Novel Functions for Neuronal RNA Processing Bodies in the Control of Axon Terminal Growth in Drosophila Melanogaster

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Novel functions for neuronal RNA processing bodies in the control of axon terminal growth in \textit{Drosophila melanogaster}

A Dissertation

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by

Sarala Joshi Pradhan

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Advisor: Dr. Scott A. Barbee
Abstract

In this thesis we first characterized neuronal functions for HPat/Pat1, a core component of RNA processing bodies or “P bodies”. We show that hpat mutants exhibit a strong synaptic hyperplasia at the developing and acutely stimulated Drosophila larval neuromuscular junctions (NMJs). The synaptic defects observed in hpat mutants are associated with rearrangement of the axonal microtubule cytoskeleton suggesting that HPat negatively regulates presynaptic microtubule-based growth during NMJ development. Interestingly, we also found that both pre-and postsynaptic HPat expression controlled rapid axon terminal growth in response to acute spaced synaptic stimulation. We also demonstrate that HPat interacts genetically with the catalytic subunit of the deadenylase complex (twin/CCR4) and the miRNA pathway (Argonaute 1) to control bouton formation. We propose that HPat is required to target mRNAs involved in the control of microtubule architecture and synaptic terminal growth for repression, presumably in P bodies, via both general and miRNA-mediated mechanisms.

Next, we investigated whether HPat interacts with the Drosophila Fragile X Mental Retardation Protein (dFMR1), to regulate neuronal structure in a Drosophila melanogaster fragile X model. First, we demonstrated that HPat interacts biochemically with dFMRP in an RNAse independent manner. Second, we show that HPat genetically interacts with dFmr1 in the Drosophila eye although the phenotype is weak, however we did not see any interaction of hpat and dfmr1 to control synaptic structure at the NMJ.
Finally, we screened additional P body components that might have function in FMRP mediated translation regulation. Interestingly, a luciferase-based translational repression tethering assays in *Drosophila* Schneider 2 (S2) cells showed the function of GW182 in FMRP-mediated translation regulation.
Acknowledgements

The completion of this dissertation brings the much-needed respite from the long-lasting effort. First of all I would like to thank Dr. Scott Barbee for giving me this opportunity and for his support, direction and mentoring that contributed to this beautiful outcome. I would also like to thank members of my thesis committee including Dr. Joseph Angleson, Dr. Todd Blankenship and Dr. Daniel Linseman for their invaluable suggestions and guidance. I also greatly appreciate Dr. Matthew Taylor for his role as the chair of my oral defense committee.

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This dissertation is dedicated to my parents.
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Chapter One: Relevant Background

1.1 Introduction

Both activity-dependent synaptogenesis and the establishment of long-term memory (LTM) in mammals require immediate new protein synthesis in response to acute synaptic stimulation. This is mediated in part by the local translation regulation of specific synapse-localized mRNAs in dendrites and axons (Flexner et al., 1963). In neurons, local mRNA translation is important for the regulated development or growth of axons and dendrite structures; and the regulation of synaptic plasticity, a cellular correlate of learning and memory (Davis et al., 1996, Ashraf et al., 2006). Local mRNA translation is regulated by sequence motifs in mRNAs in association with specific RNA binding proteins and noncoding RNAs (ncRNAs). Together, the composition of these ribonucleoprotein (RNP) particles determines whether mRNAs are transported to a specific location (e.g. to the synapse), locally translated, or targeted for storage and/or degradation (Sossin and DesGroseillers 2006, Kindler et al., 2005).

A significant amount of detail remains unknown about the mechanisms of local mRNA translation regulation in neurons and its effect on synaptic structure and function. Abnormalities in RBPs (RNA binding proteins) are associated with various neurological diseases like FMRP (Fragile X mental Retardation protein) in Fragile X Syndrome (FXS), TDP-43 (Tar DNA Binding Protein-43) in amyotrophic lateral sclerosis (ALS), RNA binding protein FUS (Fused in Sarcoma) in sarcoma, angiogenin and ataxin-2 in
ALS, ataxin-2 in spinocerebellar ataxia (SCA) and SMN (survival of motor neuron protein) in spinal muscular atrophy (SMA; Liu–Yesucevitz et al., 2011). TDP-43 is a principle component of inclusions in many cases of fronto-temporal lobar degeneration (FTLD-U) and ALS, the most common motor neuron disease in adults. Increasing evidence indicates that TDP-43 regulates mRNA metabolism by interacting with mRNA binding proteins that are known to associate with RNA granules (Liu-Yesucevitz et al., 2011). Understanding the mechanisms underlying these diseases will help researchers design drugs to intervene the neuronal pathology involving the control of these processes.

1.2 The requirement for local translation in axons and dendrites

Axons and dendrites are molecularly and functionally distinct compartments within neurons. Generally speaking, directional information first flows from the cell body to axon terminals. Dendrites integrate synaptic inputs, triggering the generation of action potentials at the level of the soma; action potentials then propagate along the axon of the second cell, which subsequently makes presynaptic contacts onto target cells.

1.2.1 Axons: Mechanisms of axon terminal growth and synaptogenesis

During development, axons in the form of axonal growth cone, emerge from cell bodies after an initial polarization stage, elongate, and travel towards target cell to establish a synaptic connection, guided by a range of intrinsic and extrinsic environmental cues including netrin, Slit, Semaphorin A, BDNF (Brain Derived Neurotrophic Factor), NGF (Nerve Growth Factor) and NT3 (neurotrophin 3) (Tahirovic and Bradke 2009). The establishment and maintenance of neuronal polarity, seen as a long, thin axon and several short, thicker dendrites, depend on coordinated cytoskeletal rearrangements and directed membrane trafficking (Hall and Giovanna 2010). The axonal
growth cone is composed of a central region filled with organelles and microtubules and a peripheral region of lamellipodia and filopodia filled with highly dynamic, actin (Tahirovic and Bradke 2009). Actin polymerization and depolymerization is important for morphological changes in neurons and establishment of long-term potentiation (LTP) involving receptor trafficking. These actin dynamics are regulated by actin nucleating (Arp2/3, formin), severing (cofilin), branching (Arp2/3, formin), and bundling (fascine) proteins (Tahirovic and Bradke 2009). Beside actin nucleators, other regulators of growth cone dynamics include proteins that modulate actin dynamics like WAVE (Wiskott-Aldrich syndrome protein [WASP]-family verprolin-homologous protein), Ena (enabled)/VASP (vasodilator stimulated phosphoprotein), profilin, and ADF (actin-depolymerizing factor)/cofilin. The WAVE protein promotes axon growth by regulating actin polymerization through Arp2/3 or profilin II. The Ena/VASP proteins, localized to tips of lamellipodia and filopodia, accelerate actin polymerization by their anticapping activity and bundle actin filaments. The Rho and Ras families of small GTPases are critical signal transducers at all stages of axonogenesis including the dynamic assembly, disassembly and reorganization of the actin and microtubule cytoskeletons involving above mentioned proteins (Hall and Lalli 2010). Actin- and microtubule-based intracellular transport is also required for neuronal polarity/axon terminal growth. Specific molecular motors like myosin move unidirectionally along actin filaments and microtubules, and regulate intracellular trafficking and neuronal polarity. The microtubule directed motors, kinesin and dynein, are mostly plus-end and minus-end directed respectively, giving a polarized stability of microtubule controlled axonal trafficking (Tahirovic and Bradke 2009).
1.2.1.1 Local protein expression and axon specification

Local translation in axons may be integral to aspects of synaptogenesis, LTP, memory storage, and growth cone navigation in response to environmental stimuli in developing vertebrate/invertebrate axons (Jung et al., 2012). Local translation provides a rapid supply of proteins in axonal compartments that can potentially affect long-term responses to transient stimuli. The presence of mRNA and the translation machinery in developing axons has been well established, and their role in local protein synthesis is emerging as an important molecular mechanism underlying synaptic plasticity and axon guidance. Ribosomes, tRNA initiation factors and mRNA have been identified in vertebrate axons (Jung et al., 2012). The cytoskeletal mRNAs β–actin and the microtubule-associated protein Tau and ADF (Actin Depolymerizing Factor) have also been identified in axons suggesting that their local translation could play a functional role in axonogenesis (Jung et al., 2012). Stimulation of neurons with the NT-3 (Neurotrophin-3) elicit a rapid localization of β-actin mRNA to axonal growth cones. This localization requires the formation of a complex between the zipcode binding protein 1 (ZBP1) and a cis-acting regulatory region in the 3’UTR of the β-actin mRNA, called the ‘zipcode’ (Jung et al., 2012). This has also been shown with the neuronal RNA-binding protein, HuD, which mediates axonal localization of Tau mRNA. The dependence of directed mRNA transport upon conserved components of neuronal RNPs including ZBP1 and HuD focuses attention on the role of RNA-binding proteins in mRNA localization. Much of the local axonal mRNA translational regulation is via the mTOR (mammalian target of rapamycin) pathway, which activates translational initiation by phosphorylating 4EBP1 (eIF4E binding protein) and the S6 kinase (Jung et al., 2012, Piper and Holt 2004).
1.2.2 Dendrites: Mechanisms of LTM

Local protein synthesis in dendrites regulates neuronal development, synaptic plasticity and the establishment of LTM. Studies of LTP and long-term depression (LTD) in mammals have identified two distinct phases of plasticity: an early phase (typically lasting 1–3 h) that is independent of new protein synthesis but dependent on kinases and a late phase (lasting >8 h) that is dependent on new protein synthesis (Sutton and Schuman 2006). Kinases, cytoskeletal components, receptors, transcription factors and other components are involved in activity-dependent changes associated with LTM. This is supported by the following published evidence: first, changes in synapse strength were observed due to LTP-induced increases in AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors in dendritic spines (Malinow et al., 2002); second, LTP-associated structural changes in rat hippocampal dendrites were inhibited when an actin antagonist was applied (Krucker et al., 2000); third, the LIM-containing protein kinase (LIMK), necessary for activation of actin polymerization, was shown to be essential in activity dependent spine morphogenesis and memory formation associated with LTP (Luo et al., 2002). Finally, it has been determined by several studies that elimination of the transcription factor CREB (Camp Response Element Binding Protein) abolishes activity-dependent LTP and LTM-associated changes (Benito and Barko 2010).

The molecular mechanisms associated with LTM have been shown to have regulatory input from the microRNA (miRNA) pathway as well (Ashraf et al., 2006, Berdnik et al., 2008, Kosik 2006). Evidence has suggested that the RISC (RNA-induced silencing complex)-mediated miRNA pathway is required for early phase LTM, by regulating local protein synthesis at the synapse. Recently it was shown that one of the
more important regulators of synaptic plasticity, the calcium/calmodulin-dependent protein kinase II (CaMKII), was translationally regulated by a RISC-dependent event locally at synapses in *Drosophila* (Ashraf et al., 2006). miRNA target predictions of the CaMKII 3’-UTR revealed possible binding sites for miR-280 and miR-289. Furthermore, studies by Fiore, et al., have demonstrated that an activity-dependent miRNA successfully regulated *pumilio* in rat cortical neurons, a conserved RNA-binding protein responsible for aiding in long-lasting changes at synapses. This suggests that miRNA function is regulated by neuronal activity and miRNA are important component of translational control of gene expression with local changes at the dendrites. Thus, miRNA genes undergo activity-induced transcription and in turn, mature miRNAs act to regulate the translation of mRNA transcripts associated with activity-dependent LTM associated changes (Fiore et al., 2009).

Besides miRNAs, other molecular mechanisms associated with different steps of translation have been identified in the control of LTM. During the translation initiation step, phosphorylation of the translation initiation factor eIF2 (Eukaryotic Initiation Factor 2) inhibits the formation of the ternary complex (the preinitiation complex consisting of the initiator, GTP, eIF-2 and the 40S subunit). Hyper-phosphorylated 4E-BPs (eIF4E-binding protein) bind with low affinity to eIF4E and can no longer inhibit translation. The major protein kinase that phosphorylates 4E-BPs and e1F4E (Eukaryotic Initiation Factor 4E) is the mTOR (Costa-Mattioli et al., 2009). Translation can also be regulated at the mRNA 3’end by controlling the length of the poly (A) tail through the RNA-binding protein CPEB (cytoplasmic polyadenylation element-binding protein) that has well defined binding site at the 3’UTR. At synapses, neuronal activity can activate CPEB
phosphorylation leading to an increase in the length of the polyA tail and mRNA translation (Costa-Mattioli et al., 2009). Neurons can also use cap independent translation via IRES (internal ribosome entry site) driven translation to upregulate the translation of a subset of mRNAs during a learning experience (Costa-Mattioli et al., 2009). Local translation depends on transport of mRNAs from the cell body to distal dendrites. In neurons, transport of mRNAs to dendrites has a major impact on their translational regulation. If mRNAs are transported in mRNA granules containing stalled polysomes, translation could also be blocked at the elongation step. Although initiation is usually the rate-limiting step in translation, elongation can also be regulated. The major mechanism for regulating elongation is phosphorylation of eEF2. During mRNA transport to dendrites, translation is also repressed by RNA binding proteins FMRP, CPEB, ZBP, Staufen etc (Costa-Mattioli et al., 2009).

1.3 The role of neuronal granules in mRNA transport and local translation

Neurons contain RNA transport granules that are usually 100-300 nm in diameter and consist of mRNAs, non coding RNAs and highly conserved RNA binding proteins including Staufen, FMRP, and motor protein, Kinesin (Krichevsky and Kosik 2001). These granules are highly heterogeneous and components may overlap (Hillebrand et al., 2007). Some examples of mRNAs found in RNPs are, CamKII (Ca^{2+}/calmodulin-dependent protein kinase II), Arc (activity-regulated cytoskeleton-associated protein), MAP2 (Microtubule-associated protein 2) and MAP1b (Microtubule-associated protein 1B). Staufen, a double-stranded RNA-binding protein, plays a pivotal role in mRNA transport, localization, translation and synaptic plasticity in both mammalian hippocampal and Drosophila neurons (Kiebler et al., 1999, Barbee et al., 2006).
1.3.1. Types of cytoplasmic RNPs

Diverse groups of cytoplasmic RNA granules or RNPs exist in neurons, somatic cells, germ line cells and embryos during development; each of which share some protein components and use similar mechanism of mRNA regulation (Noble et al., 2008). These granules “subclasses” include RNA processing bodies (P bodies), stress granules, RNA granules, neuronal granules (NG), RNA transport granules, and maternal granules. Each of these are characterized by the presence (or absence) of specific protein components (Kiebler et al., 2006, Hillebrand et al., 2007).

Many cytoplasmic RNPs are structurally and functionally related in different organisms ranging from plants to humans. For example, NGs in cultured Drosophila neurons contain conserved components of mammalian NGs and other cytoplasmic RNPs (Table 1, 2).

1.3.1.1 Transport RNPs

Also known as neuronal RNA granules and RNA transport granules, transport RNPs are motile granules that transport translationally repressed RNAs to distal regions of the cell (axons or dendrites). These particles include mRNAs that are associated with various regulatory non-coding RNAs and RNA binding proteins and are usually not associated with the ribosome. Some of the hallmark proteins in these granules are the double stranded RNA-binding protein Staufen, FMRP, and/or ZBP1. However, these particles can exist in highly heterogeneous population (Hillebrand et al., 2007, Kiebler et al., 2006).
Table 1. List of conserved components of P bodies and NG (modified from Hillebrand et al., 2007). NGs are structurally and functionally related.

<table>
<thead>
<tr>
<th>Protein class</th>
<th>Mammalian, Fly, Yeast P bodies</th>
<th>Drosophila NGs</th>
<th>Mammalian NGs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conserved NG components</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fragile X like</td>
<td>FMRP</td>
<td>dFMR1</td>
<td>FMRP, FXR1, FXR2</td>
</tr>
<tr>
<td>RNA transport</td>
<td>Staufen</td>
<td>Staufen, Btz</td>
<td>Staufen, Btz</td>
</tr>
<tr>
<td>Deadbox RNA helicase</td>
<td>RCK/Me31B/Dhh1p</td>
<td>Me31B</td>
<td>Me31B</td>
</tr>
<tr>
<td>Core P body components</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5’ to 3’ exonuclease</td>
<td>XRN1p</td>
<td>Pcm</td>
<td>?</td>
</tr>
<tr>
<td>Sm like involved in repression</td>
<td>RAP55/Tral/Scd6p</td>
<td>Tral</td>
<td>?</td>
</tr>
<tr>
<td>Enhancers of decapping</td>
<td>Edc3p, pat1p/pat1/pat1</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Decapping enzyme</td>
<td>Dcp1p/Dcp2p</td>
<td>Dcp1</td>
<td></td>
</tr>
<tr>
<td>Sm-like involved in decapping</td>
<td>Lsm1-7</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Deadenylase</td>
<td>CCR4/Pop2/NotTwin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RISC component</td>
<td>mAGO1, mAGO2</td>
<td>Ago-2</td>
<td>?</td>
</tr>
<tr>
<td>NMD components</td>
<td>Upf-1</td>
<td>Upf-1</td>
<td>?</td>
</tr>
<tr>
<td>RNA binding proteins and repressors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cap binding</td>
<td>e1F4E&lt;sup&gt;a&lt;/sup&gt;</td>
<td>e1F4E also&lt;sup&gt;×&lt;/sup&gt;</td>
<td>some conditions</td>
</tr>
<tr>
<td>e1F4E-binding</td>
<td>e1F4E-T&lt;sup&gt;i&lt;/sup&gt;</td>
<td>Cup</td>
<td>?</td>
</tr>
<tr>
<td>Poly(A)binding</td>
<td>PABP&lt;sup&gt;d&lt;/sup&gt;</td>
<td>PABP&lt;sup&gt;×&lt;/sup&gt;</td>
<td>?</td>
</tr>
<tr>
<td>Zip code binding</td>
<td>?</td>
<td>imp</td>
<td>ZBP1</td>
</tr>
<tr>
<td>Puf domain</td>
<td>?</td>
<td>pum</td>
<td>?</td>
</tr>
<tr>
<td>Zn finger domain</td>
<td>?</td>
<td>nanos&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;i&lt;/sup&gt;</td>
<td>?</td>
</tr>
<tr>
<td>Y box</td>
<td>?</td>
<td>Yps</td>
<td>?</td>
</tr>
</tbody>
</table>

