Role of *Drosophila* Orb2 (CPEB) in Synaptic Protein Synthesis

By

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Role of *Drosophila* Orb2 (CPEB) in Synaptic Protein Synthesis

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Abstract

How a transient experience creates a persistent memory remains a fundamental unresolved issue in neuroscience. One of the molecular processes that is believed to be critical for long- lasting memory is synthesis of new protein at the synapse. However, how synaptic protein synthesis is regulated and how these new proteins confers persistence of memory is largely unknown. Previous studies in *Drosophila* and *Aplysia* have implicated that a family of mRNA binding proteins known as Cytoplasmic Polyadenylation Element (CPE) binding proteins is essential for persistent change in experience-dependent synaptic efficacy and persistence of memory. Moreover specific CPEB family members demonstrate biophysical properties that are associated with prion-like proteins. They exist in two distinct physical states: a monomeric and a dominant self-sustaining amyloidogenic aggregated state. This suggested a model in which a transient experience creates persistence molecular alteration in the nervous system via recruiting a stable and self-sustaining amyloidogenic aggregates of neuronal CPEB. The primary objective of this thesis is to determine how *Drosophila* neuronal CPEB, Orb2, regulates protein synthesis and how conversion to the aggregated state effects its function. Combining *in vitro* and *in vivo* studies we find that the monomeric Orb2 represses, while the amyloidogenic oligomeric Orb2 enhances translation and imparts its translational state onto the monomer. The monomer removes, whereas the oligomer stabilizes and elongates the polyA tail of mRNA. In support of these findings, we have identified a two novel proteins: CG13928, which binds only to monomeric Orb2, promotes deadenylation, and CG4612, a putative polyA binding protein, promotes oligomeric Orb2-dependent translation. We posit that monomeric Orb2 keeps target mRNA in a translationally dormant state and experience-dependent conversion of Orb2 to the stable amyloidogenic state activates translation, resulting in persistent alteration of synaptic activity and stabilization of memory. This study also provides an example of an amyloid-based protein switch that turns a repressor into an activator.

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Table of Contents

Introduction 1
- Role of protein synthesis in synaptic plasticity and memory 1
- Local Protein Synthesis in Neurons 4
- Translational control by cytoplasmic polyadenylation 7
- Translational control by mRNA deadenylation: 10
- CPEB Proteins in Neuron 13
- CPEB and Synaptic Plasticity 14
- Prion hypothesis of long-term memory 17
- Prion-like state of Orb2 and long lasting memory: 19
- Goal of this study: 22

Materials and Methods: 24

Results 47
- Orb2 is a translational repressor: 47
- Orb2 monomer represses and oligomer activates translation: 58
- Orb2A isoform induces Orb2B oligomerization dependent translation *in vitro* and *in vivo*: 68
- Orb2 monomer and oligomer have distinct roles in mRNA metabolism: 74
- Orb2 monomer and oligomer form distinct translation regulatory complexes: 78

Discussion 89
- Orb2-mediated translation: Role of monomeric and oligomeric Orb2 89
- Orb2 monomer deadenylates and Orb2 oligomer stabilizes and polyadenylates target mRNA 94
- The molecular basis of Orb2 monomer- and oligomer-mediated translation regulation 95
- A model for Orb2-mediated synapse-specific local protein synthesis 99
- Translation regulation by mammalian CPEB 100
- Amyloids as a conformation-based protein switch 101
- A putative biochemical "trace" of long-lasting memory 102

Bibliography: 104

Introduction

The cellular basis of memory storage is believed to be experience-dependent changes in synaptic efficacy, often referred to as synaptic plasticity (Kandel et al., 2014; Martin et al., 2000b; Martin and Morris, 2002). The synapse is a communication bridge between neurons, and the minimal unit of change. Since strength of an individual synapse influences the nature of communication in a network of neurons, it was envisioned that one of the ways information can be encoded in the nervous system is by changing the function of the synapse in an experience-dependent manner (Kandel et al., 2014). The discovery of long-term facilitation and long-term depression of synaptic function in response to behavioral training provided experimental credence to the synaptic plasticity hypothesis of memory (Bliss and Collingridge, 1993; Malenka and Bear, 2004; Martin et al., 2000b; Takeuchi et al., 2014). The synaptic plasticity hypothesis of long-term memory also raised some fundamental questions (Takeuchi et al., 2014). How does a synapse change its function? Do all synapses of a given neuron change when animal form a memory? If indeed changes in synaptic strength underlie memory, how does the synaptic change persist for long period of time, even for months and years?

Role of protein synthesis in synaptic plasticity and memory

Some memories last for minutes, while others persist for days and months (Carew et al., 1972; Dudai, 2002, 2004). These temporally distinct forms of memories also have distinct biochemical requirements. While short-term memory depends on the modification of the existing proteins in the synapse, long-term memory requires the de novo synthesis of mRNAs and proteins (Bliss and Collingridge, 1993; Kandel et al., 2014; Malenka and Bear, 2004).

