can be used to study null mutations in candidate genes, as long as the mutations do not cause cell lethality. The assay appears more sensitive than typically used cell-based assays on the evidence of our prior analysis of Me31B, whose requirement for miRNA function, clearly seen in the in vivo assay, is only evident in double-knockdown experiments in the more commonly used cell-culture assays (Barbee et al., 2006; Chu and Rana, 2006; Eulalio et al., 2007).

Our in-vivo experiments revealed no requirement for the sev-dfmr1 interacting proteins Dco, Orb2, Rm62 and SmD3 in miRNA repression. For reasons explained above, it is unlikely that this reflects a weakness in the experimental assay for miRNA function. A bigger surprise was our finding the dFMR1 itself appeared dispensable for
miRNA function *in vivo*. Because the allele used is a well-characterized null allele, and the absence of dFMR1 in the mutant clones is confirmed by antibody staining, our conclusion that dFMR1 is not a core, essential component of the RISC/miRNA pathway is strong. This conclusion is not inconsistent with any of the existing data showing biochemical association between RISC and FMRP and genetic interactions between Ago1 and FMRP (Gao, 2008; Ishizuka et al., 2002; Jin et al., 2004; Li et al., 2008). However, it is also consistent with recent observations indicating the dispensability of FMRP for RISC function in cultured cells (Didiot et al., 2009). We suggest that the function of dFMR1 and, by extension, FMRP may be restricted to a subset of transcripts, for instance those with UTRs containing both FMRP binding motifs and miRNA target element. Indeed similar models that account for the mRNA specificity of FMRP have been previously proposed (Li et al., 2008; Siomi et al., 2004).

Data presented here provide a foundation on which to design further experiments to understand the specific roles of FMR1 and its interacting proteins in translational control.
CHAPTER SIX

CONCLUSIONS
Brief summary of findings

The main findings of this work are listed below with a short description of the implications and significance.

**RNP s exist in *Drosophila* neurons**

The first major finding of this dissertation work was that, like in mammals, yeast, *Drosophila* and *Xenopus* oocytes, ribonuclear particles (RNPs) also exist in *Drosophila* neurons. This is significant because of *Drosophila*’s genetic amenability and motor neuron culture system and will allow much progress in the field of neuronal translational control. It also underscores the evolutionary conservation of translational control mechanisms.

**RNP s in *Drosophila* neurons are functionally homologous to yeast P-bodies, mammalian neuronal granules and maternal granules.**

Using colocalization studies, similar components are found in both *Drosophila* neuronal RNA granules and yeast P-bodies. Conserved components hint at conserved function across granule types. This implies that neuronal RNA granules may have roles in storage, repression, decay and translational activation of neuronal RNAs.

**Trailer Hitch and Me31b localize to neuronal RNA granules and act as translational repressors**
Two of the conserved components identified in neuronal RNA granules of \textit{Drosophila} are Trailer Hitch and Me3b protein. These proteins demonstrate strong genetic interaction with the known mRNA regulator, dFMRP. Additionally, Me31b is required for efficient function of the miRNA and RNAi pathways. Finally, both proteins are important for developing normal dendritic branches. Together, these results are consistent with the idea that Me31b acts as a translational repressor. Further studies into the mechanism of repression will give insight into translational control in neurons.

\textbf{Candidate-based approach identified novel components of RNA granules.}

Using a candidate gene based approach, five novel FMRP interactors were identified. Four out of the five candidates were demonstrated to colocalize with FMRP in neuronal RNA granules of \textit{Drosophila}.

\textbf{Fragile X Protein genetic interactors overexpression affects dendritic branching.}

Several known translational repressors (FMRP, Me31b, Nanos, Pumilio) cause a decrease in complexity when overexpressed in \textit{Drosophila} sensory neurons. Overexpression of four out of the five identified FMRP interactors alters sensory neuron branching. This suggests a role in the development and or maintainance of dendrites.

\textbf{Future directions and applications}

Determining the exact mechanisms of translational repression will give insight into neuronal translational control.
More detailed investigations of the identified proteins (Me31b, Trailer Hitch, Doubletime/Discs Overgrown, Orb2, PABP, Rm62/Dmp68, SmD3) in *Drosophila* will lead to a more in-depth knowledge of translational control in neurons. As apparent from descriptions in Chapter 2, the mechanisms of targeting, repression, transport and activation are complex. Elucidation of the mechanism will further our understanding of translational control in neurons, and perhaps, dendrites. Determining the potential role of these proteins in synaptic plasticity will further our knowledge molecular mechanisms underlying synaptic plasticity, and perhaps, learning and memory.