<sup>a</sup> Ortholog present, but association with RNA granules has not been described.
<sup>b</sup> Eulalio et al., 2007a, Mazroui et al., 2002, Sheth and Parker 2003, Andrei et al., 2005, Liu et al., 2005a, Pillai et al., 2005
<sup>c</sup> (except PABP) Barbee et al., 2006
<sup>d</sup> Kiebler et al., 1999, Kanai et al., 2004, Antar et al., 2005, Macchi et al., 2003
<sup>e</sup> eIF4E and PABP can associate with P-bodies in yeast under conditions of translational arrest induced by glucose deprivation. Teixeira et al., 2005
<sup>f</sup> Ye et al., 2004
<sup>×</sup> Ye et al., 2004
<sup>×</sup> Ye et al., 2004
<sup>i</sup> UPF1 has also been shown to interact with Stau in a Stau-mediated NMD pathway. Kim et al., 2005
<sup>+</sup> Pat1p acts as a general repressor of translation in yeast. Coller and Parker 2005, Yang et al., 2006, Barbee et al., 2006
<sup>j</sup> Andrei et al., 2005
1.3.1.2 Stress granules

Stress granules are large aggregates of translationally repressed mRNAs, translation initiation factors and 40S small ribosomal subunits that generally accumulate in the cytoplasm of cells undergoing translational arrest due to various environmental cues including heat shock, oxidative stress and energy deprivation (Anderson and Kedersha, 2006). Some of the key components of stress granules are the RNA binding proteins TIA-1, TIA-R, PABP (poly-A binding protein), and G3BP (Ras GTPase-activating protein-binding protein) (Gilks et al., 2004 and Touriere et al., 2003, Kedersha and Andersen 2002, Mazroui et al., 2006 and Dang et al., 2006). Stress granules are formed by phosphorylation of eIF2a which prevent mRNAs already bound to the small 40S ribosomal subunit to be translated. These RNAs are subsequently stored in stress granule and will be translated again once there is no more stress (Kimball et al., 2003). Based on FRAP (Fluorescent recovery after photo bleaching) analysis, components of stress granules are dynamic and stress granules disappear in cells treated with translation elongation inhibitors. This suggests that stress granules are assembled from translationally competent mRNAs (Kedersha et al., 2005). Not much is known about the function of stress granules in neurons. However, the Staufen and the mammalian Pumilio2 proteins can be transferred from neuronal transport RNPs into dendritic stress granules in response to stress (Vassey et al., 2006). This is also true for FMRP (Kim et al., 2006). FMRP moves out of polyribosomes and into stress granules upon oxidative stress. The presence of stress granules in dendrites near synapses suggests that mRNAs may be temporarily stalled for translation upon cellular stress. Finally, Several recent studies have documented that cytoplasmic TDP-43 aggregates co-localize with neuronal
stress granule markers although the significance is unclear (Colombrita et al., 2009).

1.3.1.3 Processing bodies

P bodies are large RNP complexes assembled in the cell body located in the close proximity of mitochondria and ribosomes, in which regulatory factors exhibit differential localization depending on their activity on mRNAs. P body organization has depicted a dense core, where decay enzymes are concentrated; on which peripheral protrusions made of p54 (RNA helicase), let7 (miRNA) and ribosomes are anchored as seen by immunogold labeling with electron tomography (Cougot et al., 2012). Study of P bodies started when the occurrence of XRN1 the 5’-3’ exoribonuclease was found inside granular structures in mouse fibroblast cell line (Bashkirov et al., 1997). Later the presence of GW182 in these foci gave them the name as ‘GW bodies /mRNA decapping bodies’ (Estathioy et al., 2002). These GW bodies in humans also contained Dcp2 (Decapping complex), XRN1, Lsms1-7 (Lykke Andersen 2002, Sheth & Parker 2003, Cougot et al., 2004) and were subsequently named “RNA processing bodies” or “P bodies” (Sheth and Parker 2003). P bodies are believed to be sites of mRNA translation repression and /or 5’ to 3’ decay and are found in neurons, somatic cells and oocytes. P body components Me31b and HPat (Xenopus homologues Xp54 and P100 respectively) have been found to participate in the storage of maternal mRNA in Xenopus oocytes (Ladomery et al., 1997) and Me31B localize to maternal RNA transport and storage granules in Drosophila egg chambers (Nakamura et al., 2001).

The core components of mammalian P bodies are Dcp1 and 2, Lsms (Sm-like proteins; enhancers of decapping), rck/p54 (RNA helicase), GW182 (RISC component) that are again heterogeneous in composition. P bodies also contain the decapping
enhancers Edc3, Hedls, Ro52, Pat1 and the deadenylating enzymes including the Ccr4–Caf1 and Pan2–Pan3 complexes. In essence, all the enzymes required for degrading mRNAs via the deadenylation–decapping 5′–3′ decay pathway come together inside P bodies (Marnef et al., 2010). Mammalian P body components also include the RISC machinery such as Ago (Argonaute) proteins. Thus mRNAs in P bodies may undergo regulation via both miRNA and general pathways (Hillebrand et al., 2007).

P bodies and stress granules are distinct structures. However, there is strong evidence that the two compartments are functionally linked and stress granules emerge next to P-bodies in *S. cerevisiae*, and in these cells the formation of stress granules is dependent on the presence of P bodies (Olszewska et al., 2012). Recent results suggest that stress granules and P bodies physically interact and that mRNAs may move between these two compartments. When mammalian cells were treated with arsenic, both stress granules and P bodies increased, and the P bodies often docked to the stress granules (Kedersha and Anderson 2002, Wilczynska et al., 2005).

1.3.1.3.1 Function

P body components function both in translation repression and mRNA degradation pathways (Decker and Parker 2012, Cougot et al., 2004, Brengues et al., 2005, Coller and Parker 2005, Liu et al., 2005a, Teixeira et al., 2005, Coller et al., 2001, Gavin et al., 2006, Krogan et al., 2006). In the former, mRNAs are deadenylated by the CCR4/Not1 complex, targeted for translational repression and stored within larger P body aggregates (Teixeira et al., 2005). In the latter, mRNAs are targeted for deadenylation by CCR4/Not1 complex followed by decapping by Dcp1/Dcp2 and then 5′-to-3′ exonucleolytic degradation by XRN1 exonuclease (Cougot et al., 2004, Sheth and Parker
Evidence suggests P bodies disappear after inhibition of deadenylation by deletion of CCR4 in *S. cerevisiae* and mouse, thus deadenylation is a prerequisite for P body formation (Kulkarni et al., 2010). However, it is also possible that locally deadenylated mRNAs can co- or post-translationally be directed inside the P bodies as repressed mRNAs with the help of translation repressors like Me31B. Later, in response to a local signal, these mRNAs could then exit P bodies and reinitiate translation (Kulkarni et al., 2010, Pradhan et al., 2012). Once mRNAs are deadenylated, they can also be directed towards decay pathway where enhancers of decapping like HPat, Lsms, EDC3 come to play to help recruit decapping complex Dcp2/1 which removes the cap and then subsequently degraded by XRN1 enzyme. P bodies increase in number and size when mRNA decay is inhibited in an XRN1 deleted yeast and human cells (Kulkarni et al., 2010, Marnef et al., 2010).

### 1.3.1.3.2 P body assembly

mRNPs that recruit RNA decapping machinery (Edc3, Lsm-Pat1, Dcp1/2) acquire the ability to assemble into P bodies (Franks and Lykke-Andersen 2008). P body formation is directly proportional to the cytoplasmic concentration of translationally repressed mRNPs (Franks and Lykke-Andersen 2008). This is supported by a number of observations. First, the entrapment of mRNAs in polysomes through treatment with drugs such as cycloheximide, depletes the cellular pool of ribosome-free mRNPs and causes rapid disappearance of visible P bodies. Second, in human, yeast, and *Drosophila* cells when mRNA were manipulated to release them from polysomes, it resulted in enhanced P body formation (Franks and Lykke-Andersen 2008). Third, slowing down enzymatic steps of mRNA decay lead to accumulation of P bodies (Franks and Lykke-Andersen 2008).
2008). The integrity of P body is critically dependent on RNA, as treatment with RNase in vitro or in permeabilized cells causes the disassembly of P bodies. These observations demonstrate a direct correlation between the cellular concentration of translationally repressed mRNPs and the extent of P body assembly in the cytoplasm and suggest a model in which P bodies form by the self-assembly of repressed mRNPs (Figure 1; Teixeira et al., 2005, Liu et al., 2005a/b, Franks and Lykke-Andersen 2008).

In *Saccharomyces cerevisiae*, the cAMP-dependent protein kinase A (PKA) is a key regulator of the assembly of P bodies. PKA inhibits the formation of the larger P body aggregates by phosphorylating Pat1 (yeast homologue of HPat), a conserved constituent of these foci that functions as a scaffold during the assembly process (Marnef et al., 2010). HPat plays role in P body assembly in yeast and mammals. In humans the DEAD box RNA helicase Rck and the scaffold protein Pat1b, both proteins are required for the assembly of P bodies. The C-terminal RecA-like domain of Rck interacts with the N-terminal acidic domain of Pat1b. In HeLa cells, Pat1b (vertebrate orthologue for HPat) assembles P bodies in the absence of Rck binding. In contrast, Rck requires the Pat1b binding site in order to promote P body assembly and associate with the decapping enzyme Dcp2 as well as Ago2 and TNRC6A, two core components of the RNA induced silencing complex. This indicates that P body assembly occurs in a step-wise manner, where Rck participates in the initial suppression of mRNA translation, whereas Pat1b in a second step triggers P body assembly and promotes mRNA decapping (Ozgur and Stoecklin 2013). P bodies are not required for mRNA silencing; but blocking siRNA or miRNA silencing pathways at any step prevents P body formation thus indicating that P bodies arise as a consequence of silencing (Eulalio et al., 2007a).
1.3.2 Neuronal P bodies

Neuronal P bodies consist of the mRNA decapping machinery, including the decapping enzyme (Dcp1/Dcp2), the activators of decapping complex (Dhh1p/RCK/p54/Me31B, Pat1p, Scd6p/RAP55, Edc3p, the Lsm1p-7p), and the 5′ to 3′ exonuclease, XRN1p (Barbee et al., 2006). These components are highly heterogeneous and homologues have been found in different species. Distinct types of P bodies have recently been identified in fly and mammalian neurons that exhibit motorized movement towards distal synapses in response to chemical stimulation (Barbee et al., 2006, Zeitelhofer et al., 2008a).

1.3.2.1 Motility of neuronal P-bodies

FRAP experiments show that P bodies in mammalian cells are highly dynamic and motile structures mostly confined to a particular region within the cytoplasm (Andrei et al., 2005, Kedersha et al., 2005). Some P bodies travel longer distances from the cell periphery towards the nucleus, whereas others appear immobile (Aizer et al., 2008). Dual color imaging shows that most stationary P bodies associate with actin bundles, whereas mobile P bodies are connected to the microtubule network (Aizer et al., 2008). Drugs that interfere with microtubule polymerization such as nocadazole and vinblastine significantly increase the number of P bodies and reduced their mobility (Aizer et al., 2008). Dynein may facilitate the transport of proteins and/or RNA into P bodies, yet it is not clear whether the actual movement of P bodies along microtubules is also dependent on dynein (Aizer et al., 2008). The conventional kinesin (KIF5, kinesin heavy chain) is the molecular motor of transport RNPs in neurons (Kanai et al., 2000). P bodies are transported to dendrites by active transport mechanism using the kinesin motor protein.
and localize to synaptic regions in response to neuronal activities, carrying a specific set of mRNAs, involved in dynamics of actin cytoskeleton, to local translational machinery in dendrites (Oh-Jh et al., 2013).

1.3.2.2 P bodies in the control of synapse structure

The dendritic localization of mRNAs and their subsequent translation at stimulated synapses contributes to the experience-dependent remodeling of synapses and the establishment of LTM. Dendritic P bodies may regulate local translation by storing repressed mRNAs in unstimulated cells, and releasing them on synaptic activation (Cougot 2008). Chemically induced neuronal activity results in a 60% decrease in the number of P bodies in dendrites, suggesting that P bodies disassemble after synaptic stimulation releasing mRNAs and possibly to be translated. This supports a model where neuronal P bodies are required for the transport specific translationally repressed mRNAs to the synapse. Examples of P body components involved in regulating aspects of long-term synaptic plasticity include Me31B and Tral in sensory neuron dendrite morphogenesis and Ago1, HPat, and CCR4 at the NMJ (Barbee et al., 2006, Jin et al., 2004, Pradhan et al., 2012). Studies by Jin et al., and Pradhan et al., have shown that knocking down Ago1 and HPat and/or CCR4 modulate synaptic structural changes at the Drosophila NMJ.

1.3.2.3 P bodies and the miRNA pathway

P bodies are believed to be sites of miRNA-mediated mRNA regulation where P bodies act as a center for scaffolding miRNA function. miRNAs are short (~21-22 nucleotides) non-coding regulatory RNAs that modulate gene expression post-transcriptionally (Li and Jin 2009). miRNAs generally down-regulate expression of target
mRNAs by binding specifically with target sequences in the 3’ UTR, although there is evidence that they can bind to sequences in the 5’ UTR and protein coding region (Breving et al., 2010).

Briefly, miRNAs are transcribed in the nucleus as primary transcripts (pri-miRNAs). The pri-miRNA forms a secondary hairpin structure that is recognized and cleaved by the RNAse III enzyme ‘Drosha’ that yields a precursor miRNA (pre-miRNA) of about 80 nucleotides. The pre-miRNA is exported to the cytoplasm where it is cleaved by the RNAse III enzyme ‘Dicer’ into the mature miRNA duplex. One strand of the miRNA duplex is selected and then incorporated into the RISC. Generally speaking, the other strand is degraded. It is believed that the miRNA guides the RISC to its target mRNA, where perfect complimentary base pairing between the 5’-most 7-8 nucleotides in the miRNA (the “seed region”) and the target mRNA leads to down-regulation of target mRNA expression. This is believed to occur either by translational repression or by mRNA degradation (Kim 2005).

The Ago proteins, which are signature components of the RISC, have also been found to localize to mammalian P bodies (Jakymiw et al., 2007). mRNAs that are targeted for translational repression by miRNAs concentrate in P bodies in a miRNA-dependent manner (Liu et al., 2005a). miRNAs silence gene expression by either repressing translation or by inducing decay of the mRNAs to which they are bound. Bhattacharyya et al. showed that the endogenous CAT-1 (cationic amino acid transporter-1) mRNA together with miR-122 localizes to P-bodies in liver cells. When mRNA silencing was prevented by knocking down components of the miRNA biogenesis pathway, it led to the loss of P bodies. And interestingly, re-introduction of siRNAs
(small interfering RNAs) caused P bodies to re-emerge (Kulkarni et al., 2010). Another important player in P body formation is the miRNA RISC component GW182 (Figure 2). GW182 interacts with its functional partner Argonaute proteins (AGO) via multiple domains to exert its silencing activity in the miRNA pathway. There is evidence that a knock down of GW182 disrupts P body formation. Although the precise mechanism of action of GW182 proteins in P body formation is not fully understood, these proteins have been shown to interact with the cytoplasmic PABP and with the PAN2-PAN3 and CCR4-NOT deadenylase complexes (Ding and Han 2007, Huntinzer and Izaurralde 2011).
Figure 1. The kinetic model for P body formation (taken from Franks and Lykke-Andersen 2008)

Model illustrating the hypothesis that the extent of P body (PB) formation is directly proportional to the cytoplasmic levels of repressed mRNPs and depends on competing rates of translational repression, P body factor recruitment, mRNP multimerization, and mRNA decay. Before mRNAs can assemble into P body, they must be translationally repressed. Translational silencing factors then promote the formation of a P bodies “monomer” by recruiting decapping factors and/or other P bodies assembly components. If mRNA decay enzymes are abundant and hence mRNA decay is rapid, mRNAs may be degraded prior to P bodies assembly. However, if decay enzymes are limiting, the repressed mRNAs assemble into P bodies where they can be degraded or released back into the translational pool.
miRISC containing Ago, miRNA and a GW182 family protein recognizes mRNA target guided by miRNA. The physical association of miRISC with mRNA releases ribosomes (orange ovals) and initiates changes in this complex, such as recruiting additional factors or triggering modification (illustrated as a green circle). This change leads to relocalization of the whole complex to P bodies or formation of P bodies. Inside P bodies decapping and deadenylation might be involved in degradation of some mRNA targets. Other mRNAs might retain a translation repression condition and use P bodies as a storage compartment. Under certain circumstances, repressed mRNAs in P bodies can be released into the cytoplasm, where their translation resumes. Potentially, miRISCs can also be recycled.
1.4 Summary

In review, the temporal and spatial regulation of mRNAs locally at the synapses are important for neuronal morphology and physiology. In neurons translationally repressed mRNAs are actively transported to their destinations in axons and dendrites in a variety of ribonucleoprotein particles (RNPs). A subset of these neuronal RNPs have been shown to contain proteins associated with mRNA processing bodies (P bodies), a distinct class of highly conserved cytoplasmic granules that have been linked to both mRNA decay and translational repression via general and miRNA mediated pathways. There is growing amount of evidence of components of P bodies having role in neural physiology. In this thesis, we provide evidence that P bodies have important functions in *Drosophila* neurons.

For the first question in this thesis, we postulated that HPat/Pat1p a core P body component implicated in both mRNA repression and 5’ to 3’ decay pathways, had a key function in the control of synaptogenesis or axon terminal growth at the larval *Drosophila* NMJ. Interestingly, we found that *hpat* mutants exhibit a strong synaptic hyperplasia at the NMJ both in a developing larva and acutely stimulated larva. Experiments done with *hpat* overexpression and mutants showed that *hpat* is a negative regulator of bouton formation in a developing larva. We further determined that this phenotype was associated with the rearrangement of the axonal microtubule cytoskeleton. Mechanistically, our findings show that HPat interacts genetically with the catalytic subunit of the deadenylase complex (twin/CCR4) and the miRNA pathway (Argonaute 1) to control bouton formation. Together, these data suggest that HPat is required to target mRNAs involved in the control of microtubule architecture and synaptic terminal growth.
for repression, presumably in P bodies, via both general and miRNA-mediated mechanisms. This role of HPat is true in both developing larva and acutely stimulated larva as we were able to see a similar phenotype in an acutely stimulated larva soaked in high potassium solution. We saw that pre and post synaptic overexpression of hpat suppressed ghost bouton formation in these acutely stimulated larva.