The necessity of protein synthesis in memory was first suggested by the work of Flexner et al. (Flexner et al., 1963). They observed that injection of protein-synthesis inhibitor puromycin into the temporal lobe of mice from day 1 to 3 after learning blocked long-term memory. This observation suggested that after training animals need new protein synthesis in the brain to consolidate the newly acquired information. Following this initial observation a number of studies in mice, rats, fruit flies, and honeybee, in which animals were fed protein synthesis inhibitors before or immediately after training, resulted in a similar disruption of memory (Lagasse et al., 2009; Remaud et al., 2014; Wittstock et al., 1993; Wustenberg et al., 1998). A general problem with these studies is that feeding protein synthesis inhibitors results in a global disruption of animal physiology and it was therefore difficult to distinguish whether the inability to form long-term memories is due to the disruption of memory processes or a general disruption of the animal physiology (Rodriguez-Ortiz et al., 2008). However, a number of behavioral observations argued against a general disruption of the animal physiology or function of the nervous system. For example injection of protein synthesis inhibitors prior to training did not interfere with learning or short-term memory. Even for long-term memory, protein synthesis inhibitors only interfered with very specific forms of long lasting memory (Naghdi et al., 2003; Scharf et al., 2002). For example the animals can be subjected to the same behavioral training in two different manners. If a training paradigm consists of multiple training sessions they can be delivered successively (massed) or with gaps between individual sessions (spaced) (Mauelshagen et al., 1998). Both massed and spaced training produce long-term memory, but memory produced by the spaced training often persist longer than that produced by the massed training. Remarkably injection of protein synthesis inhibitors only interfered with spaced training-induced memory but not that of massed training (Collatz et al., 2006; Hermitte et al., 1999; Scharf et al., 2002). These observations had two major

implications. One, long-term memories of different durations have distinct molecular requirements, and second, only spaced training produces a form of long-lasting memory that requires new protein synthesis.

While the requirement of protein synthesis in long-term memory was evident, it was unclear what specific function protein synthesis serves in long-lasting memory. The discovery of synaptic plasticity as the cellular substrate of memory prompted a number of groups to ask which aspects of synaptic function is dependent on new protein synthesis. Similar to memory processes, the activity-dependent change in synaptic activity has multiple phases that mirror the behavioral memory; short, intermediate and long-term (Carew et al., 1972; Castellucci et al., 1978; Nonaka et al., 2014) . While the short or intermediate-term change in synaptic activity is primarily due to change in the efficacy of preexisting synapses, long-term synaptic plasticity involves growth of new synapses (Bartsch et al., 1995). These various phases of synaptic plasticity also have distinct molecular requirements similar to behavioral memory (Bailey et al., 2000; Casadio et al., 1999; Sutton and Carew, 2000). Application of inhibitors of mRNA and protein synthesis revealed various phases of the activity-dependent long-term potentiation (LTP) of hippocampal CA1-CA3 synapses (Krug et al., 1984) or long-term facilitation of *Aplysia* sensory-motor neuron synapse. While transcriptional inhibitors did not have an effect on early phases of LTP (60-90 mins) but did have an effect on late-phase LTP, protein synthesis inhibitor anisomycin blocked both early and late phases of LTP. These and other subsequent studies suggested that there is a protein synthesis-dependent but transcription-independent early phase and both transcription- and protein synthesis-dependent late phase (Huang and Kandel, 2007).

Local Protein Synthesis in Neurons

Synapses are made of functionally diverse proteins and these proteins are required not only for synaptic transmission and regulation of synaptic transmission but also for building and maintaining a synapse (Miniaci et al., 2008). Since proteins have a finite half-life compared to the life-time of a synapse, the synapse requires continuous feedback of proteins to maintain synaptic protein content (Cohen et al., 2013). In addition to this homeostatic maintenance of synaptic content, synaptic plasticity involves alteration in both function and size of the synapse and therefore a significant change in the protein composition of the synapse (Cline, 2003; Davis and Bezprozvanny, 2001). The maintenance of synaptic protein homeostasis and changes in synaptic proteins are particularly challenging for a neuron because of its organization (Davis and Bezprozvanny, 2001). The number of synapses formed by a given neuron is large. A typical mammalian neuron forms on average 1000 synapses (Cernuda-Cernuda et al., 2003). The relative distance of these synapses from cell body varies significantly. Moreover, synapses that are just a few microns apart can be functionally and morphologically independent of each other (Martin et al., 1997; Miniaci et al., 2008). These neuronal features raise several questions: how does a neuron maintain the protein composition of a synapse? How does it restrict changes in protein composition to a subset of synapses when they share the same cell body, the source of mRNAs and most proteins?

Most proteins are synthesized in neuronal cell bodies (Steward and Schuman, 2003). However, over time it became evident that in neurons new proteins are synthesized outside the cell body (Steward et al., 2014). The synthesis of new proteins outside the cell body was first observed from incorporation of radiolabeled amino acids in isolated preparation of axons (Arch, 1972; Wilson, 1971). Moreover, in a narrow time window, which is considered insufficient for transport