**Interactors of Fragile X protein may give insight into potential therapeutics**

Identifying interactors of the FMRP not only provides insight into mechanisms facilitating translational control but also into the function of FMRP in the nervous system. Understanding molecular interactions of FMRP and their neuronal function will open avenues of research for potential interventions to treat or even prevent Fragile X Syndrome in humans.

**Technical limitations/major obstacles in field**

**Difficulty in inhibiting or modulating translation exclusively in the dendrite**

Many studies have now demonstrated that the local protein synthesis in the dendrite is required for long-lasting forms of synaptic plasticity and synaptic tagging (Frey and Morris, 1997; Martin et al., 1997). However, genetic manipulations to study the role of various translational regulators are limited by the ability to separate somatic
and dendritic translational control. One highly artificial way to circumvent this problem is to physically separate the dendrites from the cell body. This approach, however, is only possible in cultured neurons and leads to a very short window in which changes from experimental manipulations can be assessed. Advances in this area would greatly increase our understanding of local translational control.

**Difficulty in defining a translational repressor**

A major problem of studying translational control is the difficulty in assessing exact roles of proteins in translational regulation. Because of the complexity of steps required for appropriate localization, repression and eventual translational activation of a specific mRNA (transcript A), assigning an appropriate function is extremely difficult. For example, protein X represses transcript A by masking. However, protein X is required for the binding of an adaptor protein for transport to the dendritic site where it is required, and therefore, it is responsible for preventing transcript A’s degradation when it is not localized to the appropriate location. A classical genetic approach to remove or destroy protein X may lead to a misleading function of protein X. Without protein X, transcript A never gets localized to the only place it is translated and instead it is degraded. Therefore in the classical single mutant approach, one assumes that protein X is required for the translational of transcript A, and is in fact an activator.

Genetic analysis of proteins implicated in RNA regulation is confounded by the formal possibility that a regulatory protein could serve dual, or even opposite functions. For example, a protein may serve as a direct repressor of an mRNA transcript through a mechanism by which it masks (the 3'UTR). The same protein may also be critical for
binding the adaptor protein required for transport of the mRNA to a dendritic site and in
the same context, serves to prevent that mRNA's degradation during the transport
process.

From a classic genetic analysis, one might draw the mistaken conclusion that,
since the mRNA does not get translated, the protein under scrutiny must serve as an
activator when the relationship is far more complex in nature. Therefore, one cannot
easily draw a conclusion from a genetic analysis alone without an understanding of how
the protein interacts with its target mRNA.

To definitively assign a function to a specific protein will likely require
transcript-specific and multi-disciplinary approaches (as has been done in the oocyte),
using a combination of biochemistry, genetics, immunocytochemistry, proteomics, and
expression profiling of a specific neuronal phenotype.

**Problems in interpretation of data**

Another hurdle in the study of translational regulation is the various affects on
translation. One example of a pharmacological inhibitor causing varied effects on
translation is from the superior colliculi of the young rat. Application of cyclohexamide
to the cells leads to a general decrease in translation measured by S-methionine
incorporation while simultaneously causing a 2-fold increase in the CAMKII transcript
translation (Scheetz et al., 2000).

Phosphorylation of EF2α has also varied effects on translation, leading to a
general decrease in translation but to an activation of a specific type of transcript. EF2α
once phosphorylated leads to a dominant inhibition of the 40S ribosomal subunit, thus
acting as a general repressor of translational initiation (Dever, 2002). However, for mRNAs containing an upstream open reading frame (uORF), phosphorylation of EF2α actually causes an increase in translation. The reason for this, is the titration of the ternary complex to levels where most 40S subunits omit the upstream uORF. The result is higher levels of transcripts with the uORFs to be translated (Govind et al., 2007).

The implications of these results is that the role as a translational repressor or activator may not be quite so easy to assign. In the context of memory and synaptic plasticity application a protein synthesis inhibitor may actually be increasing the translation of certain transcripts.

**Conclusions**

In this dissertation I have identified several novel components of RNA granules in neurons that are putative regulators of protein translation. Further investigations of these components will provide insights into the mechanism controlling local translation in dendrites, and will help elucidate how memory is formed.
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