For the second question, we hypothesized that HPat and/or other P body components were required for FMRP-mediated translational repression based on published studies and our preliminary results. In addition to HPat, we screened most of the core P body components in Drosophila, for their function in FMRP-mediated translation repression. We used an in vitro luciferase reporter system, with FMRP tethered to Fluc reporter and knocking down P bodies with individual dsRNAs. Our results show that the P body component GW182, is required for the function of FMRP-mediated repression and knock down of two components Tral and Dcp1 enhance FMRP mediated repression. The requirement of GW182 for FMRP mediated translation repression is a novel finding and needs further investigation to address questions such as if FMRP recruit GW182 to a target mRNA. However, we did not see any biochemical interaction of FMRP and GW182 despite seeing some colocalising particles in S2 cells.
Chapter Two: Methods

2.1 Drosophila stocks

Fly stocks were raised and maintained at 25°C on standard Bloomington media containing corn syrup. Strains were obtained from: Canton-S, w^{1118}, hpat^{EY10289}, hpat^{(3)06442}, dFmr1^{Δ50M}, elav^{C155}-Gal4, C380-Gal4, D42-Gal4, B24-Gal4, Df(3R)RD31, Df(3R)Exel8165, Δ2-3 transposase, Dcp2^{BG01766}, Ago1^{1(2)k00208}, dFmr1^{Δ50M} (Bloomington); sev-dFmr1 (P. Jin); C380-Gal4, cha-Gal80 (S. Sanyal); UAS-YFP:FMRF (D. Zarnescu); TWG:HPat (S. Barbee); Dcp2^{GS3219}, Dcp2^{GS0176}, Twin^{GS12209}, Twin^{GS8115}, CG3995^{LL03178}, hpat^{LL03060} (Drosophila Genomics Resource Center); UAS-HPat^{hpn}^{27378} and UAS-HPat^{hpn}^{100872} (VDRC).

2.2 Generation of hpat^{d3} allele

Two new alleles for hpat were generated by imprecise mobilization of transposon P{EPgy2}patr-1^{EY10289} in Barbee lab. patr-1^{EY10289} (hpat^{EY10289}) is a homozygous lethal P element insertion line, in which the P{EPgy2} element is inserted in the 5’ UTR of HPat at a position 66 bp distal to the first initiation codon. Excision lines were generated by crossing virgin patr-1EY10289 flies to males possessing Δ2-3 transposase. From 14 independent excision events, one allele (hpat^{d3}) was found to not carry P element markers (w+ and y+) and retain homozygous lethality. A second allele (hpat^{d17}) was found to not carry P element markers but was homozygous viable. Finally, a precise excision allele (hpat^{d15}) was generated for use as a control for genetic background. The molecular
lesions in all *hpat* alleles where characterized by PCR and DNA sequencing by Dr. S. Barbee, R. Dhatt and SJ Pradhan.

2.3 Construction of *hpat* transgenic lines

Gal4-responsive transgenic HPat lines were constructed previously as follows by Dr. S. Barbee. The 2907 bp open reading frame of *hpat* was PCR amplified from EST clone RE36948 and cloned into pTW (which contains the pUAST promoter) or pTWG (which contains the pUAST promoter and an carboxyl terminal EGFP tag). RE36948, pTW, and pTWG were all obtained through the *Drosophila* Genomics Resource Center. Both transgenes (pTW-HPat and pTWG-HPat) were used as substrates for P element-mediated germline transformation (Genetic Services, Inc.). Transformed lines were crossed to the indicated GAL4 driver stocks. A transgenic construct for rescue of *hpat* mutants was constructed as follows. A ~21 kb genomic fragment containing the entire *hpat* locus plus about 5.4 kb of upstream and 10.4 kb of downstream DNA ([Pacman] BAC CH322-21M20) was obtained through the BACPAC Resource Center. The entire BAC clone was used for PhiC31-mediated germline transformation (BestGene, Inc.) into an attP landing site on chromosome 2 (Bloomington *Drosophila* Stock Center stock #9736). One line (*hPat21M20*) was recovered and used to rescue *HPat^d3* phenotypes. The rescue line was balanced in the lab and later turned homozygous.

2.4 *hpat* antibodies

*hpat* antibodies were gift from Akira Nakamura, RIKEN Center for Developmental Biology, Kobe, Japan. Basically, the full-length HPat coding region was cloned into pProExHTa (Invitrogen) to produce a 6His-tagged HPat protein. The protein was expressed in *E. coli* BL21, and purified using Ni-NTA Agarose (Qiagen) under the
denaturing condition. Purified protein was further purified by SDS-PAGE, dialyzed against PBS containing 2M urea, and used to elicit polyclonal antibody production in rabbits and rats (MBL, Japan). Rabbit polyclonal antisera were affinity-purified with the same antigens immobilized on a HiTrap NHS-activated HP column (GE Healthcare).

2.5 Larval immunohistochemistry and RNA in situ hybridization

To study NMJ morphology, wandering third-instar larvae were dissected in calcium-free HL3 (hemolymph-like) buffer (Stewart et al., 1994). Larval body wall preparations were processed as previously described (Sanyal et al., 2003). Unless otherwise indicated, control genotypes used for analysis were F1 larvae from w^1118 crossed to Canton-S. Heterozygous larvae were F1 larvae from the indicated genotype crossed to w^1118. Primary antibodies used were mouse anti-Dlg or anti-Futsch 22C10 (Developmental Studies Hybridoma Bank) and goat anti-HRP-Dylight-594 (Jackson Labs). Secondary antibodies used were Alexa 488-conjugated anti-mouse IgG (Molecular Probes). To study HPat localization or co-localization with FMRP, CNS (central nervous system) and larval body wall preparations from Canton-S flies were dissected and processed as described above. Primary antibodies used were rabbit or rat anti-HPat (this study) or mouse anti-FMRP 6A15 (Abcam) and goat anti-HRP-Dylight-633 (Jackson Labs). Secondary antibodies used were Alexa 488- or 568-conjugated anti-rat, rabbit, or mouse IgG (Molecular Probes). Quantification of HPat- and FMRP-containing punctae was done from 10 random ROI using the JACoP plugin for ImageJ v1.45 (NIH).

RNA in situ hybridization was carried out as described previously (Park et al., 2003). RNA probes were labeled with DIG (digoxigenin) and visualized with NBT/BCIP.
2.6 High Potassium Stimulation

High potassium stimulation for activity assays was done by K. Nesler using protocols previously described (Ataman et al., 2008). Briefly, non-wandering third instar larvae were partially dissected leaving CNS intact. The larvae then received 2, 2, 2, 4 and 6-minute stimulation pulses with osmolarity adjusted 90mM KCl HL-3 (Roche et al., 2002). Each stimulation paradigm was separated by 15 minutes rest steps in 5mM KCl HL-3 (Stewart et al., 1994) and a final resting step of 74 minutes. Upon completion of the stimulation paradigm, larval preparations were processed as described above for NMJ morphology. Psuedo-stimulated control experiments were done in the same manner, except that the 5mM KCl HL-3 replaced the 90mM KCl HL-3 during mock stimulation steps. Ghost boutons were identified by the presence of HRP and absence of Dlg.

2.7 NMJ and CNS imaging for morphological analysis

Laser scanning confocal microscopy was performed on an Olympus FluoView FV1000 microscope. Projections from confocal z-series stacks were obtained using a 60X (N.A. 1.35) or 100X (N.A. 1.4) objective and generated from stacks collected at intervals of 0.8 µm. Images were combined using FV1000 imaging software. Analysis of boutons was done essentially as previously described (Rohrbough et al., 2000). For each genotype, the indicated synaptic boutons were counted in both hemisegments of abdominal segment A3 for a minimum of 10 larvae (unless otherwise indicated; 20 paired NMJs). For each experiment, images were randomized blindly and then scored blindly using the Cell Counting plugin for ImageJ v1.45 (NIH). There was no obvious difference in muscle size between mutant or transgenic groups and controls. Briefly, 1b and 1s boutons were identified based on size and intensity of Dlg staining. 1bs are bigger and
more intensely stained with DLG compared to 1s boutons. HRP stain was used as a neuronal marker (Zhang et al., 2001, Ataman 2008).

2.8 Statistical Analysis

Statistical analysis including graphing was performed using Anova Tukey’s one way posthoc test or Kruskal-Wallis; Dunn’s post-hoc test; and statistical significance was determined to be at p < 0.05. Where indicated, data are normalized to controls and are presented as mean ± SEM. The numbers indicated in the columns of all graphs are the number of individual NMJs from which measurements were taken for that genotype. All indicated stocks were crossed to a w^{118} genetic background to generate heterozygotes for analysis.

2.9 Real time PCR

2.9.1 RNA isolation from the larval CNS

RNA was extracted from 25 CNSs from third instar larva of respective genotype in RNAse-free condition. CNS samples were homogenized using a TissueRuptor (Qiagen) in lysis solution (Exiqon A/S). Total RNA was isolated using the spin column-based miRCURY ™ RNA isolation kit (Exiqon A/S). Eluted in 50µl volume and stored at -80°C. RNA quantity and integrity was measured using BioRad RNA std sen kit and Experion automated electrophoresis system (BioRad).

2.9.2 qPCR

Quantitative real-time PCR (RT-qPCR) was performed on an iQ5 Real-Time PCR Detection System (Biorad) using HPat-specific Quantitect primers and the SYBR Green RT-PCR Kit (Qiagen). hpat expression levels were analyzed in triplicate and compared to three ubiquitously expressed reference genes: glyceraldehyde-3-phosphate
dehydrogenase (GAPDH), α-tubulin 84B, and ribosomal protein L13A (RpL13A) and dad1 as negative control. Melt curve analysis of all primer sets showed no amplification of non-specific products.

2.10 Immunoprecipitation of HPat and FMRP

For coimmunoprecipitation experiments, UAS-HPat:GFP flies were crossed to a pan-neuronal driver (elav<sup>Cl55</sup>-GAL4). Adult head extract was generated as described previously (Barbee et al., 2006). Briefly, F1 adults (~150 total) were selected against balancer and allowed to mature for 3 days, harvested in 15ml tube, snap frozen in liq. N<sub>2</sub> and vortexed three times for 10-15 sec to remove heads (re-immersed in liq. N<sub>2</sub> in between each vortex). Flies were then filtered through a wire mesh ( precooled in liq. N<sub>2</sub>) to separate heads (in middle mesh) and body. Fly heads were either stored at -80<sup>0</sup> C or processed immediately to make extract. Heads were homogenized five times with loose plunger (dounce homogenizer) and five times again with tight plunger in 3 ml freshly made extraction buffer, DXB-100 without RNase inhibitor (DBX -100 extraction buffer: 8.1 ml H<sub>2</sub>O, 1ml 1M KCL, 500µl of 50mM Hepes, pH 7.3 with KOH, 10µl of 1M MgCl<sub>2</sub> 2.5µl of 1M sucrose, 20µl of 500mM DTT, 100µl of 10% tritonX in water, 1 Tablet EDTA free protease inhibitor) spun 3000g/10 min to remove debris and made aliquots of 300µl and snap frozen in liq. N<sub>2</sub> and stored at -80<sup>0</sup> C. The RCDC protein assay kit (Biorad Cat. No. 500-0114) was used to estimate total protein.

Immunoprecipitation experiment was done according to Invitrogen Dynabead protein G Cat no 100.03D protocol. All procedures done at 4<sup>0</sup> C on ice in pre-cooled tubes and instruments unless otherwise stated. Briefly, 20 µg of either antibody or normal IgG (mouse or rabbit; Santa Cruz Biotech) was bound to protein G-conjugated magnetic
dynabeads (Invitrogen Cat. No. 100-03D) for 2 hours at 4°C. 300 µl of head extract (0.8 – 1.0 µg/ml) was added in the presence or absence of an RNAse cocktail (1U RNAse A; 40U RNAse T1; Ambion AM2286) and incubated overnight at 4°C with gentle agitation. Bound complexes were eluted by boiling in Lamelli buffer + 100mM DTT (BioRad) for 10 minutes. 30 µl of supernatant was collected by magnetic separation, separated by SDS-PAGE, transferred to PVDF membranes (BioRad Cat. No. 162-0177), and analyzed by western blotting. The indicated proteins were detected by western blot signal enhancer (Thermoscientific Cat. no. 21050). Primary antibodies used were mouse anti-dFmr1 (Abcam cat. No. 6A15) and rabbit anti-GFP (Torrey Pines Biolabs Cat. no. 401). Secondary antibodies used were HRP conjugated anti-mouse IgG or anti-rabbit IgG (Jackson Labs).

2.11 Western analysis

Protein extracts were made as follows. Briefly, \( w^{118} \), homozygous \( hpat^{d3} \), and homozygous \( hpat^{Ey10289} \) wandering third-instar larvae were homogenized using a motorized disposable microtube pestle in PBS containing EDTA-free protease inhibitor tablets (Roche Cat. no. 04693159001). Immediately after homogenization, an equal volume of 2x Laemmli sample buffer + 100mM DTT (BioRad) was added and the sample was passed through a QIAshredder (Qiagen) and boiled for 10 min. The sample was clarified by centrifugation and the supernatant separated by SDS-PAGE. Protein was transferred to a PVDF membrane (Amersham), blocked with 5% milk in TBS (Tris-buffer saline), blotted with rat anti-HPat (this study) and mouse anti-beta-Actin (Abcam Cat. no. ab8224) antibodies, and detected using western blot signal enhancer (Thermoscientific Cat. no. 21050). Relative HPat and Actin expression levels were
quantified using ImageJ v1.45 (NIH) image analysis software. \textit{hpat} mutants were normalized to controls.

2.12 Denaturing polyacrylamide gel electrophoresis of RNA

RNA was denatured and run on 5% TBE urea gel and run at 100V as per protocol described in Biorad Cat. No. 161-1133 and detected by staining with ethidium bromide.

2.13 Neuron culture

Neuron culture of CNS dissected was as per protocol described in Barbee et al 2006. Briefly, CNS from third instar \textit{Drosophila} larva were dissected and placed into a liberase enzyme (Roche 1988409) solution and incubated at room temperature for 1 hr. Tissues were then rinsed in S2 culture medium and subjected to two mechanical trituration consisting of 56 and 90 times each. Cells were plated onto coverslips coated with concanavalin A (Sigma C-2010) and laminin (VWR 47743 734) in tissue culture dishes and allowed to grow at 25°C for 72 hrs and immunostained. Gal4-C155 driver was used to drive expression of a functional HPat:GFP fusion protein. Cells were imaged using confocal microscopy and sought for the presence of HPat:GFP (or dFMR1-positive) punctae.

2.14 Analysis of rough-eye phenotypes by SEM

Rough-eye phenotypes were examined using a JEOL 5800LV scanning electron microscope after fixation as described previously (Barbee et al., 2006). Genotypes used for SEM were as follows: \textit{dFmr1} overexpression, +/-sev-\textit{dFmr1}; HPat suppression, +/-sev-\textit{dFmr1}; +/-\textit{hpat}\textsuperscript{FY10289} and +/-sev-\textit{dFmr1}; +/-\textit{hpat}\textsuperscript{3}; HPat rescue, (\textit{hpat}\textsuperscript{21M20}/sev-\textit{dFmr1}; +/-\textit{hpat}\textsuperscript{3}. All indicated stocks were crossed to into a \textit{w}\textsuperscript{1118} genetic background to generate heterozygotes for analysis.
2.15 Luciferase assay in S2 cells to study general translation repression

2.15.1 Creation of dsRNA for P bodies

dsRNA for individual P bodies of interest was designed using ‘Snap Dragon’ program (http://www.flyrnai.org/cgi-bin/RNAi_find_primers.pl). Primers designed to make the dsRNA were used to amplify from respective cDNAs. PCR products were cleaned up using Cycle Pure kit (Omega Biotek Cat. no. D6493-02) and transcribed into RNA using Ambion Megascript Kit (AM1333) and cleaned up using LiCl precipitation that came with the kit.

2.15.2 S2 cells maintenance

*Drosophila* S2 cells were maintained in in 250 ml Suspension Culture Flasks with vented caps (Celltreat Cat. no. 229520) in Complete Schneider’s *Drosophila* Medium (89% Schneider’s *Drosophila* Medium (1X), (liquid + L-Glutamine, Gibco Cat. no. 11720), 10% fetal bovine serum (Gibco Cat. no. 10082), 1% penicillin-streptomycin, (liquid, prepared with 5,000 units/mL Penicillin G sodium and 5,000 µg/mL streptomycin sulfate in 0.85% saline) (Gibco Cat. no. 15070) and fungizone (Gibco Cat. no. 15290-018). Complete media was filter sterilized using corning 500ml filter system (0.22µm PES 431097) and stored at 6°C. Cells were passaged every 72 hr and split the day before transfection to make sure cells were in exponential growth phase. For transfection, media did not contain fungizone.