of newly synthesized protein from cell body to the distant synapses, radiolabeled proteins were detected in synaptic fractions immediately after synaptic stimulation (Steward et al., 1991). These studies however failed to establish unequivocally whether these new proteins are indeed synthesized within the axon or from some other cells that are attached to the isolated axons or that a small amount of contaminating cell bodies accounts for the incorporation of radiolabeled amino acids. Discovery of ribosomes in the axon and dendritic processes confirmed the idea that protein synthesis can occur outside the cell body (Steward and Levy, 1982). Bodian et al. first noticed the presence of ribosome particles in the proximal dendrites adjacent to synaptic "knobs" in monkey spinal cord motor neurons (Bodian, 1965). Subsequently a series of studies by Steward and Levy, demonstrated that ribosomes, including polysomes, are present in the dendritic spines in granule cells of rat dentate gyrus (Steward and Falk, 1986; Steward and Levy, 1982). Since polysomes represent ribosomes bound to translating mRNA, the presence of polysomes indicated active translation at the synapse (Steward and Ribak, 1986; Sutton and Schuman, 2006). More compelling evidence of local protein synthesis in dendrites came from the studies with cultured neurons (Aakalu et al., 2001). For example, in hippocampal neurons, myristolated GFP tagged reporter with the 3'UTR of CAMKII mRNA was used to entrap the translated proteins in site of synthesis (Rook et al., 2000). Stimulation with brain derived neurotrophic factor (BDNF), showed enhanced GFP fluorescence in the dendrites. Taken together with the observation that a number of mRNAs are localized in the dendrites and axons of neurons in various species, these observations suggested that mRNAs are indeed translated locally in the synapse (Moccia et al., 2003).

What is the function of local protein synthesis in the nervous system? The first functional role of local protein synthesis was shown by Kang et al. (Kang and Schuman, 1996). In this study they noticed that local application of the growth factor BDNF not only causes local proteins

synthesis in the dendrites but also that this newly synthesized protein is required for the rapid enhancement of synaptic transmission. Kang et.al found that BDNF increased synaptic efficacy in neurites that were dissociated from the cell bodies and application of protein synthesis inhibitors such as emetine inhibited the synaptic enhancement. Since the neurites were separated from the primary source of proteins, the cell body, this study provided the first causal link between local protein synthesis and synaptic plasticity. More elegant experiments in *Aplysia* sensory-motor neuron co-cultures demonstrated that synapse specific long-term facilitation requires local protein synthesis in the presynaptic cell (Martin et al., 1997). In these experiments a bifurcated *Aplysia* sensory neuron was used which made synaptic contact with two separate target motor neurons. Repeated application of serotonin (5-HT) to one set of synapses produced a CREB-dependent, synapse-specific, long-term facilitation, which can be captured at the opposite synapse by a single pulse of 5-HT (Casadio et al., 1999). Perfusion of protein synthesis inhibitors locally in the synaptic region did not change short-term facilitation, but selectively interfered with long-term facilitation. Furthermore this system also revealed that a transient and neuron wide CREB-mediated long-term facilitation can stabilized at specific synapses by local application of serotonin in the synapse and this stabilization requires local protein synthesis (Casadio et al., 1999). However, prior application of protein synthesis inhibitors blocked the ability to capture facilitation via single pulse of 5-HT. Likewise, repeated application of 5-HT only to the cell body of sensory neurons produced a CREB-dependent, cell-wide long-term facilitation. However, this cell wide facilitation was not associated with any new synaptic growth and the synapse-specific facilitation did not persist more than 24 hours. This transient cell-wide facilitation can be converted to persistent synapse-specific facilitation by a single pulse of 5-HT at the synapse (Casadio et al., 1999). Again application of protein synthesis inhibitor at the synapse blocks the capture of cell

wide facilitation. A similar synaptic tagging was also reported in other systems including mouse hippocampus (Alarcon et al., 2006). These results suggested two very specific roles of local protein synthesis. First, local protein synthesis is required for a retrograde signal from synapse to the cell body that activates transcriptions in the cell body. Second, local protein synthesis also "tags" the activated synapse and the tag allows the activated synapses either to capture and/or utilize the globally distributed gene products (Frey and Morris, 1997; Reymann and Frey, 2007).

Translational control by cytoplasmic polyadenylation

These observations suggested that local protein synthesis is a key molecular event in generating synapse-specific long-term change in synaptic efficacy and raised a number of questions. How is local protein synthesis in the synapse regulated? What proteins are locally synthesized and how do they stabilize synaptic growth and function? Protein synthesis in the synapse can be regulated by a number of ways, such as the availability of the mRNA, modification of the mRNA to make it translationally competent or the availability of protein synthesis machinery (Davis et al., 1987; Si et al., 2003a; Steward and Levy, 1982; Steward and Reeves, 1988). Among these plausible mechanisms regulation, changing the polyA tail length of the mRNA has emerged as one of the principal mechanisms of synaptic protein synthesis regulation (Si et al., 2003a; Udagawa et al., 2012).