2.15.3 Tethering and complementation assays in S2 cells

For this, plasmids pAc5.1-lambdan-HA (ID 21302), pAc5.1-Fluc-Stop-5BoxB (ID21301) were obtained from Addgene. Full FMRP sequence was cloned in frame at C
terminus of HA tag. In order to normalize and compare the reporter expression levels, *Renilla* luciferase vector was used. *Renilla* luciferase was cloned into pAc5.1 (Invitrogen) driven with an actin promoter by Dr. S. Barbee. RNA interference was performed as described previously (Eulalio et al. 2007b). S2 cells were transfected in six or twelve-well plates (1.5x10^6 cells/well for a 12 well plate) using effectene transfection reagent (Qiagen cat no 301427). All transfections were performed in triplicate (i.e. 3 biological replicates). For the λN-tethering assay in 12 well plates, the following plasmids were cotransfected: 0.05 µg reporter plasmid (F-Luc–5BoxB), 0.2 µg pAc5.1–R-Luc as transfection control, and 0.5 µg of plasmids expressing λN-HA–protein or λN-HA:FMRP fusion. For knock down of P body components 1µg (2µg used for one experiment of GW182) individual dsRNAs were transfected along with the plasmids, F-Luc and R-Luc activities were measured using the Dual-Luciferase reporter assay system (Promega Cat. No. E2940). Western blot was performed from cells to check knockdown of P body levels. On the day of transfection cells were vigorously re-suspended, and concentration of cells was determined using a neubauer haemocytometer. Volume of cell suspension to be used (1.5x10^6 cells /well to be transfected) was calculated. Cells were spun down, washed once with 1xPBS, and resuspended in an appropriate volume of media (0.8ml/well). Total DNA was prepared in Effectene as described in the kit (Qiagen Cat. no. 301427). 0.55 ml complete media was added to DNA mixture and mixed by pipetting up and down. 0.8 ml cell suspension was seeded drop wise into each well (Costar 12 well, Corning Cell-bind Cat. 3336) containing cells, while gently swirling plate. Cells were incubated for approximately 72 hours at 25°C in a humidified incubator.
2.15.4 Luciferase assays

In order to quantitatively assay changes in F-luc reporter repression we utilized a dual-glow luciferase reporter system. Three samples (technical replicates) were measured from each triplicate (biological replicate) transfection. Firefly and *Renilla* luciferase luminescence values were measured in a 96 well plate (flat bottom, non-treated, white polystyrene; corning Cat. no. 3912) using a BIO-TEK Synergy HT plate reader. Reagents Luciferase Assay Reagent II and Stop and Glow solution were prepared following manufactures instructions. 75µl of cells was added to each well, then 75µl LARII reagent was dispensed into each well. Firefly luminescence measurement was taken with sensitivity set to 100 or higher (S=100). If S100 did not work the settings were changed as per need. 0.75µl Stop and Glo reagent was added to each well then *Renilla* luminescence level was measured (S=100). The remaining cells were snap-frozen in liq N₂ and stored at -80°C for future analysis.

2.16 S2 cell stain for colocalisation studies

S2 cells were harvested after 72h of transfection with 0.5µg/well each of pAc5.1B-EGFP-DmGW182 plasmid (Addgene 22419) and pAc5.1B-3HA-3FLAG-dFmr1 (Breanna Symmes) or pAc5.1B-dFmr1 (Breanna Symmes). Cells were allowed to stick on poly-d-lysine coated dish and staining was done as per protocol described in Eulalio et al., 2007a using mouse anti-dFmr1 (Abcam Cat. No. 6A15) and rabbit anti-GFP (Torrey Pines Biolabs Cat. no. 401 pines) and imaged using confocal microscopy to look for punctate co-localization of GW:GFP and FMRP particles.
2.17 Immunopreicpitaion of GW182:GFP and FMRP

Interaction between GW182 and dFmr1 was detected by co-immunoprecipitation from S2 cells extracts harvested after 72 h of transfection with 0.5μg/well of pAc5.1B-EGFP-DmGW182 (Addgene 22419) and pAc5.1B-3HA-3FLAG-dFmr1 (Breanna Symmes). Immunoprecipitation was done using Pierce™ Magnetic HA-Tag IP/Co-IP kit (Thermo scientific Cat. No.88838). The lysis buffer used was as in the protocol described in Huntzinger et al., 2010 and consisted of 50mM Tris at pH 7.4, 150mM NaCl, 1mm EDTA, 0.1% Triron X, supplemented with pepstatin, RNase inhibitor and protease inhibitor.
Chapter Three: Results

New evidence suggests that dendritic RNPs in *Drosophila* and mammalian neurons share highly conserved translational repression machinery with cytoplasmic RNA processing bodies (Barbee et al., 2006; Cougot et al., 2008, Zeitelhofer et al., 2008). Initially thought to be sites of mRNA decay, P bodies have been shown to be involved in both general- and microRNA (miRNA)-mediated translational repression pathways. With this in mind, the underlying objective of this thesis is to identify and characterize functions for P bodies and P body components in synaptic translational control. First, we focused on the function of *Drosophila* HPat/Pat1, a highly conserved P body component involved in translational repression, P body assembly, and mRNA decapping pathways. Next, we used a reverse genetic approach to screen HPat and other known P body components for their ability to modulate FMRP-mediated translational repression. FMRP has been shown to associate closely with P bodies in fly and mammalian neurons (Jin et al., 2004, Cougot et al., 2008, Barbee et al., 2006, Pradhan et al., 2012). The main hypothesis underlying the work presented in Section 3.1 is that HPat is an important regulatory factor controlling mRNA transport or translation and structural plasticity in *Drosophila* neurons. The main hypothesis in Section 3.2 is that HPat and other P body components control axon terminal growth via FMRP- and/or miRNA-mediated translational repression pathways. The molecular mechanisms underlying basic forms of learning and memory are conserved amongst species. Bearing this in mind, we predict
that the findings made here in *Drosophila* can be applied to analogous processes in mammals.

### 3.1 Role of HPat in the regulation of synapse structure

As indicated previously, P body assembly is believed to be a stepwise process where key P body components, notably Pat1 and a complex of Lsm proteins, are first recruited to an mRNA to form a P body monomer. Under certain cellular conditions these monomers can recruit additional P body components to form visible P body aggregates (Figure 1; Decker and Parker 2012). The Pat1 protein has been proposed to act as a key scaffolding molecule during this assembly process (Braun et al., 2010, Pilkington and Parker, 2008) and can interact with many other P body components via its different regions (Ozgur et al., 2010). In *Saccharomyces cerevisiae* P body assembly and disassembly can be regulated by the direct phosphorylation of Pat1p by the cAMP-dependent protein kinase, PKA (Ramachandran et al., 2011). Loss of both, Dhh1p and Pat1p, results in a failure to repress translation and prevents P body formation in response to glucose starvation (Hurto et al., 2011). Although Pat1 has no recognizable functional domains or motifs, it plays essential roles in both the translational repression and mRNA decay pathways (Pilkington & Parker 2008, Marnef and Standart, 2010). As such, the single yeast and invertebrate Pat1 orthologs have dual functions in the control of deadenylation and decapping (Boag et al., 2008, Haas et al., 2010, Pilkington and Parker, 2008). In contrast, gene duplication in vertebrates has led to the evolution of two Pat1 paralogs (named Pat1a and Pat1b), with distinct functions in translational repression and decapping (Braun et al., 2010, Ozgur et al., 2010). Together, these data suggest that Pat1 proteins may be functioning at a pivotal point where the decision is made between
targeting a specific mRNA for repression and storage in P bodies or for decapping followed by 5’-to-3’ exonucleolytic degradation.

Aside from their roles in mRNA metabolism, very little is known about the physiological functions of either P bodies or most P body components in neurons in vivo. In this study, we ask if HPat, the fly ortholog of Pat1, plays a role in the control of synaptic growth at a model glutamatergic synapse, the larval *Drosophila* NMJ. **In summary**, we find that HPat is a strong negative regulator of synaptic growth both during development and following acute chemically induced synaptic stimulation. Specifically, we show that HPat has both a pre- and postsynaptic function in the control of synaptogenesis during these processes. Synaptic hyperplasia observed in *hpat* mutants correlates strongly with a disruption in the organization of the axonal microtubule cytoskeleton suggesting that HPat negatively regulates a pathway required for presynaptic microtubule-based growth. Finally, we demonstrate that HPat interacts genetically with catalytic components of the deadenylase but not the decapping machinery to control synapse formation. Our findings suggest a model where HPat is directing some specific neuronal mRNAs required for growth of synaptic boutons for translational repression, presumably within neuronal P bodies. It remains unclear if this function is in the soma or locally in axon terminals.

### 3.1.1 HPat is an essential gene

To check if *hpat* is an essential gene and to study its function, different alleles of *hpat* mutants were generated. Most alleles of *hpat* (also known as Patr-1 or protein associated with topo II related-1) were recessive lethal and homozygous *hpat* mutant individuals died at the late third-instar or early pupal stage without obvious
morphological defects (Figure 3A). By mobilizing a P element insertion located within
the hpat 5' UTR (hpat\textsuperscript{EY10289}), two partial deletions of the EY10289 insertion (hpat\textsuperscript{d17} and hpat\textsuperscript{d3}; Figure 3A.) were isolated. The hpat\textsuperscript{d17} allele was found to be homozygous viable.
In contrast, hpat\textsuperscript{d3} was recessive lethal. In hpat\textsuperscript{d17}, a ~10.2 kb deletion occurred within the central region of the P element leaving ~600bp of flanking insert sequence. The deleted regions were identified by PCR with primers flanking the P-element insert at the 5'UTR and sequencing the PCR product. In hpat\textsuperscript{d3}, a ~3.9kb deletion occurred within the central region of the P element leaving ~6.9kb of flanking P element sequence. The lethality of hpat\textsuperscript{d3} was not complemented by either hpat\textsuperscript{EY10289} or a second lethal P element insertion, hpat\textsuperscript{(3)06442} (Figures 3A; 4A). Moreover, hpat\textsuperscript{d3} was lethal in trans to a genetic deficiency, Df(3R)Exel8165, which deletes the entire hpat locus (Figure 4B). The lethality of hpat\textsuperscript{d3} was rescued by the introduction of a genomic DNA fragment (P21M20) that contained the entire hpat locus plus flanking genomic sequence that included two uncharacterized upstream genes (CG3995 and CG5220; Figure 4B). hpat\textsuperscript{d3} was not lethal in trans to a recessive lethal allele of the nearest gene, CG3995 (CG3995\textsuperscript{LL03178}; unfortunately no alleles were available for CG5220). Together, based on this genetic evidence, we conclude that hpat\textsuperscript{d3} specifically affects hpat function, and that hpat is an essential gene.

To further characterize lethal hpat alleles and examine HPat protein expression in vivo, we used two polyclonal antibodies against the HPat protein. As predicted, these antibodies recognized bands of approximately 102 kDa and 108 kDa on immunoblots of extracts from whole Drosophila third instar larvae (Figure 5A). Evidence from gene prediction algorithms and cDNA analysis suggest that these bands likely represent two
isoforms of hpat that result from two alternative transcriptional start sites (Figure 3B). The expression level of both isoforms is significantly reduced, but not completely eliminated, in extract from hpat\textsuperscript{d3} and hpat\textsuperscript{EY10289} whole homozygous third-instar larvae (the 102 kDa band is reduced to 38% and 2% of controls respectively; the 108 kDa band is reduced to 66% and 15%; Figure 5A and B). In contrast to whole larval immunoblots, HPat antibody staining indicates that HPat expression is severely disrupted in hpat\textsuperscript{d3} and hpat\textsuperscript{EY10289} mutant ventral ganglia and muscle (Figure 6A and B). This suggests that the hpat\textsuperscript{d3} allele may have a strong effect on the tissue-specific expression of HPat and that the HPat antibodies are specific for the HPat protein. Together, these data also indicate that hpat\textsuperscript{d3} and hpat\textsuperscript{EY10289} are hypomorphic, and not null alleles of HPat.

3.1.2 HPat is expressed in the larval central nervous system (CNS).

Neuronal expression of HPat was further verified by in situ hybridization, quantitative real-time PCR (RT-qPCR) and immunohistochemistry (Figures 7A and B). First, when probed with an antisense riboprobe (Figure 4A), the presence of hpat mRNA was detected throughout the larval CNS when compared to sense controls. hpat mRNA was also abundant in total RNA isolated from the larval CNS when analyzed by RT-qPCR. This analysis showed strong hpat mRNA expression in the larval CNS relative to reference “housekeeping” genes (Figure 9).

As expected, when larval brains where stained with polyclonal antibodies against HPat, the HPat protein exhibited a specific and punctate staining pattern in the cytoplasm and neuropile of most neurons within the third-instar larval ventral ganglion (Figures 6A; 7B). We also observed high levels of HPat expression in the optic lobes and larval eye imaginal discs (data not shown). To examine synaptic enrichment at the NMJ, we
performed double labeling with antibodies against HPat and a postsynaptic marker (Discs Large or “Dlg”; the fly ortholog of postsynaptic density protein 95 or “PSD-95”). Interestingly, HPat was not enriched at peripheral NMJ synapses (Figure 6C). However, HPat was moderately expressed in muscle and exhibited a specific punctate staining pattern with distinct perinuclear enrichment (Figures 6B; 7C). Thus, HPat is a cytoplasmic protein that is expressed in neurons and muscle but is not detectably enriched at the neuromuscular synapse. We further examined subcellular localization of the HPat protein in neurons in vivo by determining if HPat co-localized with FMRP, a component of neuronal transport granules. As predicted, HPat strongly co-localized to FMRP-expressing neurons in the larval ventral ganglion (merged image in Figure 6B). Both HPat and FMRP also partially co-localize within punctae in peripheral nerves exiting the larval CNS (merged image in Figure 7D). 48% (±11%; n=203) of HPat-positive foci also contained FMRP. Conversely, 33% (±10%; n = 182) of FMRP-positive foci also contained HPat. Surprisingly, both HPat and FMRP-containing granules disappear from peripheral nerves prior to reaching the NMJ.

To confirm intracellular HPat localization, the experiment described in Figure 6 was also done using a transgenic line that expressed a HPat:GFP fusion protein under control of the UAS/GAL4 expression system. When HPat:GFP (UAS-HPat:GFP) expression was driven by a motor neuron-specific Gal4 driver (C380-Gal4), HPat:GFP localized to large particles within the cell body of GFP expressing cells in the ventral ganglion (Figure 8) and to peripheral nerves exiting the larval CNS. The percent colocalization between HPat:GFP and FMRP in peripheral nerves was not specifically assayed but appeared to be substantially lower than that observed between wild-type HPat- and FMRP-containing
granules (data not shown).

3.1.3 HPat regulates synaptic terminal growth during development of the larval NMJ

3.1.3.1 D. melanogaster as a model for studying functions for neuronal P bodies

*Drosophila* is an excellent model system in which to study development, physiology and behavior parallel to human beings. Genetic manipulations in many organisms have been used to investigate gene/protein functions. However, the extent to which genetic methods and tools have been developed for *Drosophila melanogaster* far exceeds that of other animal models. For example, *Drosophila* neurons from genetically modified animals can be easily cultured and stained to study neuronal granules. Neurons and synapses in the olfactory circuit, in the adult are very well characterized in terms of their connectivity and function. It is also a well-defined system for the study of memory and local protein synthesis using the olfactory/electric shock paradigm (Ashraf et al., 2006).

The *Drosophila* larval NMJ is an excellent genetic model system for the study of glutamatergic synapses in the mammalian brain (Schuster, 2006). The larval neuromuscular junction (NMJ) of *Drosophila* is very easy to dissect and is an excellent system to study synaptic development and function (Keshishian et al., 1996). The abdomen is divided into eight segments, each having about sixty uniquely identifiable muscles and each muscle innervated by one or more motor neurons (Hoang and Chiba 2001). Glutamate is the primary excitatory neurotransmitter at the NMJ, (thus mimicking the human brain) but subsets of synaptic terminals also contain octopamine (the invertebrate equivalent of norepinephrine) and neuropeptides. The larval NMJ is comprised of axon branches that terminate on the surface of the muscle that are termed as
synaptic boutons. Based on size, three types of boutons can be distinguished at the larval NMJ (Johansen et al., 1989, Jia et al., 1993, Atwood et al., 1993). Type I boutons are the largest, are subdivided into type 1b (big) and type 1s (small); and are present at all muscles (Rohrbough et al., 2000). Type 1b and 1s are again easily distinguishable that 1b is bigger, more plastic and contains more Dlg the fly homologue for PSD95 (Menon et al., 2004). Type II boutons are much smaller, contain octopamine and innervate nearly all muscles (Monastirioti et al., 1995). Type III boutons are also small, contain insulin-like peptide and are restricted to a single muscle in each segment (Gorczyca et al., 1993). Most body wall muscles are innervated by more than one motor neuron, but no muscle is innervated by two motor axons with the same bouton type (Hoang and Chiba 2001). These boutons are extremely plastic both during development and in response to acute synaptic stimulation (Collins and DiAntonio 2007). So, the function of P bodies can be studied at the Drosophila NMJ in genetically manipulated flies for those P bodies.

Here, we examined the paired synapse (at both hemi-segment) at muscle 6/7 in abdominal segment 3 of wandering third instar larva. This NMJ contains 1b and 1s boutons that are derived from distinct neurons. We also examined the NMJ at muscle 4. This NMJ is innervated by a single neuron, contains only type 1b boutons, and allows for the quantification of synaptic branching, an additional measure of synaptic complexity (Zhang et al., 2001).

### 3.1.4 HPat loss-of-function causes structural defects at the NMJ

Third instar larva of $hpat^{d3}$, $hpat^{d17}$, and $hpat^{FY10289}$ mutants were double-stained with Dlg and HRP and the number of type 1b synaptic boutons was assayed at NMJ in each genotype. Interestingly, $hpat^{d3}$, $hpat^{d17}$, and $hpat^{FY10289}$ mutants showed a robust
and highly significant hyperplasia of muscle 6/7 synaptic terminals compared to controls (Figure 10A-B). First, in hpat\textsuperscript{d3} homozygotes, there was a 85% increase over controls (p < 0.0001). Importantly, the increase in bouton numbers observed in hpat\textsuperscript{d3} mutants was significantly suppressed by the introduction of two copies of an hpat genomic rescue construct (P21M20; described in Figure 4B; Figure 10A-B; p < 0.0001). The overgrowth phenotype observed in hpat\textsuperscript{d3} homozygous mutants was confirmed by examining 1b bouton numbers in combination of other alleles of hpat. hpat\textsuperscript{EY10289} homozygotes showed a 54% increase, hpat\textsuperscript{d3}/hpat\textsuperscript{EY10289} showed 59% increase, and hpat\textsuperscript{d3}/hpat\textsuperscript{d17} mutant backgrounds showed a 43% increase (all at p < 0.0001). Similar increases were observed in type 1s synaptic boutons (Figure 10C). As a control for genetic background effects, we also generated a genetic revertant line by the precise excision of the hpat\textsuperscript{EY10289} insertion (called hpat\textsuperscript{15p}). When crossed to the homozygous viable hpat\textsuperscript{d17} allele (hpat\textsuperscript{15p}/hpat\textsuperscript{d17}), there is a small, but significant, increase in bouton numbers (Figure 10B, C; 1b boutons = 26% increase over controls; p < 0.05). When crossed to the hpat\textsuperscript{d3} allele (hpat\textsuperscript{15p}/hpat\textsuperscript{d3}), there is a slightly greater increase (Figure 10B; 34% increase; p < 0.001). Together, these data do suggest that genetic background may have a weak effect on bouton formation. However, this effect on 1b bouton numbers was not significantly greater than that observed in hpat\textsuperscript{d3} or hpat\textsuperscript{EY10289} heterozygotes (Figure 10B; hpat\textsuperscript{15p}/hpat\textsuperscript{d17} = 3% increase over hpat\textsuperscript{d3}/++; and hpat\textsuperscript{15p}/hpat\textsuperscript{d3} = 10% increase over hpat\textsuperscript{d3}/+). Moreover, both 1b and 1s bouton numbers in both lethal hpat mutants (hpat\textsuperscript{d3} and hpat\textsuperscript{EY10289}) remain significantly higher than hpat\textsuperscript{15p}/hpat\textsuperscript{d17} mutant larvae.