All newly made mRNAs receive a polyA tail in the nucleus through transcription-coupled polyadenylation (Bilger et al., 1994; Rigo and Martinson, 2009). Polyadenylation in the nucleus requires a cis-acting polyadenylation signal sequence, AAUAAA in the 3'UTR, and a polyadenylation complex composed of cleavage and polyadenylation specificity factors (CPSF) and the enzyme poly (A) polymerase (PAP) (Murthy and Manley, 1995; Proudfoot and Brownlee, 1976). CPSF directly binds to the hexanucleotide sequence at the 3' UTR and recruits PAP to form

an active polyadenylation complex (Bienroth et al., 1991; Murthy and Manley, 1995). As a result of generalized polyadenylation in the nucleus, most pre-mRNAs have a poly (A) tail of approximately ~70-90 nucleotides long in *Saccharomyces cerevisiae* (Brown and Sachs, 1998; Groner et al., 1974) and ~250 in mammalian cells (Brawerman, 1981). Once transported out of the nucleus to the cytoplasm the mRNA can undergo additional modifications and some of these modifications in the cytoplasm control mRNA translation (Barnard et al., 2004; Kim and Richter, 2006). One such cytoplasmic modification of mRNA is elongation of the polyA tail. In eukaryotes the presence of a long polyA tail at the 3' end of the messenger RNA is an important regulatory step in translation (Sachs et al., 1997). The longer polyA tail can stabilize or enhance translation of the mRNAs (Munroe and Jacobson, 1990). The polyA tail-dependent translation regulation is particularly interesting in the context of synaptic protein synthesis because it was discovered as one of the key mechanisms of spatio-temporal regulation of translation (Groisman et al., 2000).

That polyA tail length is important for translation was first discovered in *Xenopus* oocyte by Joel Richter and colleagues in an attempt to understand how translationally dormant maternal mRNAs are translated upon exposure to oocytes maturating factors (Groisman et al., 2002). In immature oocytes, dormant mRNAs usually have short poly (A) tail of ~40 bases, but when these mRNAs are polyadenylated up to ~150 bases they then become translationally active, which results in meiotic division and oocyte maturation (Ivshina et al., 2014). Richter and colleagues subsequently discovered that cytoplasmic polyadenylation is dependent on the presence of U-rich sequence element UUUUAU at 3'UTR (CPE-element) and a CPE-element binding protein CPEB (Hake et al., 1998). Pre-mRNAs which have a cytoplasmic polyadenylation element (CPE) at their 3'UTR upstream of the AAUAAA poly (A) selection site are bound by CPSF and transported to the cytoplasm (Lin et al., 2010). In the cytoplasm a complete polyadenylation complex is

assembled upon the joining of symplekin, a scaffolding protein, germline development 2 poly (A) polymerase, or Gld2, and the deadenylating enzyme poly (A) ribonuclease or PARN (Barnard et al., 2004; Kim and Richter, 2006). Both Gld2 and PARN are enzymatically active but the more robust nuclease activity of PARN keeps the mRNA transnationally silent by maintaining a short poly(A) tail (Kim and Richter, 2006). In addition, Maskin, which is a poly (A) binding protein, simultaneously binds CPEB and eukaryotic initiation factor 4E (eIF4E) and prevents eIF4E's interaction with the 5' cap structure, stopping the assembly of the translation initiation complex (Cao and Richter, 2002; Richter and Sonenberg, 2005; Stebbins-Boaz et al., 1999).

Hormone such as progesterone causes unmasking of dormant mRNA by removing the inhibitory constraints and elongating the polyA tail (Sarkissian et al., 2004). The first step in this process is the activation of Aurora A kinase which phosphorylates CPEB protein (Mendez et al., 2000a; Sarkissian et al., 2004). CPEB phosphorylation has multiple effects: first, it causes PARN expulsion from the RNA-protein complex thereby stops deadenylation; second, it enhances interaction between CPEB and CPSF complex (Kim and Richter, 2006; Mendez et al., 2000b), and finally, phosphorylated CPEB has a low affinity for Maskin resulting in dissociation of Maskin from CPEB-eIF4E complex and allowing the formation of translation initiation complex (Cao et al., 2006; Kim and Richter, 2007) (Fig 1.1).

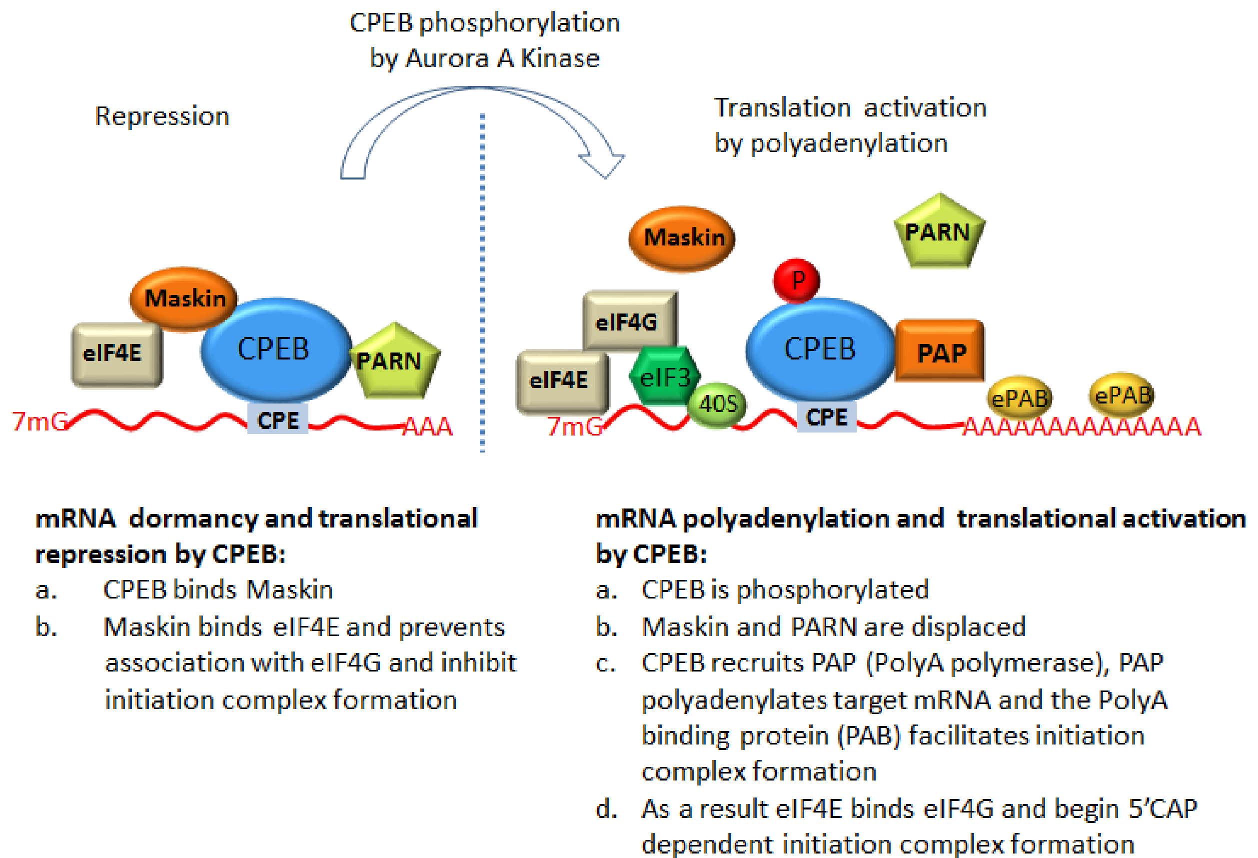


Figure 1.1: Mechanism of canonical CPEB mediated translation in *Xenopus* oocytes. In immature oocytes the translationally dormant mRNA is bound by CPEB and Maskin. Maskin binds both eIF4E and CPEB and prevents interaction with eIF4G.This occupancy of eIF4E by Maskin restricts formation of 5'CAP dependent translation initiation complex. On the other hand when CPEB is phosphorylated Maskin and PARN are displaced from the complex, polyA polymerase elongates polyA tail, eIF4E and eIF4G interact each other and form translation initiation complex.

Translational control by mRNA deadenylation:

The polyA tail length of mRNAs in the cytoplasm is regulated by two opposing activities: polyadenylation and deadenylation. As mentioned before, long polyA tails often protect mRNAs from degradation and enhance translation of the mRNA. On the other hand shortening of polyA tail length is mostly associated with reduction in translation and decay of an mRNA. After

deadenylation the mRNAs are degraded in two ways: degradation in the 3' to 5' direction by cytoplasmic exosome complex or deadenylation followed by decapping and degradation in 5' to 3' direction by Xrn1 nuclease (Houseley and Tollervey, 2009; Meyer et al., 2004; Parker and Song, 2004).

What are the mechanisms of cytoplasmic deadenylation? Two protein complexes, Ccr4-Not and Pan2-Pan3, are known so far to deadenylate mRNA in the cytoplasm. Ccr4-Not protein complex is one of the primary deadenyaltion complexes in eukaryotes, and mediates complete deadenylation of the target mRNA. This complex consists of five conserved subunits: Ccr4, Caf1/Pop2, Not1, Not2 and Not3/5 (Wahle and Winkler, 2013). In mammals there are two Ccr4 orthologues, Ccr4a and Ccr4b (Morita et al., 2007; Wang et al., 2010). Ccr4 has Mg+ dependent 3'exonuclease activity (Chen et al., 2002; Tucker et al., 2002). Ccr4 protein has two well characterized domains: a nuclease domain for exonuclease activity and a leucine-rich repeat (LRR) domain which mediates interaction with other subunits in the complex such as Caf1/Pop2 (Draper et al., 1995; Dupressoir et al., 2001; Malvar et al., 1992). The second conserved catalytic subunit of Ccr4-Not complex is Caf1 (Schwede et al., 2008) and it degrades polyA sequences with high efficiency (Bianchin et al., 2005; Viswanathan et al., 2004). Not1 acts as a central scaffolding agent and it interacts with most of the subunits in the complex (Bai et al., 1999; Maillet and Collart, 2002; Maillet et al., 2000). The function of the Not2 subunit is unknown, though it is highly conserved. In yeast two other Not subunits, Not3p and Not5p, have been identified according to their sequence similarity (Chen et al., 2001; Liu et al., 1998; Oberholzer and Collart, 1998). However in other organisms, instead of Not3 and Not5, there is only the Not3 subunit (Albert et al., 2000; Lau et al., 2009; Temme et al., 2004). The function of Not3 is unclear.