These results were also confirmed at the muscle 4 NMJ (Figures 11A, B). At this synapse, hpat\textsuperscript{d3} homozygotes exhibited a 53% increase in type 1b bouton numbers when
compared to controls (p < 0.0001). Again, we validated this result by examining bouton numbers in $hpat^{EY10289}$ homozygotes (32% increase; p < 0.05) and $hpat^{d3}/hpat^{EY1089}$ mutants (49% increase; p < 0.001). While introduction of two copies of the P21M20 rescue construct did substantially reduce the number of type 1b bouton numbers compared to $hpat^{d3}$ homozygotes, this decrease was not statistically significant (p = 0.108). This might be due to the generally weaker effect HPat appears to have on the muscle 4 NMJ compared to muscle 6/7. Surprisingly, the quantification of muscle 4 synaptic branches did not show significant increase over controls (Figure 11C). This observation suggests that $hpat$ may specifically be involved in the control of new bouton growth at the NMJ and not axon terminal branching.

3.1.5 HPat has a function on both sides of the synapse in the control of synaptic terminal development

An analysis of bouton numbers in transgenic lines where the HPat protein was over-expressed using either a motor neuron- or muscle-specific Gal4 driver ($C380$-Gal4 and $24B$-Gal4 respectively) was done. Presynaptic over-expression of transgenic $hpat$ revealed the opposite phenotype as $hpat$ reduction-of-function mutants (Figure 12A-B). HPat neuronal over-expression NMJs displayed a modest, but statistically significant, decrease in type 1b boutons (13% decrease; p < 0.01). A second motor neuron-specific Gal4 driver ($D42$-Gal4) exhibited a similar decrease in bouton number (12%; p < 0.05). In contrast, postsynaptic over-expression ($24B$-Gal4) of HPat resulted in no significant change in type 1b bouton numbers when compared to controls (Figure 12A-B). Unlike in 1b boutons, quantification of type 1s boutons did not follow a similar trend for the neuronal drivers (Figure 12C-D). Surprisingly, postsynaptic over-expression of $hpat$
using 24B-Gal4 resulted in a significant increase in 1s boutons compared to controls (37% increase; p < 0.05). Unfortunately, HPat levels could not be sufficiently depleted by RNAi so these results could not be confirmed by cell autonomous HPat reduction-in-function. Experiments with available hpat hairpin RNAi lines alone (and in combination with UAS-dicer) were done but significant results were not observed (Figure 13A-B). Taken together, the lack of a strong and consistent phenotype following HPat overexpression suggests that hpat does not have a specific role on either side of the synaptic cleft. Thus, both muscle and neuronal hpat function is likely required for normal synaptic terminal development at the NMJ.

3.1.6 HPat regulates organization of the presynaptic microtubule cytoskeleton

In the previous Figure 10A; we noticed that hpatΔ3 mutants had the presence of clusters of terminal synaptic boutons, a phenotype that is suggestive of abnormal presynaptic overgrowth (Figure 10A; arrows). At the Drosophila NMJ, terminal synaptic boutons can also be identified by the presence of distinct “loop”-like organization of the microtubule cytoskeleton (Roos et al., 2000). These loops are believed to identify sites of active bouton division. Based on the synaptic terminal overgrowth phenotype we observed in hpat mutants (Figure 10A-D), we predicted that we would see a similar increase in the number of microtubule loops per NMJ in hpat mutants (muscle 6/7; abdominal segment 3) when compared to controls. Hence the microtubule cytoskeleton was examined in hpatΔ3 and hpatE10289 mutant larvae by double-staining with antibodies against presynaptic HRP and Futsch, the Drosophila ortholog of microtubule-associated protein 1B or “MAP1B”. Futsch staining tightly co-localizes with microtubule markers and can be used to readily identify synaptic microtubule loops (Roos et al., 2000). hpatΔ3
homozygous larvae exhibited a robust and highly significant increase in the number of Futsch-positive loops compared to controls (Figure 14A, B; mean number of Futsch loops per NMJ: control = ± 1.1; hpat\textsuperscript{d3} mutants = 18.3 ± 1.1; 53% increase; p < 0.0001). These loops were often found in clusters off the main synaptic arbor (Figure 14A; inset). The hpat\textsuperscript{d3} mutant phenotype was completely suppressed by the introduction of two copies of an HPat genomic rescue construct (Figure 14B; P21M20 rescue = 10.6 ± 0.7; p < 0.0001). Interestingly, while we did see an increase in the total number of Futsch loops in hpat\textsuperscript{EY10289} homozygous mutant larvae compared to controls, this increase was not statistically significant (hpat\textsuperscript{EY10289} mutants = 14.8 ± 1.0; 23% increase; p =0.141). However, this is consistent with the generally weaker phenotype observed in hpat\textsuperscript{EY10289} mutants when examining bouton development (Figures 10B; 11B). We also analyzed the distribution pattern of these Futsch loops in the main synaptic arbors, synaptic terminal junctions, and terminal boutons. There was a significant increase in the number of Futsch loops at branch junctions in both hpat\textsuperscript{d3} mutants (5.8 ± 0.5; 140% increase; p < 0.001) and hpat\textsuperscript{EY10289} mutants 4.2 ± 0.5; 75% increase; p < 0.05 compared to controls (2.4 ± 0.3; Figure 14D). Despite this increase, the relative frequency of Futsch loops occurring in each synaptic region remained same (Figure 14E). In contrast, hpat\textsuperscript{d3}/hpat\textsuperscript{d3}; rescue larvae did show a significant redistribution in the number of Futsch loops in each synaptic region compared to control and hpat mutant genotypes (Figure 14D-E). This may be because two copies of the P21M20 transgene only partially rescue specific synaptic phenotypes (Figure 10B). Finally, Futsch staining in hpat\textsuperscript{d3} mutants was generally weaker and more punctate than in controls (compare Futsch staining in Figure 14A). Together, these data suggest that directly or indirectly, the mRNAs that control the
microtubule cytoskeleton are being regulated by HPat.

### 3.1.7 HPat regulates activity-dependent synaptic growth

Because HPat regulates synaptic growth during larval development, we speculated that it might also be involved in the control of activity-dependent synapse formation at the larval NMJ. To test this hypothesis, we used a model system that allowed us to assay activity-dependent plasticity at this synapse (Ataman et al., 2008). Treatment of partially dissected larval NMJ preparations with a spaced high K+ depolarization paradigm induces the formation of immature synaptic boutons known as “ghost boutons” (Ataman et al., 2008). These presynaptic varicosities have been shown to contain synaptic vesicles but lack active zones and postsynaptic structures (Ataman et al., 2006). We analyzed transgenic lines that expressed HPat under the control of either a motor neuron-specific (C380-Gal4) or muscle-specific driver (24B-Gal4) and stained larval preparations with antibodies against HRP and Dlg (Figure 15A-B). In our hands, stimulated control larval preparations exhibited a significant increase in the number of ghost boutons compared to pseudo-stimulated controls (Figure 15A-C; mean number of ghost boutons per NMJ: C380/+ unstimulated control = 1.9 ± 0.3 or stimulated control = 6.9 ± 0.8; p <0.0001; 24B/+ unstimulated control = 1.1 ± 0.2 or stimulated control = 5.3 ± 0.8; p < 0.001). As we predicted, both pre- and postsynaptic over-expression of HPat suppressed this phenotype. Following presynaptic expression, the number of ghost boutons observed per synapse was completely reduced to baseline levels (Figure 15A, C; unstimulated C380-Gal4; UAS-hpat = 1.5 ± 0.2; stimulated C380-Gal4; UAS-hpat = 2.2 ± 0.4). Similarly, postsynaptic over-expression of HPat significantly suppressed ghost bouton formation (Figure 15B-C; unstimulated 24B-Gal4; UAS-hpat = 1.9 ± 0.4;
stimulated 24B-Gal4; UAS-hpat = 3.1 ± 0.5). While there is some genotypic variation, the ratio of unstimulated to stimulated ghost boutons drops to similar levels using both drivers (C380-Gal4; UAS-hpat =3.6 to 1.4; 24B-Gal4; UAS-hpat = 4.8 to 1.6). Based on these data, we conclude that HPat is a strong negative regulator of activity-dependent synaptic growth at the larval NMJ. Furthermore, these results support our model that hpat has a function on both sides of the synaptic cleft during development and following acute synaptic activity.

3.1.8 Neuronal hpat interacts genetically with components of the deadenylation machinery.

Again, it has previously been shown that HPat and its orthologs have important cellular functions in the control of mRNA storage and decay (Marnef and Standart, 2010). Thus HPat might be regulating synaptic terminal growth at the NMJ through one (or both) of these pathways. We speculated that HPat could interact genetically with twin (the fly CCR4 ortholog) or dcp2, key catalytic components of the deadenylase and decapping complexes respectively, to control synaptic terminal growth during development (Figure16A; Rehwinkel et al., 2005; Temme et al., 2004). As shown previously, hpat<sup>d3</sup> heterozygotes did not exhibit an increase in 1b bouton numbers compared to controls (Figures 10B; 16B). Similarly, twin (the CCR4 homologue) and dcp2 heterozygotes did not show a significant increase over baseline numbers of boutons (Figure 16B). However, if either twin or dcp2 interact with hpat in the same (or parallel) genetic pathway to control bouton growth, there should be an enhanced synaptic hyperplasia in trans-heterozygous larvae. As predicted, in hpat and twin trans-heterozygotes, a highly significant overgrowth compared to controls was seen (Figure
16B; twin$^{GS8115/+}$; hpat$^{d3/+}$ = 20% increase; twin$^{GS12209/+}$; hpat$^{d3/+}$ = 39% increase; p < 0.05 and p < 0.0001 respectively; both twin$^{GS8115}$ and twin$^{GS12209}$ are lethal hypomorphic alleles of twin; Zaessinger et al., 2006). Nearly identical results were observed in twin$^{GS8115}$/ twin$^{GS12209}$ mutants (20% increase; p < 0.01). The synaptic terminal overgrowth phenotype observed in trans-heterozygote and twin$^{GS8115}$/ twin$^{GS12209}$ mutant larvae was not as robust as observed in hpat mutants alone. This suggests that, while hpat can interact genetically with twin/CCR4 to control synaptic terminal growth, twin is not a limiting factor for hpat function. In contrast to twin, no significant increase was observed in hpat and dcp2 transheterozygotes compared to controls (Figure 16B). Two lethal alleles of dcp2 were examined: dcp2$^{BG01766}$ and dcp2$^{GS3219}$. Only dcp2$^{BG01766}$ homozygous mutants resulted in viable third-instar larvae and they did not exhibit a significant synaptic hyperplasia at the NMJ (Figure 16B; dcp2$^{GS3219}$ homozygotes and dcp2$^{BG01766}$/dcp2$^{GS3219}$ mutants did not survive to the third-instar stage; data not shown). In both twin and dcp2 mutants, type 1s boutons showed a substantial amount of variability (Figure 16C). Together, these data suggest that HPat does not require the decapping machinery to regulate synaptic terminal growth during development at the Drosophila NMJ. More specifically, this indicates that HPat may be involved in targeting some key mRNAs involved in the control of new synaptic bouton growth for translational repression, presumably in P bodies, and not for 5’-to-3’ mRNA decay. At this point, it remains unclear if this function is in the soma, peripheral nerves, or locally at the synapse.
3.2 P body components interact with FMRP

The findings outlined above as well as published data indicate that a subset of FMRP-containing RNA granules in *Drosophila melanogaster* and mammalian neurons colocalize with proteins classically associated with somatic P bodies. Moreover, FMRP interact with some of the P body components biochemically, physically and genetically. Namely, HPat, Me31B, Tral, and Ago1 biochemically purify with FMRP, presumably in a subset of neuronal RNP granules. Finally, *dfmr1* function in developing eye imaginal discs is regulated by P body components HPat, Me31b, Tral and AGO1 (Figure 17, Barbee et al., 2006, Jin et al., 2004).

In this second section we address two questions. First, why does FMRP associate with components of P bodies in neurons? Second, which of these P body components are required to regulate the function of FMRP as a translational repressor? We know that FMRP bind to neuronal mRNAs for repression, but the exact mechanism and role of P body involvement in this process is not fully understood.

3.2.1 FMRP as the causative gene for Fragile X Syndrome

Fragile X Syndrome (FXS) is the most common form of inherited mental retardation in humans and is a major genetic cause of autism (Bassell and Warren, 2008). The disease is manifested with IQ values typically between 20 and 70, cognitive dysfunction, delay in speech, hyperactivity, hypersensitivity to sensorial stimuli, attention deficit and autism. Individuals affected have a characteristic, facial morphology of macrocephaly, with a long narrow face and prominent forehead, jaw, and ears. Macroorchidism are commonly seen in post pubescent male patients.

The cellular phenotype of FXS is an increase in immature long and thin dendritic
spines (Irwin et al., 2000). In humans, FXS is caused by a CGG trinucleotide expansion within the 5’-untranslated region (UTR) of the Fragile X Mental Retardation 1 gene (FMR1) which leads to DNA condensation hence a reduction or loss of expression of the Fragile X Mental Retardation Protein (FMRP) (Santoro et al., 2012).

FMRP is a RNA-binding protein that is highly expressed in the brain where it is predicted to regulate roughly 4% of neuronal mRNAs (Ashley et al., 1993, Siomi et al., 1993). FMRP has been shown to act as a translational repressor (Laggerbauer et al., 2001, Li et al., 2001) presumably through a direct association with structural elements in the UTRs of target mRNAs (Darnell et al., 2005, Darnell et al., 20011). The FMRP protein has two sequence motifs characteristic of RNA binding proteins, a heterogeneous nuclear ribonucleoprotein K Homology (KH) domain and a cluster of Arginine-Glycine-Glycine, residues known as ‘RGG’ box that bind to a G quartet structure formed by some mRNAs. The G quartet is an RNA loop structure with a planar conformation of four guanine residues. The heterogeneous nuclear ribonucleoprotein K homology (KH) domains are shown to bind a RNA tertiary structure known as a “kissing complex. There is also evidence that FMRP can interact with some target mRNAs via the novel noncoding RNA BC1. FMRP has been found to have both biochemical and genetic interactions with components of the miRNA pathway. Thus, FMRP may regulate neuronal translation via or in concert with miRNAs (Penagarikano et al., 2007).

A current hypothesis is that FMRP regulates mRNA transport and local protein synthesis in response to stimulation of metabotropi glutamate receptors (mGluRs). Excessive signaling through mGluR5 can account for multiple cognitive and syndromic features of fragile X syndrome, When FMRP is absent, as in FXS, protein synthesis is
constitutively elevated, which lead to overactive AMPAR internalization and exaggerated LTD, even in the absence of mGluR activation (Santoro et al 2012).

Most FMRP is thought to form ribonucleoprotein (RNP) particles that associate with polyribosomes, a fraction of FMRP can be found in RNA granules that traffic with repressed mRNAs in dendrites (Kiebler and Bassell, 2006, De Rubeis and Bagni, 2010). There is a growing amount of evidence that the latter type of RNP also contains conserved components of P bodies (Barbee et al., 2006, Jin et al., 2004, Pradhan et al., 2012). In this section we have sought to further characterize the ability of HPat to interacting with FMRP-containing neuronal granules by examining colocalization, biochemical and genetic interactions at the NMJ and eye. Finally, we have also attempted to identify and characterize specific P body components required for FMRP-mediated repression by developing an in vitro FMRP tethering assay in Drosophila S2 cells. In summary, our finding show that HPat and FMRP interact both biochemically and genetically although the latter is very weak at the NMJ. Furthermore, in a screen of most known P body components, we identify the miRNA pathway effector protein, GW182, as a novel regulator of FMRP-mediated translational repression in vitro.

3.2.2 HPat is a dominant modifier of dFmr1 gain-of-function in the developing eye

Based on above background, we next asked if hpat interacted genetically with dfmr1. Ectopic over-expression of dfmr1 in the developing eye directed by sevenless gene enhancer elements (sev-dFmr1) has been shown to cause a strong and consistent rough-eye phenotype in the adult fly caused by apoptotic cell death during eye development (Wan et al., 2000). This system has proven to be useful for identifying genes that are required to help facilitate FMRP function. For example, it has previously
been shown that a reduction in the expression of genes encoding for the P body proteins, me31b, trailerhitch (trl), and ago1, can completely suppress this rough eye phenotype (Jin et al., 2004, Barbee et al., 2006). These data strongly suggest that the genes encoding these proteins are required to regulate dfmr1 function in vivo.