Another well characterized protein complex that removes the polyA tail of mRNAs is the PAN complex. Both in yeast and mammals the poly (A)-specific nuclease PAN complex consists of two subunits, PAN2 and PAN3 (Boeck et al., 1996; Brown et al., 1996; Uchida et al., 2004). The catalytic PAN2 subunit has 3'-exonuclease activity and it requires divalent cations for enzymatic function (Uchida et al., 2004; Zuo and Deutscher, 2001). PAN2 requires poly (A) binding protein for its enzymatic activity, such as PABPC in mammals and Pabp1 in yeast, (Lowell et al., 1992; Sachs and Deardorff, 1992; Uchida et al., 2004). PABPC is recruited to the PAN complex via its interaction with PAN3. PAN3 has two binding motifs that mediate protein-protein interaction: PABP-interacting motif 2 or PAM2 at the N-terminal end that mediates interaction with PABPC, and a C-terminal motif that mediates interaction with PAN2 (Siddiqui et al., 2007). *In vitro* studies suggested that PAN2-PAN3 complex deadenylates the target RNA incompletely, leaving 25 nucleotides (Lowell et al., 1992). Similar observations were also made *in vivo* in yeast strains where accumulation of RNA with similar tails was found (Tucker et al., 2001).

Both Ccr4-Not and Pan2-Pan3 complexes localize in the cytoplasm (Cougot et al., 2004; Tucker et al., 2001). Initially, it was thought that deadeynylation functions are redundant in the cytoplasm due to the presence of two deadenylation complexes. However, several studies suggested that these two complexes serve distinct functions(Bonisch et al., 2007). It was proposed that Pan2-Pan3 performs initial phase of deadenylation and the remaining bulk deadenylation is mediated by Ccr4-Not complex. Recruitment of both Ccr4-Not and Pan2-Pan3 complexes to the target mRNA are mediated by two mechanisms. One, a generalized deadenylation mechanism in which these complexes are recruited by polyA binding protein C1 (PABC1) and second, a targeted mRNA deadenylation mechanism in which these complexes are recruited to the target mRNA by element specific RNA binding protein. In the case of general deadenylation, Pan3 subunit, through

its PAM2 motif, interacts with the C-terminal domain of PABPC1. However, none of the Ccr4-Not complex subunit proteins have PAM2 motifs, and recruitment of this complex to PABPC1 is mediated, in mammals, by mammalian TOB proteins that are known to have PAM2 motif in their C-terminal region (Ezzeddine et al., 2007; Ezzeddine et al., 2012; Funakoshi et al., 2007). There are several examples of Ccr4-Not involvement in targeted mRNA deadenaylation. In yeast *S. cerevisiae* Ccr4-Not complex is recruited by Mpt5 protein, which belongs to the PUF family of RNA binding protein, similar to the *Drosophila* Pumilio protein. Mpt5 binds specific sequence elements of target mRNAs and recruits the Ccr4-Not complex through binding the Caf1 subunit of the complex (Goldstrohm et al., 2007). In human cells the Ccr4-Not complex is recruited through short A/U rich elements (ARE) present in a number of mRNAs with very short half-lives, for example mRNA of cytokine tumor necrosis factor (TNF) α or the inducible transcription factors c-Fos and c-Myc. The specific ARE element of these mRNAs is bound by a zinc-finger containing Tristetraprolin (TTP) protein, which in turn binds Ccr4-Not complex via the CNOT1 subunit of the complex (Sandler et al., 2011).

CPEB Proteins in Neuron

Although CPEB proteins were discovered in developing oocytes, based on studies both in invertebrates and mammals, proteins of the CPEB family emerged as potential candidates for regulator of synaptic protein synthesis (Richter and Klann, 2009). In mammals CPEB has four family members, CPEB1-4. Though all of them are expressed in the brain, CPEB1 and CPEB2-4 are thought to form different functional groups of CPEB family proteins (Mendez and Richter, 2001). They all contain a common RNA binding domain and a Zn-finger domain at the C-terminal end, but they differ in the N-terminal end (Richter, 2007). Intriguingly CPEB3 and CPEB2 contain an N-terminal unstructured domain similar to prion-like domains (discussed in detail later). *In vitro*

iterative binding assays suggested that CPEB2-4 do not interact with the canonical CPE, instead they bind a U-rich stem-loop structure (Huang et al., 2006). CPEB3 neither requires the AAUAAA polyadenylation signal nor does it bind to the CPSF polyadenylation factor (Huang et al., 2006). There are two CPEB proteins in *Aplysia* (Liu et al., 2006). Similar to mammalian CPEB, the two ApCPEBs differ in the N-terminal end, one of them having a poly glutamine (Q) stretch at the N-terminus (Fig 1.2), along with the propensity to form amyloid-like structures. In *Drosophila* there are two CPEB orthologues, Orb1 and Orb2, and both of them are expressed in the nervous system (Hafer et al., 2011; Pai et al., 2013). Similar to *Aplysia* (ApCPEB), Orb2 also possess a poly-glutamine (Q) stretch at its N-terminus (Fig 1.2).

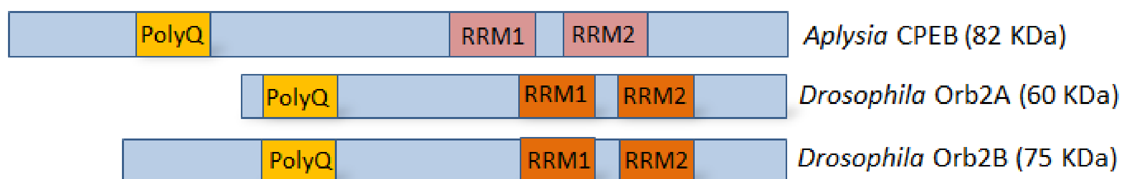


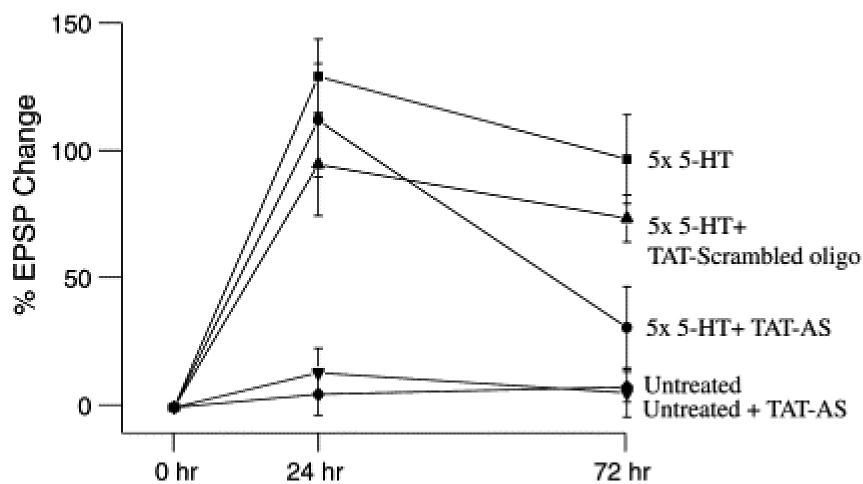
Figure 1.2: *Drosophila* Orb2 and *Aplysia* CPEB have structural similarity. RRM refers to RNA recognition motif. PolyQ refers to polyglutamine stretches of 25 and longer.

CPEB and Synaptic Plasticity

Several studies reported that CPEB-mediated translational regulation is essential in early development (Fox et al., 1989; McGrew et al., 1989; Minshall et al., 1999; Tay et al., 2000). However, CPEB mediated translational regulation also exists beyond development. Although earlier studies in mouse suggested CPEB1 (mCPEB, a 62 KDa protein) is expressed in testis, ovary and kidney (Gebauer and Richter, 1996), subsequent studies found moderate level of CPEB protein in the brain (Huber et al., 2000; Kang and Schuman, 1996; Martin et al., 2000a; Martin et al., 1997;

Wu et al., 1998). CPEB1 is present in the post-synaptic density, and activation of NMDA receptors enhances the translation of Ca^{2+}-calmodulin dependent kinase (α-CaMKII), a molecule that is important for LTP (Du and Richter, 2005; Huang et al., 2003; Huang et al., 2002; Wells et al., 2001; Wu et al., 1998). Inactivation of CPEB-1 impaired some forms of LTP; however, it is not clear whether CPEB1 is a primary regulator of long-term memory-related protein synthesis (Alarcon et al., 2004; Wu et al., 1998; Zearfoss et al., 2008). Among other CPEB family members, CPEB3 regulates neuronal protein synthesis and either ubiquitination or truncation of CPEB3 relieves its translation inhibitory function (Pavlopoulos et al., 2011). CPEB4, similar to CPEB1, seems to be involved in a number of cellular processes and is abundantly expressed in the nervous system (Tsai et al., 2013). However, constitutive knock out of CPEB4 has no discernable effect on synaptic function or memory.

The role of CPEB in synaptic plasticity has been well characterized in *Aplysia* (ApCPEB) (Si et al., 2003a). ApCPEB protein is upregulated in the stimulated synapse and is required for the persistence of serotonin-induced long-term facilitation (Fig 1.3).



Si et al. 2003

Figure 1.3 Maintenance of synaptic facilitation requires ApCPEB in the activated synapse. Long-term facilitation is disrupted due to the local inhibition of ApCPEB by applying TAT–ApCPEB anti-sense oligo.

The studies in *Aplysia* suggested that the neuronal CPEB has properties required of a synaptic tag: 1) ApCPEB is upregulated only at the activated synapse in a stimulus-dependent manner (Si et al., 2003a); 2) It is required not for initiation but for the persistence of long-term facilitation and stabilization of newly formed synapses; 3) ApCPEB can activate translation of dormant mRNAs (Bally-Cuif et al., 1998; Schroeder et al., 1999; Stebbins-Boaz et al., 1996; Tan et al., 2001); and finally 4) CPEB target mRNAs are involved in synaptic growth (Chang et al., 2001; Groisman et al., 2002).

In *Drosophila*, CPEB has two family members: Orb1 and Orb2. Orb1 is known to be involved in establishing polarity axes in the developing egg as well as early embryo (Hafer et al., 2011). Beyond the germline, the role of Orb1 in somatic tissues are unclear (Hafer et al., 2011). However, one recent study reported that *Drosophila* Orb1 is expressed in the adult nervous system and is required for the conversion of labile olfactory memory into stable long-term memory (Pai et al., 2013).

Unlike Orb1, Orb2 mRNA and protein are expressed both throughout development and in somatic tissues (Hafer et al., 2011). The Orb2 localization in embryonic central nervous system (CNS) is restricted to cell bodies and mostly absent in the axonal tracts (Hafer et al., 2011). However, in the adult fly brain Orb2 protein can be detected in the cell bodies, axonal tracts as well as dendritic terminals (Hafer et al., 2011; Kruttner et al., 2015; Majumdar et al., 2012). Orb2 has been implicated in development of embryonic nervous system and mesoderm through asymmetric division of stem cells and other precursor cells (Hafer et al., 2011). In adult flies,

removal of Orb2 does not interfere with short-term memory but impairs long-term memory (Majumdar et al., 2012).