Based on these observations, we predicted that hpat might also be required to regulate dFmr1 function. To test this hypothesis, we asked if loss of a single copy of lethal alleles of hpat (hpat<sup>EY10289</sup> and hpat<sup>d3</sup>) could suppress the rough-eye phenotype caused by dfmr1 over-expression. As predicted, both alleles of hpat consistently showed a consistent but weak suppression as indicated by a partial reversion back to the wild-type phenotype (Figure 17). This ability to suppress is dependent upon HPat function. Introduction of a single copy of a genomic rescue construct caused eyes to revert back to the rough-eye phenotype (hpat<sup>21M20</sup>; Figure 17). Interestingly, eyes in the rescue construct appeared to be rougher than that observed following dfmr1 overexpression alone suggesting a possible enhancement of the phenotype. Together, these data are suggestive of a dominant genetic interaction between hpat and dFmr1 and indicate that HPat may be required to regulate FMRP function in vivo.

### 3.2.3 HPat colocalizes with FMRP in cultured neurons

FMRP is also known to colocalize with some P-body components in neurites of primary cultures of fly larval motor neurons and mammalian hippocampal neurons (Barbee 2006; cougat et al., 2008). We have already shown that HPat partially colocalizes with FMRP-containing granules in vivo (Figure 7). Based on this, we also sought to examine the localization of HPat with FMRP in cultured Drosophila neurons. In one of the representative cell, we saw moderate colocalization, that 27.5% of
HPat:GFP(+) granules also contain FMRP (1 cell; 80 granules) (Figure 18a). When more (4) cells were analysed the total number of HPat:GFP granules ranged from 86 to 222. Out of these granules the percentage of colocalization with FMRP ranged from 11% to 27.5%. In neurons cultured from FMRP:YFP overexpression flies we saw that 55.9% of FMRP:YFP (+) granules also contain HPat (1 cell; 59 granules)(Figure 18b). When more (5) cells were analysed the total number of FMRP:YFP granules ranged from 24 to 99. Out of these granules the percentage of colocalization with FMRP ranged from 21% to 55.9%.

3.2.4 HPat co-immunoprecipitates with FMRP

The colocalization and genetic interaction between hpat and dfmr1 suggested that HPat might also interact biochemically with FMRP in neurons. To test this hypothesis, HPat:GFP containing particles were immunoprecipitated from adult Drosophila head extract with antibodies against GFP following pan-neuronal expression using the C155-Gal4 driver. Immunoprecipitates were separated by SDS-PAGE and immunoblotted with antibodies against FMRP. As shown in Figure 19, HPat and dFMRP coimmunoprecipitated under these, and converse, conditions. Control experiments show that neither protein was immunoprecipitated by a normal IgG. Because both FMRP and HPat have been shown to associate with large RNP particles, we speculated that they might be interacting in RNAse-sensitive manner. To test this, we examined the effects of RNAse treatment on formation of HPat-FMRP complexes. Interestingly, the presence of RNAse had no impact on complex formation (Figure 19). Together, these data suggest that HPat and dFMRP are not interacting via RNA and suggest that they may associate either directly or indirectly through protein-protein interactions.
3.2.5 HPat is not required for regulation of synaptic structure by dFmr1

Published evidence suggests that both HPat and FMRP are expressed in the larval nervous system and both HPat and dFMRP are required for the control of synapse growth at the larval NMJ (Figure 10; Zhang et al., 2001, Jin et al., 2004, Pradhan et al., 2012). Based on this and the data presented above (Figures 7, 10,11,13, 17,18,19), we next asked if hpat interacted with dfmr1 in the regulation of synaptic structure. In our hands, we found that dfmr1Δ50M homozygotes exhibited the strongest, albeit weaker than published, effect on NMJ size (Figure 20). In our lab we compared the NMJ phenotype of dfmr1Δ50M and dfmr1Δ113M null fly lines, and we always found a stronger phenotype of dfmr1Δ50M null contrary to published results where dfmr1Δ113M nulls showed the strongest phenotype (Jin et al., 2004, data not shown, Leslie Rozeboom); and this was the reason why we chose dfmr1Δ50M line for all our analysis.

Compared to heterozygous hpatd3/+ controls, hpatd3/+; dfmr1Δ50M /+ transheterozyote larvae exhibited a modest but statistically insignificant overgrowth of synaptic terminals (Figure 20). This was confirmed by counting the number of type 1b synaptic boutons (10.79% increase; p = 0.3783). We did find that type 1b bouton number for dfmr1Δ50M/dfmr1Δ50M homozygous larvae compared to dfmr1Δ50M/+ controls was statistically higher (Figure 20; 19.15% increase; p < 0.05). Together, these data suggest that hpat and dFmr1 do not interact genetically to regulate synaptic growth at the NMJ during larval development.

3.2.6 HPat does not interact with FMRP to regulate the microtubule cytoskeleton

As described in Figure 14, we also examined the effect of HPat and FMRP interaction in the cytoskeletal architecture by staining trans-heterozygous larvae with
anti-Futsch antibodies. Interestingly, we did not see any significant change in Futsch loop numbers in controls versus experimental genotypes (Figure 21). Thus, in support of our NMJ size analysis, hpat and dfmr1 do not appear to interact genetically to control cytoskeletal at the Drosophila NMJ.

3.2.7 The requirement for P body components in FMRP-mediated translational repression

Us and others have found colocalization, biochemical, and genetic interactions between FMRP and HPat (this study) and other P body components (Barbee et al 2006, Jin 2004). Based on these observations, we wanted to know: a) Why does FMRP interact with P body components; and b) What does this interaction have to do with translation regulation in neuron? To directly address this, we sought to identify specific components of P bodies that interact with FMRP and are required for FMRP-mediated repression. As a first step towards addressing these questions, we took advantage of experimental protocols using a firefly luciferase (FLuc) reporter assay in Drosophila S2 cells to screen P body components for their requirement in FMRP mediated repression (Table 2). S2 cells were co-transfected with vectors designed to tether FMRP to a FLuc reporter construct (Figure 22A). The transfection mix contained fly expression vectors for Renilla luciferase (RLuc), a Fluc reporter containing 5 repeats of a BoxB sequence in it’s 3’UTR, and a control empty vector ‘λN-HA’ as a control (Figure 22B; Rehwinkel et al., 2005). The ‘λN-HA is a bacteriophage protein tag that binds with very high affinity to a short hairpin structure called a 5BoxB sequence (Collar and Wickens 2007). We predicted that, if FMRP acts as a translational repressor when
bound to a mRNA target, then tethering \(\lambda N\)-FMRP to the FLuc-5xBoxB reporter should significantly down-regulate FLuc expression. As expected we were able to see a reduction of luciferase levels when the FLuc-5xBoxB reporter was co-transfected with \(\lambda N\)-HA: FMRP to about 40% to 80% when normalized to empty vector controls (Figure 23). **Thus, we were able develop an assay to potentially screen for P body components that are required to help facilitate or mediate FMRP-mediated translational repression in vitro.** We also did a ‘dose response’ experiment to determine the optimum concentration of \(\lambda N\)-HA: FMRP to use in this assay. When repression levels were compared for 1\(\mu\)g, 0.5\(\mu\)g and 0.1\(\mu\)g of \(\lambda N\)-HA: FMRP, we found that 0.5\(\mu\)g \(\lambda N\)-HA: FMRP/well of a 12 well assay plate showed the highest repression (data not shown). Hence this concentration was used for all subsequent experiments.

The Fluc/Rluc values for P body knock down experiments were compared in two ways, first normalized to a transfection control of \(\lambda N\)-HA, Fluc5XboxB, Rluc and second with the control of \(\lambda N\)-HA, Fluc5XboxB, Rluc plus the individual P body dsRNA. Conclusions were based on looking at Fluc/Rluc levels when normalized both ways. Unless the P body component has an effect independent of FMRP we should not see any difference in these two controls. However, we did see a small and insignificant change in Fluc/Rluc values for these two controls which could be just technical variation.

**3.2.8 GW182 is required for FMRP mediated repression**

To identify P components that are required for FMRP mediated repression we knocked down the individual P body components in co-transfected with components of the luciferase reporter assay described above (Figure 23) with dsRNA targeting
individual components of P bodies. We screened all the genes found in Table 2 except for CCR4, PCM, (we received wrong cDNAs from DGRC) and Ge1 (fly homologue does not exist). In summary, out of all the components screened, we consistently found that reducing levels of GW182 by RNAi resulted in the elimination of λN-HA:FMRP-mediated translational repression. In these experiments, the luciferase level were similar to those observed in λN-HA empty vector controls (Figure 24). These data suggest that GW182 is required for the function of FMRP in this reporter assay.

One possibility is that introduction of dsRNA into this system could indirectly affect the ability of λN-HA:FMRP to repress the reporter construct. Bearing this in mind, we also used dsRNA against GFP as an additional negative control RNA (Figure 23) and saw no effect. Thus we concluded that the effect we saw with dsRNA for GW182 is having a specific effect on FMRP-mediated translational repression. The amount of Effectene transfection reagent (Qiagen) used in transfection is proportional to the amount of total nucleic acids used in the experiment according to published protocols. Using high levels of Effectene reagent in transfections has been speculated to be toxic to cells. Therefore, we also did a transfection control for Effectene, using the amount of reagent that would have gone in the experiment if co-transfected with dsRNA. Our results showed that amount of effectene did not affect the luciferase readings (Figure 23).

Interestingly, knocking down other P body components by RNAi including hpat, staufen, me31b, lsm7, dcp2, ago1, ago2, edc3 showed no effect (Figure 25), as we did not see any significant negative effect on FMRP-mediated repression of FLuc reporter expression in the presence of dsRNA that knocked down these components. However to
our surprise, P body components Dcp1 and Tral showed an enhancement (0.19 and 0.1 respectively normalized to control) of repression much more that of FMRP by itself when these components were knocked down (Figure 26). One caveat to this experiment is that western blot of lysates of S2 cells needs to be done to verify the efficiency levels of dsRNA knock down.

3.2.9 GW182:GFP colocalise with FMRP

Because we saw an interaction of FMRP and GW182 in general translation repression assays in S2 cells (Figure 24), we examined the subcellular localization of Drosophila GW182:GFP and endogenous FMRP or tagged FMRP in S2 cells transfected with pAc5.1B-EGFP-DmGW182 (Addgene 22419) and pAc5.1B-3HA-3FLAG-dFmr1 or of pAc5.1B-dFmr1. The GW182:GFP and FMRP signal was detected throughout the cytoplasm and also as some discrete cytoplasmic foci of varying number and size (Figure 27), however the endogenous FMRP signal was weak. These foci seem to colocalize with some endogenous FMRP foci as well as pAc5.1B-dFmr1 and tagged FMRP foci (Figure 27 a,b,c). However for cells transfected with both GW:GFP and FMRP tagged plasmids the signal is too overwhelming to do any quantitation. Staining of endogenous FMRP and GW182 in untransfected cells were hard to analyse too.

3.2.10 GW182:GFP does not Co-IP with FMRP

To further verify the results we saw in figure 27, section 3.2.9, we did a coimmunoprecipitaion assay of S2 cell lysates transfected with GW182:GFP and HA-FLAG-dfmr1, we did not see any coimmunoprecipition of GW182:GFP with HA-FLAG-dfmr1 (Figure 28 lane 2). Thus, although we saw some physical interaction of these two particles in figure 27, they do not seem to associate biochemically.
Table 2. Core P body components analyzed for interaction with FMRP in translation repression assays (modified from Parker et al., 2007; Hillebrand et al., 2007)

<table>
<thead>
<tr>
<th>P body</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPat/Pat1</td>
<td>Decapping activator/repressor</td>
</tr>
<tr>
<td>Tral</td>
<td>Repressor</td>
</tr>
<tr>
<td>Lsms1-7</td>
<td>Sm like protein involved in decapping</td>
</tr>
<tr>
<td>EDC3</td>
<td>Decapping activator</td>
</tr>
<tr>
<td>Me31B</td>
<td>Dead box helicase/ repression decapping activator</td>
</tr>
<tr>
<td>Dcp1</td>
<td>Decapping component</td>
</tr>
<tr>
<td>Dcp2</td>
<td>Catalytic subunit of decapping</td>
</tr>
<tr>
<td>CCR4(^a)</td>
<td>Cytoplasmic deadenylase</td>
</tr>
<tr>
<td>GE1(^b)</td>
<td>Mammalian decapping complex</td>
</tr>
<tr>
<td>Staufen</td>
<td>RNA binding</td>
</tr>
<tr>
<td>AGO2</td>
<td>siRNA silencing</td>
</tr>
<tr>
<td>AGO1</td>
<td>miRNA silencing</td>
</tr>
<tr>
<td>Pcm(^a)/XRN1</td>
<td>5(^{-}) to 3(^{+}) exonuclease</td>
</tr>
<tr>
<td>GW182</td>
<td>RISC component</td>
</tr>
</tbody>
</table>

\(^a\) Assay not done as cDNAs from DGRC was not correct.

\(^b\) Assay not done as fly homologue does not exist.
Figure 3. Drosophila hpat alleles and isoforms

(A) Diagram of the EY10289 insertion and the effect of each hpat allele on viability and synaptic morphology. In hpat[a3] a 3929 bp fragment spanning the central region of the P element was excised. In hpat[d17] a 10189 bp fragment was excised. In both cases, flanking P element sequence remains in the hpat 5’UTR. In contrast, hpat[15p] represents a precise excision of the EY10289 insertion.

(B) Diagram of the 5’ end of the hpat gene. Alternative transcriptional start sites (red arrowheads) can lead to the production of two isoforms of the HPat protein.
Figure 4. Drosophila hpat alleles

(A) Diagram of the hpat gene showing intron and exon boundaries including the insertion sites for two P elements (hpat<sup>EY10289</sup> and hpat<sup>(3) 0442</sup>) in the hpat 5’UTR and the region targeted by the hpat anti-sense in situ construct. hpat<sup>d3</sup> and hpat<sup>d17</sup> are deletions that occurred within hpat<sup>EY10289</sup> P element such that the hpat coding sequence was unaffected, instead, the size of the P element inserted in the hpat 5’ UTR was reduced. hpat<sup>15p</sup> is a precise excision of hpat<sup>EY10289</sup> (Dr. S Barbee).

(B) Diagram of the hpat gene region. Shown are sequences included in the P21M20 rescue transgene and missing from the Df(3R)Exel8165 deficiency line (Dr. S Barbee).
Figure 5. Western blot showing HPat expression levels

(A) Western blot of whole larval extracts from whole w1118, hpatd3 and hpatEY10289 homozygous larvae. HPat antibodies recognize 108 kDa and 102 kDa bands corresponding to two predicted isoforms. Both bands are significantly reduced in hpatd3 mutants and almost eliminated in hpatEY10289 homozygotes. Smaller bands in the w1118 control and hpat mutants are likely the proteolytic fragments of the HPat protein.

(B) Relative expression levels of the 102 and 108 kDa isoforms of HPat in extract from whole hpatd3 and hpatEY10289 homozygous mutant larvae. HPat protein levels were normalized to a loading control (actin) and then to w1118 controls for both isoforms.
Figure 6. Specificity of HPat antibodies

(A) Staining w^{1118} control, hpat^{d3} and hpat^{EY10289} homozygous mutant larval ventral ganglion with HPat antibody. In contrast to western blots of larval extracts, overall HPat levels appear to be equally reduced in hpat^{d3} and hpat^{EY10289} mutants. Punctate cytoplasmic staining in the soma and neuropile of most neurons is significantly reduced. Finally, the pattern of HPat staining in the ventral ganglion is disrupted.

(B) w^{1118} control, hpat^{d3} and hpat^{EY10289} homozygous mutant NMJs stained with rat polyclonal antibodies against HPat (green) and postsynaptic Dlg (in NMJs only; magenta). All larvae from each genotype were dissected and processed collectively in the same dish to maintain consistent fixation and antibody staining conditions. Punctate perinuclear staining in muscle is equally reduced in hpat mutants. There is also a distinct nuclear staining compared to controls. This is consistent with data suggesting that HPat orthologs can have a nuclear function (Marnef et al., 2011). Very similar results were observed with rabbit polyclonal antibodies against HPat (data not shown). Scale bar = 20 µm. All images were collected using the same confocal settings.
Figure 7. HPat is expressed in larval CNS

(A) Whole-mount RNA in situ hybridization of w^{1118} larval brains stained with either anti-sense or sense riboprobes directed against the hpat mRNA.

(B) Larval ventral ganglion stained with rat antibodies against HPat (green) and/or FMRP (red) showing punctate cytoplasmic staining in the soma of most neurons. Note there is a significant amount of overlap between HPat and FMRP in the merged image. Scale bar = 20 µm.

(C) NMJs double stained with antibodies against HPat (green) and postsynaptic Dlg (red). HPat exhibits punctate perinuclear localization in muscle but is not enriched at the NMJ. Scale bar = 20 µm.

(D) Peripheral nerves exiting the ventral ganglion showing localization of both HPat (green) and FMRP (red). The merged images in (B) and (D) yellow spots indicate significant co-localization between HPat- and FMRP-containing punctae. Scale bar = 10 µm.
Figure 8. HPat forms discrete cytoplasmic foci in neurons and peripheral axons projecting from the CNS

(A-C) HPat:GFP in neuron cell bodies within the ventral ganglion stained with antibodies against GFP (green) and FMRP (red). Background fluorescence using anti-GFP antibodies made it impossible to accurately assess co-localization of FMRP in HPat-containing granules.

(D) HPat:GFP (green) localizes to cytoplasmic particles in nerves emerging from the larval ventral ganglion. Scale bar = 5 µm.

(E-F) By eye, when stained with antibodies against GFP (green) and FMRP (red), HPat-containing granules in peripheral nerves do not appear to contain FMRP.
Figure 9. Quantitative real-time PCR analysis of hpat mRNA expression in the Drosophila larval CNS

The level of hpat expression was analyzed by RT-qPCR in triplicate and compared to three ubiquitously expressed reference genes: GAPDH, α-tubulin 84B, and RpL13A. The graph indicates the number of cycles at which amplification of all PCR products reaches the cycle threshold (Ct; threshold calculated by the iCycler software). These data suggest that hpat and all target reference genes are very abundant in the larval CNS.
Figure 10. HPat negatively regulates synaptic growth during larval development at muscle 6/7

(A) Representative images of muscle 6/7 from abdominal segment A3 labeled with antibodies against postsynaptic Dlg (only). Arrows in the hpat^{d3}/hpat^{d3} panel indicate a cluster of terminal synaptic boutons often observed in this genotype.

(B) Quantification of the number of type 1b synaptic boutons at muscle 6/7. Unless otherwise indicated, stars denote statistical significance compared to Canton-S/+ controls. (* p < 0.05; ** p< 0.01; *** p < 0.001; **** p < 0.0001; one-way ANOVA; Tukey’s post-hoc test).
Figure 10. HPat negatively regulates synaptic growth during larval development at muscle 6/7

(C) Quantification of the number of type 1s and synaptic boutons at muscle 6/7. Unless otherwise indicated, stars denote statistical significance compared to Canton-S/+ controls. (* p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001; Kruskal-Wallis; Dunn’s post-hoc test).
Figure 10. HPat negatively regulates synaptic growth during larval development muscle 6/7

(D) Quantification of the number of total synaptic boutons at muscle 6/7. Unless otherwise indicated, stars denote statistical significance compared to Canton-S/+ controls. (* p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001; Kruskal-Wallis; Dunn’s post-hoc test).
Figure 11. HPat negatively regulates synaptic growth during larval development muscle 4

(A) Representative images of muscle 4 NMJs from abdominal segment A3 labeled with antibodies against postsynaptic Dlg (only).
Figure 11. HPat negatively regulates synaptic growth during larval development muscle 4

(B) Quantification of the number of type 1b synaptic boutons at muscle 4 for each indicated genotype. Unless otherwise indicated, stars denote statistical significance compared to Canton-S/+ controls. (* p < 0.05; ** p< 0.01; *** p < 0.001; **** p < 0.0001; one-way ANOVA; Tukey’s post-hoc test).
Figure 11. HPat negatively regulates synaptic growth during larval development at muscle 4

(C) Quantification of the number of total bouton branch tips at muscle 4 for each indicated genotype. Unless otherwise indicated, stars denote statistical significance compared to Canton-S/+ controls.
Figure 12. Presynaptic HPat over-expression has a moderate negative effect on synaptic growth during larval development

(A) Representative images of muscle 6/7 NMJs from abdominal segment A3 labeled with antibodies against postsynaptic Dlg (only).

(B) Quantification of type 1b boutons at muscle 6/7 clefts. Stars indicate statistical significance compared to controls (* p < 0.05; Student’s t-test). Note that there is a modest decrease in synapse size when UAS-hpat is driven by presynaptic (C380-Gal4 and D42-Gal4) but not postsynaptic (24B-Gal4) drivers.
Figure 12. Postsynaptic HPat over-expression has a strong positive effect on 1s bouton numbers during larval development

Quantification of the number of (C) total and (D) type 1s synaptic boutons at muscle 6/7 following pre- and postsynaptic overexpression of hpat. Stars indicate statistical significance compared to controls (* p < 0.05; Student’s t-test). Note that there is no significant effect on total bouton numbers following postsynaptic HPat overexpression.
Figure 13. \( hpat^{hpn} \) over-expression does not show a strong effect on synaptic growth during larval development

Quantification of the number of (A) type 1b, (B) 1s and (C) total synaptic boutons at muscle 6/7 following pre- and postsynaptic overexpression of \( hpat^{hpn} \) RNAi lines, Vienna stocks 27378 and 100872.
A

HRP   Futsch   Merge

B

Terminal
Junction
Branch

C

# Fusion Loops/apses

- Controls: 20
- mhrp/HRP: 24
- mhrp/HRP; rescue: 20
- mhrp/HRP, rescue / Futsch: 22

p = 0.141
Figure 14. HPat regulates structure of the presynaptic microtubule cytoskeleton

(A) Representative images of muscle 6/7 NMJs from abdominal segment A3 labeled with antibodies against presynaptic HRP (red) and Futsch (green) to show microtubule loops. Arrows indicate examples of Futsch loops. The inset in the \textit{hpat}^{d3}/\textit{hpat}^{d3} panel is a zoomed-in region showing an abnormal cluster of Futsch-positive loops.

(B) Quantification of the number of Futsch-positive loops per NMJ at muscle 6/7. Unless otherwise indicated, stars indicate statistical significance compared to control NMJs (*** \( p < 0.001 \); one-way ANOVA; Tukey’s post-hoc test).

(D-E) Quantitation of Futsch positive loops of indicated genotypes at muscle 6/7. Genotypes here are as follows: “C” (CantonS/w1118; \( n = 20 \)) control; “d3” (\textit{hpat}^{d3}/\textit{hpat}^{d3}; \( n = 24 \)); “EY” (\textit{hpat}^{EY10289}/\textit{hpat}^{EY10289}; \( n = 20 \)); and “R” (\textit{hpat}^{d3}/\textit{hpat}^{d3}; \textit{P21M20/P21M20}; \( n = 24 \)) rescue.
(D) Quantification of the location of Futsch-positive loops in *hpat* mutants in each of the regions shown in (C). The most significant increase is observed at the point where two synaptic arbors meet (* p < 0.05; *** p < 0.001; Kruskal-Wallis; Dunn’s post hoc test).

(E) The relative distribution of Futsch-positive loops in each indicated synaptic region. Regions quantified are the main synaptic arbors (branch or “B”), terminal synaptic boutons (terminal or “T”), and junctions where two or more branches meet (“J”).
Figure 15. Neuronal HPat negatively regulates activity-dependent synaptic growth

(A-B) Representative images of muscle 6/7 NMJs from abdominal segment A3 labeled with antibodies against presynaptic HRP (red) and postsynaptic Dlg (green). HPat expression was driven either pre- or postsynaptically using the C380-Gal4 and 24B-Gal4 drivers respectively. Arrows indicate the presence of immature ghost boutons (HRP+ Dlg−). Middle panels are zoomed-in images of regions indicated by arrows in 0x and 5x K+ treatment groups. Stimulated [5x K+] larvae of each genotype were taken through a spaced high-K+ paradigm. Unstimulated [0x K+] larvae were taken through a low K+ pseudo-stimulation paradigm (Katherine Nesler).

(C) Quantification of the number of ghost boutons per NMJ at muscle 6/7 clefts. Stars indicate statistical significance between the indicated groups (*** p < 0.001; **** p < 0.0001; Kruskal-Wallis; Dunn’s post-hoc test). Note the 3-4 fold increase in the number of ghost boutons is observed at control NMJs following high-K+ depolarization. This increase is almost completely suppressed by both pre and postsynaptic overexpression of HPat (down to a 1.4 and 1.5-fold increase respectively).
A

General pathways  miRNA pathways

HPat  P body formation

Deadenylation (CCR4/Not1)  Decapping (Dcp1/Dcp2)  or

Repression and Storage  5’ to 3’ Decay (Xrn1)

B

Bouton Counts (% control)

Control  d3+  Twnl8115Y+  d3Twlnl8115Y+  d3Twlnl222209Y+  Dcp2l222209Y+  Dcp2l1766P  d3Dcp2l1766P d3Dcp2l322119  d3Dcp2l1766P  d3Dcp2l1766P  d3Dcp2l322119  AGO1+  d3AGO1

*  ***  ***
Figure 16. HPat interacts with components of the deadenylase complex and miRNA pathway to control synaptic growth during larval development

(A) Diagram outlining major events associated with mRNA storage and decay in P bodies. mRNAs can be targeted to P bodies via general- and miRNA-mediated pathways. HPat may interact with these pathways to direct mRNAs for deadenylation (by CCR4/Not1) followed by translational repression and storage; or deadenylation followed by decapping (by Dcp1/Dcp2) and then 5’ to 3’ decay (by Xrn1).
B) Quantification of the number of type 1b boutons at muscle 6/7 clefts in genetic interaction experiments. Trans-heterozygous larvae were generated by crossing the \( hpa^{d3} \) allele to indicated alleles of each genes (e.g. \( hpa^{d3}/+; \ dcp^{BG01766}/+ \) or \( hpa^{d3}/+; \ twin^{GS12209}/+ \)). Stars indicate statistical significance compared to the control (* \( p < 0.05 \); ** \( p < 0.01 \); *** \( p < 0.001 \)) one-way ANOVA; Tukey’s post-hoc test). There is a significant genetic interaction between HPat and both CCR4 and Ago1 in the control of synapse growth during development. In contrast, there is no significant genetic interaction between \( dcp2 \) and HPat.

(C) Quantification of the number of (A) type 1s boutons at muscle 6/7 clefts in genetic interaction experiments. Stars indicate statistical significance compared to the control (* \( p < 0.05 \); Kruskal Wallis; Dunn’s post-hoc test).

(D) Quantification of the number of total boutons at muscle 6/7 clefts in genetic interaction experiments. Stars indicate statistical significance compared to the control (** p < 0.001; Kruskal Wallis; Dunn’s post-hoc test).
Figure 17. HPat is a dominant modifier of dFmr1 function in the Drosophila eye

The rough-eye phenotype is most obvious along the posterior margin of the eye (oriented to the right). Upper panels: SEMs of adult compound eyes at 150x magnification. Lower panels: SEMs of areas indicated by a box (above) at 500X magnification.
A. Neuronal cultures from TWGHPat lines crossed to neuronal driver C155, stained with GFP and mFMRP antibodies. 20% of HPat:GFP(+) granules also contain FMRP (1 cell; 173 granules).

B. Neuronal cultures from YFP;FMRP lines crossed to neuronal driver C155, stained with GFP and HPat antibodies. 55.9% of FMRP:YFP (+) granules also contain HPat (1 cell; 59 granules).
Figure 19. Western blot of HPat:GFP and FMRP coimmunoprecipitation

Western blot of each indicated coimmunoprecipitate probed with either mouse anti-dFMRP or rabbit anti-GFP antibodies. Note that incubation of coimmunoprecipitates with RNase has no effect on the interaction between HPat and dFMRP.
Figure 20. HPat does not regulate dFmr1 function in synaptic growth and structure
A graph showing quantification of the total number of type 1b boutons at muscle 6/7 clefts in hpat^{d3}+/+, dFmr1^{d50M}+/+, dFmr1^{d50M}/dFmr1^{d50M}, and dFmr1^{d50M}/hpat^{d3} larvae. The number of boutons analyzed for each genotype is indicated for each column. The differences between bouton numbers in hpat^{d3}/+ and dFmr1^{d50M}/hpat^{d3} are not statistically significant.
Figure 21. Quantification of the number of futsch-positive loops per NMJ at muscle 6/7 clefts of indicated genotypes
Figure 22. Tethering assay in S2 cells to look at the role of translation repression by FMRP

A. Diagram to show FMRP tethered lambdaN-HA tag that bind to 5XBoxB of Fluc reporter.

B. Diagram to show transfection plan of S2 cells. Transfection mix contained vectors for Renilla luciferase (L. Rozeboom), Plasmids pAc5.1-lambdaN-HA (ID 21302) or pAc5.1-lambdaN-HA:FMRP, pAc5.1-Fluc-Stop-5BoxB (ID21301) from Addgene. Full FMRP sequence was cloned in frame at C terminus of HA tag. dsRNA against P bodies were also cotransfected.
Figure 23. FMRP repress translation in S2 cells luciferase assay

S2 cells were cotransfected with a mixture of plasmids expressing Renilla luciferase, Fluc reporter with a 5Box B sequence and a control empty vector ‘lambdaN-HA’ as a control or a ‘lambdaNHA-FMRP; vector. FMRP repressed Fluc reporter mRNA to 0.3 normalised to its empty vector control. An irrelevant dsRNA like GFP did not show any effect on FMRP. We also did a transfection control for effectene, using the amount of reagent that would have gone in the experiment if co-transfected with dsRNA. Shown are the % of Fluc/Rluc levels normalized to individual controls.

Control = λN-HA, Fluc5XboxB, Rluc
FMRP = λN-HA:FMRP, Fluc5XboxB, Rluc
Control+dsGFP = λN-HA, Fluc5XboxB, Rluc, dsGFP RNA
FMRP+dsGFP = λN-HA:FMRP, Fluc5XboxB, Rluc, dsGFP RNA
Control+Effectene = λN-HA, Fluc5XboxB, Rluc, high dose effectene
FMRP+Effectene = λN-HA:FMRP, Fluc5XboxB, Rluc, high dose effectene
Figure 24. P body component GW182 is required for FMRP mediated repression in translation assays

S2 cells were treated with the indicated dsRNAs of GW182 and were cotransfected with a mixture of plasmids expressing Renilla luciferase, Fluc reporter with a 5Box B sequence and a control empty vector ‘lambdaN-HA’ as a control or a lambdaN-HA-FMRP vector. GW182 de-repressed the FMRP mediated repression by 0.2 normalised to its control.

Control = λN-HA, Fluc5XboxB, Rluc
Control. FMRP = λN-HA:FMRP, Fluc5XboxB, Rluc
Control+ dsGW182 = λN-HA, Fluc5XboxB, Rluc, dsGW182 RNA
FMRP + dsGW182 = λN-HA:FMRP, Fluc5XboxB, Rluc, dsGW182 RNA
Figure 25. Some P body components are not required for FMRP mediated repression in S2 cells luciferase assay

S2 cells were treated with the indicated dsRNAs and were cotransfected with a mixture of plasmids expressing Renilla luciferase, Fluc reporter with a 5Box B sequence and a control empty vector ‘lambdaN-HA’ as a control or a lambdaN-HA:FMRP vector. P components Staufen, HPat, Ago2, Ago1, lsm7, EDC3, Me31B and Dcp2 did not show de-repression when the cells were treated with dsRNA against these P bodies.
Control+dsPbody = λN-HA, Fluc5XboxB, Rluc, dsRNA
FMRP+dsPbody = λN-HA:FMRP, Fluc 5XboxB, Rluc, dsRNA
Figure 26. Knock down of P body components dcp1 and Tral enhance FMRP mediated repression in S2 cells luciferase assay

S2 cells were treated with the indicated dsRNAs and were cotransfected with a mixture of plasmids expressing Renilla luciferase, Fluc reporter with a 5Box B sequence and a control empty vector ‘lambdaN-HA’ as a control or a lambdaN-HA-FMRP vector. P components Tral and Dcp1 show enhanced repression when the cells were treated with dsRNA against these P bodies.

Control = λN-HA, Fluc5XboxB, Rluc
FMRP = λN-HA:FMRP, Fluc5XboxB, Rluc
Control+dsDep1 or dsTral = λN-HA, Fluc5XboxB, Rluc, dsDcp1 or Tral RNA
FMRP+ dsDcp1 or dsTral = λN-HA:FMRP, Fluc5XboxB, Rluc, dsDcp1 or Tral RNA
Figure 27. GW:GFP and FMRP colocalise in S2 cells
Confocal micrograph of S2 cells transfected with (A,B,C) pAc5.1B-EGFP-DmGW182 (Addgene 22419), (B) pAc5.1B-dFmr1 (Breanna Symmes) or (C) pAc5.1B-3HA-3FLAG-dFmr1 (Breanna Symmes) and stained with anti-GFP antibodies (Roche) and FMRP antibody (abcam) after 72h. There are some colocalization of GW:GFP (green) with FMRP (Red) particles (merged yellow). (D) staining of endogenous FMRP and GW182.
Figure 28. Western blot of GW182:GFP and FMRP coimmunoprecipitation
Western blot of coimmunoprecipitate probed with either Rat anti-HA (Roche) or Rb anti-GFP (Torry Pines) antibodies. Western blot with guinea pig anti-GW182 was also done and did not show any coimmunoprecipitation.
Chapter Four: Discussion

4.1 Functional role of HPat

Translation regulation of synaptic mRNAs locally at the neuronal synapses play important role in the control of long-term synaptic plasticity (Jung et al., 2012; Kindler and Kreienkamp, 2012). Evidences suggests that this process is mediated by factors that are associated with a variety of neuronal RNPs, including P bodies (Zeilelhofer et al., 2008b) the functional roles of which is less known in the control of neural physiology (Barbee et al., 2006; Hillebrand et al., 2010). In this study, we provide novel data indicating that HPat/Pat1, a core component of P bodies, is required to control synaptic terminal growth at the Drosophila NMJ during development and following acute spaced synaptic activity (Figures 10,11,12, 15). These changes in the synaptic growth at the developing larval NMJ in hpat mutants correlates with reorganization of the microtubule cytoskeleton in axon terminals (Figure 14). We have identified that, hpat interacts genetically with the deadenylase, but not the decapping, pathway to control bouton formation (Figure 16). Thus these data suggest that HPat may target some key synaptic mRNAs for deadenylation and translational repression, presumably in P bodies, and not for degradation. Therefore, we can speculate that neuronal P bodies sequester and transport mRNAs from the soma to distant sites of translational activation, either in axon terminals or dendritic spines (Barbee et al., 2006; Cougot et al., 2008; Zeilelhofer et al., 2008b).
4.1.1 Functions for HPat

Two major functions for HPat orthologs have been identified in cellular process, the assembly of P bodies; and the coupling of deadenylation to decapping (Marnef and Standart, 2010). In eukaryotes, two paralogs of HPat (Pat1) have been identified depending on cell type and RNP composition. Vertebrates have two Pat1 proteins, Pat1a and Pat1b. In somatic cells, vertebrate Pat1b interacts strongly with Rck (vertebrate Me31B/Dhh1p)-positive P bodies. Pat1b is required for P body assembly, and provides a physical link between deadenylation and decapping. In oocytes, Pat1a interacts weakly with Rck and instead associates with deadenyalted mRNAs in cytoplasmic polyadenylation element binding protein (CPEB)-containing translational repression complexes (Marnef et al., 2010; Ozgur et al., 2010). In Caenorhabditis elegans, a single HPat ortholog (PATR-1) exhibits distinct tissue-specific functions. In somatic cells, PATR-1 localizes to particles containing CGH-1 (worm Me31B/Dhh1p) that are involved in mRNA decapping and degradation (Boag et al., 2008). These RNPs are thought to be analogous to P bodies that also contain DCAP-2 (Dcp2), ALG-1 (Argonaute), and AIN-1 (GW182; Ding et al., 2005), raising the possibility that they are involved in the miRNA pathway. Unlike in vertebrates, it is currently unclear if worm PATR-1 can also localize to distinct RNPs in ovaries and oocytes that are specifically involved in translational repression.

The Drosophila HPat protein has a well-defined role in the regulation of mRNA degradation. In S2 cells, epitope-tagged HPat can co-immunoprecipitate with the decapping enzyme (Dcp2), the decapping activators (Me31B, Dcp1, Edc3, Edc4 and
Lsm1-7), components of the CCR4-NOT deadenylase complex, and the exoribonuclease (Xrn1; Braun et al., 2010, Haas et al., 2010). When HPat is artificially tethered to a reporter mRNA in vitro, its binding is sufficient to trigger deadenylation followed by decapping (Haas et al., 2010). And co-depletion of both Drosophila HPat and Me31B (fly Dhh1) inhibits decapping caused by either miRNAs or tethering of GW182 to reporter mRNAs (Eulalio et al., 2007b). Therefore, Drosophila HPat appears to have a defined role in decapping and 5’ to 3’ mRNA decay.

However in our study we did not find hpat interact genetically with dcp2 or dcp1 to control synaptic terminal growth (Figure 16). So, at least at the NMJ, 5’ to 3’ decay pathway is not required for axon terminal growth. However HPat is involved in the regulation of deadenylation, which is important for synaptic terminal growth during development. Our results show that twin/CCR4 mutants (both hypomorphic alleles used in this study) by itself and in hpat mutant background cause synaptic overgrowth at the NMJ (Figure 16). In addition, deadenylase complex twin/CCR4 and NOT1 have been shown to control dendrite morphogenesis in Drosophila sensory neurons, and siRNA knockdowns of deadenlyase complex significantly increased dendritic arborization and dendrite branch length respectively (Parrish et al., 2006). In vitro data in S2 cells experiments have shown that depleting enhancers of decapping like HPat, inhibits only decapping but not deadenylation (Eulalio et al., 2007b, Haas et al., 2010). It is possible that HPat is contributing to the deadenylation of specific neuronal mRNAs involved in the control of synaptic terminal growth. This HPat-mediated deadenylation immediately targets these mRNAs for decapping and decay. This is consistent with a model for Pat1 function in yeast, where Pat1 first inhibits translation and then, in an independent step,
acts as a scaffold to recruit components of the decapping complex to repressed mRNAs (Nissan et al., 2010). Furthermore, under certain cellular conditions, repressed mRNAs that are stored within P bodies have been shown to be capable of re-entering the translating pool (Brengues et al., 2005). It is unknown if specific HPat containing granules involved in the repression of mRNAs associated with synaptic terminal growth also contain proteins required for mRNA decay.

4.1.2 Targeting of synaptic mRNAs to P bodies by the miRNA pathway

The role of Ago1 in the control of synaptic terminal growth at the Drosophila NMJ has already been established (Jin et al., 2004). Other studies have demonstrated that the miRNA pathway is involved in the control of synaptic development and plasticity (Siegel et al., 2011). Me31B/Dhh1p/Rck, a core component of fly P body, is required for both miRNA mediated repression and the control of dendrite morphogenesis in Drosophila sensory neurons (Barbee et al., 2006; Hillebrand et al., 2010). P bodies in cultured hippocampal neurons contain the GW182 protein, miRNAs, and miRNA-repressed mRNAs (Cougot et al., 2008; Cougot et al., 2012). GW182 has been shown to be an important functional component of P bodies and the miRISC. Inhibition of GW182 expression leads to the disruption of P body assembly and miRNA-mediated repression (Ding et al., 2005, Jakymiw et al., 2007, Liu et al., 2005b). GW182 also interacts directly with the Ago proteins to facilitate binding of miRNAs to target mRNAs in order to direct their deadenylation and translational silencing or their deadenylation followed by exonucleolytic degradation (Eulalio et al., 2007b). HPat has also been shown to directly co-immunoprecipitate with GW182 by interacting with its C-terminal silencing domain (Ja¨ger and Dorner, 2010). Genetic interaction linking HPat to the miRISC, indicates that
the miRNA pathway is targeting key neuronal mRNAs involved in the control of synaptic terminal growth for HPat mediated deadenylation and repression in P bodies (Figure 16). The weaker phenotype observed in $hpa^d_3+/;ago^{1(2)k00208+/}$ trans-heterozygotes compared to $hpa^d_3$ homozygotes (Figure 10, 16; 25% and 85% increase in bouton numbers, respectively) suggests that this interaction is incomplete. Genetic studies showed that HPat protein is required for the control of only 15% of all mRNAs targeted for repression or decay by Ago1 (Eulalio et al., 2007b). Thus, this and our evidence on partial interaction of HPat with the miRNA pathway suggest that may be some of the key mRNAs required for synaptic terminal growth are being regulated via the general pathway involving HPat.

4.1.3 Implications for translational regulation by P bodies

4.1.3.1 P bodies are sites of RNPs as factories for mRNA decay or repression

The data presented in this study provide support for two models of P body function in neurons. First, P bodies may assemble in both pre- and postsynaptic cells where they are required to deadenylate and package specific translationally repressed mRNAs destined for the synapse. Some of these mRNAs may have essential functions in the control of synaptic terminal growth. This model is supported by the following observations, that HPat localizes to particles in the both muscle and neurons in the larval ventral ganglion (Figures. 7, 8) and can form HPat-containing granules in peripheral nerves projecting towards the NMJ but not enriched at the NMJ (Figure 7). This suggests that these particles are dynamic, dissociating completely (or into sub-resolution P body monomers) prior to reaching the synapse, perhaps in response to synaptic activity at the NMJ (Zeitelhofer et al., 2008a). HPat overexpression may drive mRNAs into P bodies.
where they are sequestered from the translating pool resulting in an inhibition of synaptic terminal growth (Figures 12, 15). Our data also indicate that activity-dependent growth is more sensitive to HPat overexpression than synaptogenesis during larval development (Figures 15). This may be because activity dependent processes require immediate new protein synthesis. HPat overexpression may prevent key mRNAs from rapidly entering the translating pool. In contrast, synaptic development is significantly slower process that allows for alternative mechanisms to deliver key mRNAs and/or proteins required for synaptogenesis. In hpat mutants, some key mRNAs may remain in the translating pool throughout development where they can be inappropriately regulated and translated thus resulting in synaptic hyperplasia (Figures 10, 11).

4.2 HPat interaction with FMRP

This study and published literature show that Drosophila FMRP interacts biochemically (Me31B, Tral, Ago1, HPat), physically (Me31B, Tral, Dcp1, Pcm) and genetically (Me31B, Ago1, Tral, HPat) with some P body components (Barbee et al., 2006, Jin et al., 2004). A genetic interaction functionally links hpat to dFmr1 in the eye but not at the larval NMJ (Figure 17). The extent of HPat interaction with FMRP was generally weak in the eye assay and colocalization studies compared to already published P components (Barbee et al 2006, Figure 17, 18). In vivo staining of Drosophila larval ventral ganglia with HPat and FMRP antibodies showed moderate colocalization (Figure 7). Before the HPat antibody was available, this staining was also done in transgenic HPat:GFP lines (Figure 8), however this colocalisation was very hard to analyse as endogenous FMRP always gave weak staining and when HPat:GFP was overexpressed, it would mask the endogenous. FMRP stain.
Neuronal cultures from transgenic HPat:GFP and FMRP:YFP lines also showed some colocalisation but quantitation of punctate particles of HPat and FMRP were hard to analyse as the transgenes gave overwhelming signal. However at the moment we do have HPat antibody and this experiment can be repeated to look at endogenous HPat and FMRP granules for colocalisation. Another caveat to this experiment is that we have never been able to grow neurons to show extensive branching for analysis of particles.

Although the genetic interaction between HPat and FMRP in the eye was weak, we wanted to check if this interaction gave a strong phenotype at the larval NMJ. We did not see any interaction of HPat and FMRP at the NMJ (hpat^{d3}/dfmr1 showed weak phenotype of 10.75% increase in 1b bouton numbers compared to hpat^{d3}/+) (Figure 20). We looked at the genetic interaction of HPat and FMRP in another way as described previously in figure 14B section 3.1.6; looking at futsch loops, which was also insignificant (Figure 21). Although the results with HPat was not as expected we were curious to see why there was strong interaction of FMRP with other P body components and if this interaction has anything to do with the repression function of FMRP. One caveat to these experiments is that we have never been able to replicate the published dfmr1 null phenotype discussed in section 3.2.5.

It is well established that FMRP is a translation repressor in RNA transport granules in neurons (Laggerbauer et al., 2001, Li et al., 2001). However, FMRP has also been shown to be a modulator of neuronal mRNA stability. In mice, FMRP associates with the NFX2 protein to destabilize the Nxf1 mRNA (Zhang et al., 2007), via unknown mechanism. Evidences show that FMRP may act as mRNA stabilizing factor, as the general levels of some neuronal mRNAs (such as specific GABA receptor subunits) are
increased in the cortex of FMR1 KO mice and in *Drosophila dFmr1* null mutants (D'Hulst et al., 2006); and two known FMRP target mRNAs, PSD-95 and the Myelin basic protein (MBP), exhibit decreased stability in the absence of FMRP (Zalfa et al., 2007). Together, these observations illustrated the need for more work in order to understand precisely how FMRP controls target mRNA function (e.g. stability, decay, and/or repression) and how these diverse processes can impact synaptic structure and function. The role of FMRP in synaptic plasticity is already established (Zhang et al., 2001, Lee et al., 2003). It has been proposed that the diverse roles for FMRP in enhancing mRNA stability, promoting mRNA regulation are dependent upon the molecular composition of distinct FMRP-containing RNP particles (De Rubeis and Bagni, 2010). This is supported by the above-described discovery of interaction of P body components with FMRP (Pradhan et al., 2012, Barbee et al., 2006, Jin et al., 2004).

It seems that there is substantial overlap of composition of FMRP containing granules and P bodies.

### 4.3 Are P bodies sites for FMRP-mediated repression?

We have observed genetic, biochemical and physical interaction of various P body components and FMRP in my study and published work so we wanted to check if these P bodies are actually required for FMRP mediated repression. The role of FMRP in general translation repression is already established in other experimental systems and we were able to show it in S2 cells luciferase assay by tethering FMRP to a reporter, which is a novel assay to study FMRP mediated translation repression (Figure 23). Knocking down GW182 by RNAi prevented the ability of tethered FMRP to repress a FLuc reporter compared to controls. This data suggest that GW182 is required for the repression caused
by FMRP. Other P body components like Dcp1, and Tral showed a mild increment in repression when these components were knocked down (Figure 26). These experiments need to be repeated to be conclusive as the difference in ratio is very small. Dcp1 has a role in decay pathway and Tral is important for dorso-ventral patterning in *Drosophila* embryos. If these results are true it will contradict the usual function of these P body components in the degradation pathway. The effect seen when knocking down GW182 were consistent upon repeated experiment with 1 or 2 µg/ well, however a real time PCR or western blots of cell lysates need to done to check if dsRNA treatment knocked down these proteins/genes or not. A better and increased phenotype might be seen if the assay were repeated with a higher concentration of the dsRNA or doing a 10 days protocol with two times dsRNA treatments for figure 26 (Eulalio et al., 2009).

One caveat to these experiments in S2 cells is that the screening of P body components interacting with FMRP have been done only once except for GW182 and HPat; others need to be repeated with the same protocol or with modified protocols of adding more dsRNA or increasing treatment times to confirm the results (Eulalio et al 2009).

4.4 GW182 and FMRP interaction

The de-repression effect seen (0.9 normalised to control) when GW182 was knocked down is very interesting, as nobody has shown a direct interaction of P component GW182 and FMRP. GW182 family proteins are essential for microRNA-mediated gene silencing. They are recruited to miRNA targets through direct interactions with Argonaute proteins and promote target silencing by repressing translation and enhancing mRNA turnover. Although the precise mechanism of action of GW182 proteins is not
fully understood, these proteins have been shown to interact with the cytoplasmic poly
(A)-binding protein (PABP) and with the deadenylase complexes. These findings suggest
that GW182 proteins function as scaffold proteins for the assembly of the multi-protein
complex that silences miRNA targets (Braun et al., 2013). There is one report where
FMRP was found to colocalize but not Co-IP in GW bodies in mammals (Moser et al.,
2007). This is similar to our results (Figures 27, 28) where we were able to see moderate
colocalisation of GW182:GFP and FMRP but no immunoprecipitation in S2 cells. This
suggests that multiple mRNA binding proteins may complex for mRNA regulation within
the heterogeneous and highly dynamic GWB (GW bodies) microenvironment. Protein
complexes containing the GW182 protein have been shown to include Ago2, Dicer,
hDcp, Lsm4 and Ge1 (Moser et al 2007), some of which are defined P body components.
So, knocking down other P body components which may be within the GW complex;
along with FMRP might show a robust de-repression in our S2 cells assay. The Fluc
reporter we used consists of multiple miRNA binding sites outside of 5XboxB site
(Nathan Boin) so it is possible that the role of GW182 in FMRP mediated repression is
dependent on the miRNA pathway and it is not acting by itself.

There are some caveats to the colocalisation assays we did. It was very hard to
quantify the colocalising particles of GW:GFP and FMRP as the signal of GW:GFP was
too strong and masked the endogenous FMRP signal. And also that in experiments where
tagged or untagged FMRP plasmids were used, both the signals of FMRP and GW182
were overwhelming so could not see many individual puncta. These experiments need to
be repeated for endogenous GW182 and FMRP as we have both the antibodies available
in the lab now, however the signal of both the endogenous particle may be low. There
seemed to be comparatively low percentage of transfected S2 cells after 3-5 days of transfection. It could be either that the transfection efficiency is low or the cells may have died.

4.5 Future directions

In this thesis I have tried to address the specific second questions about which P body components interact with FMRP and if they are required for FMRP mediated translation regulation; by colocalization, co-IP, genetic interaction at the eye and NMJ, and translation regulation by luciferase assay in S2 cell system. In our screen, of all the P body proteins studied in the luciferase assay, only GW182 seem to affect the function of FMRP. One interesting aspect will be to investigate the physiological role that this interaction has in translation regulation in neurons. Future experiments to be done to dwell on this would be:

1. Look at the dfmr1: gw182 genetic interactions at the NMJ, eye and sensory neurons.

2. A negative control experiment to verify GW182:GFP and FMRP colocalisation. This is being done by transfecting cells with GFP vector and staining for FMRP and GFP; these two particles should not colocalise. And as GW182 antibody has recently been available in the lab look at the endogenous particles of FMRP and GW182 colocalisation in vivo or /and in neuronal cultures and/or in S2 cells.

3. Verify dsRNA knock down in S2 cells by looking at reduction or elimination of individual P body components specially GW182. This can be done by western blot or real time PCR of cell lysates saved from luciferase assay.
4. Repeat neuron culture in figure 19 to look at endogenous HPat and FMRP particles.

5. One important investigation could be searching for potential targets of HPat important in neuronal physiology. This can be done using RNA immunoprecipitation (RIP) to pull down any RNA that can immunoprecipitate with HPat and analyse RNA profiles by microarray, sequencing etc. In the mammalian brain, CamKII (calcium/calmodulin-dependent protein kinase II) is synthesized at synapses and is required for neuroplasticity and memory formation (Kelleher et al., 2004). In *Drosophila* the RISC machinery is involved in synaptic plasticity, LTM formation, and regulation of synaptic translation of CamKII (Ashraf et al., 2006). Based on this we can predict that CamKII may be a target of HPat as HPat is involved in miRNA pathway. Other potential targets involved in neural physiology could be Dlar (*Drosophila* liprin a) a protein tyrosine phosphatase implicated in synapse development (Kaufmann, et al. 2002) or proteins of the Wg pathway (Ataman et al., 2008).
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Appendix

List of abbreviations:

ADF = actin depolymerising factor
AGO1 = Argonaute 1
ALS = Amyotrophic lateral sclerosis
AMPA = $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ARC = activity-regulated cytoskeleton-associated protein
BDNF = Brain derived neurotrophic factor
CAMK11 = $Ca^{2+}$/calmodulin-dependent protein kinases II
CNS = central nervous system
CPEB = cytoplasmic polyadenylation element binding protein
Dcp1/2 = decapping complex 1/2
DGRC = Drosophila genomic resource center
DIG = Digoxigenin
DLar = Drosophila liprin $\alpha$
Dlg = Disc large
DTT = dithiotheritol
eEF2 = eukaryotic elongation factor 2
ENA/VASP = Enabled/vasodilator-stimulated phosphoprotein.
FTLD-4 = Frontotemporal lobar degeneration 4
dFMR1 = Drosophila FMR1
FMRP = Fragile X mental retardation protein
FMR1 = fragile X mental retardation 1, the gene responsible for FXS
FRAP = fluorescent recovery after photo bleaching
FUS = fused in sarcoma
GW = Gawky
GW bodies
HL3 = hemolymph like 3
HRP = horse radish peroxoixidase
Co-IP = Co-immunoprecipitation
IRES = Internal ribosome entry site
KH = K homology
LiCl = Lithium Chloride
LIMK = LIM domain kinase
LTD = long term depression
LTM = long term memory
LTP = long term potentiation
MAP1B = microtubule associated protein 1B, the mammalian homolog of futsch
miRISC = miRNA-containing RISC
miRNA = microRNAs
mTOR = mammalian target of rapamycin
NBT/BCIP = nitro blue tetrazolium chloride/ 5-Bromo-4-chloro-3-indolyl phosphate
NG = neuronal Granule
NGF = nerve growth factor
NMJ = Neuromuscular Junction
NT3 = neurotrophin3
P body = RNA processing body
PABP = Poly A Binding protein
PVDF = polyvinylidene difluoride
qRT-PCR = quantitative real-time PCR
RBP = RNA binding protein
RIP = RNA immunoprecipitation
RISC = RNA-induced silencing complex
RNAi = RNA interference
RNP = Ribonucleoprotein
RT = room temperature
SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM = Scanning electron microscopy
SMN = survival of motor neuron
TBE = Tris/Borate/EDTA
TBS = Tris-buffred saline
TDP-43 = TAR DNA-binding protein 43
Tral = Trailerhitch
UTR = untranslated region
WAVE = WASP family Verprolin-homologous protein
ZBP = Zip code binding protein