Prion hypothesis of long-term memory

Since proteins are short-lived compared to the duration of memory, it remains unclear how memory can be immune to turnover of individual proteins (Lynch and Baudry, 1984; Roberson and Sweatt, 1999). For a long time the likely solution was believed to be structural changes at the synapse that confer stability to the memory. As a corollary it was assumed that the requirement for activity-dependent molecular changes, such as synthesis of new protein was transient. However, studies in a number of model systems suggested that the maintenance of behavioral memory as well as structural alterations requires ongoing macromolecular synthesis (Kandel, 2001; Martin et al., 1997; Nader, 2015). This posed a problem: how does a transient experience produce such a persistent state of macromolecular synthesis? A potential solution to this problem of molecular turnover is to either have proteins with unusually long-half lives or a self-sustaining biochemical reaction. In 1984, Crick first addressed the possibility of a self-sustained molecular alteration as the basis of long-term memory storage using protein phosphorylation as a candidate mechanism (Crick, 1984). Subsequently a number of plausible candidate mechanisms has been proposed including auto catalytic nature of calcium-calmodulin dependent kinaseII or CamKII (Dudai, 2004; Lisman, 1994). Another plausible candidate for a self-sustaining biochemical reaction came from the analysis of the amino acid sequence of ApCPEBs. Si et al. made the surprising observation that the neuronal isoform of ApCPEB has a glutamine and asparagine (Q and N)-rich N-terminal domain, reminiscent of well-characterized prions in yeast (Si et al., 2003b). Prions are proteins that can assume at least two distinct physical states: a monomeric state and self-templating amyloidogenic aggregated state (Prusiner, 1998). Once the aggregate state is

formed it induces the conformational conversion of the monomeric state and initiates a dominant autocatalytic state. Prions were first discovered as the causative agent for a number of neurodegenerative diseases (Prusiner, 2013). However studies primarily in yeast revealed that proteins can adopt a prion-like state and be non-pathogenic and even be the basis of phenotypic variations (Shorter and Lindquist, 2005; Wickner et al., 2007). A number of assays developed for studying prions in yeast revealed that ApCPEB could exist in yeast in two distinct conformational states, very much like other yeast prion proteins. One of these states is monomeric and incapable of self-perpetuation. The other state is oligomeric and stably inherited across generations in yeast although occasional switches to the monomeric state occur (Heinrich and Lindquist, 2011; Si et al., 2003b).

Si et al. went on to explore the conformational state of ApCPEB in *Aplysia* neurons (Si et al., 2010). They found that when ApCPEB was overexpressed in *Aplysia* sensory neurons, it formed punctate structures that were amyloid-like in nature, a common characteristic of all known prions and prion-like proteins. These punctate structures were due to self-assembly of the ApCPEB protein and once formed they could recruit newly synthesized protein, a feature necessary for self-sustenance. Intriguingly, application of 5 pulses of serotonin, which produces long-term facilitation, increased the number of puncta, suggesting that the aggregation of ApCPEB could be regulated by modulators of synaptic activity. Importantly, injection of an antibody that selectively binds the aggregated form of ApCPEB did not prevent the initiation of long-term facilitation but selectively blocked its maintenance beyond 24 hours (Si et al., 2003a).

These observations led to a model that explains persistent synaptic protein synthesis at the activated synapse. According to this model ApCPEB in the sensory neuron has at least two conformational states: 1) a recessive monomeric state where ApCPEB is inactive or acts as a

repressor of translation; and 2) a dominant, self-sustaining, active multimeric state. In a naive synapse, the basal level of ApCPEB is low and the protein is in the monomeric state. Serotonin stimulation converts ApCPEB from the monomeric to the prion-like state, which might be more active or devoid of the inhibitory function of the basal state. Once the prion state is established at an activated synapse, dormant mRNAs, made in the cell body and distributed globally to all synapses, can be activated only locally through the activated ApCPEB. Because the activated ApCPEB can be self-perpetuating, it can contribute to a self-sustaining synapse-specific long-term molecular change and provides a mechanism for the stabilization of learning-related synaptic modification and growth, thereby contributing to the persistence of memory storage.

Prion-like state of Orb2 and long lasting memory:

CPEB's role and mode of action in the maintenance of long-term plasticity and hence memory is not restricted to *Aplysia*. Similar to ApCPEB, *Drosophila* Orb2 has two distinct physical states in the adult fly brain; a monomer and a stable SDS-resistant amyloid-like oligomer. The oligomers are formed at physiological concentrations of Orb2 protein and stimulation of behaviorally relevant neurons, such as the Kenyon cells of the mushroom body or octopamine or dopamine responsive neurons, increases the level of the oligomeric Orb2 (Kruttner et al., 2012; Majumdar et al., 2012). Considering the evolutionary distance between the *Aplysia* CPEB and *Drosophila* Orb2, these observations suggested that the amyloidogenic oligomers of Orb2/CPEB may act to stabilize activity-dependent changes in synaptic efficacy across species.

There are six protein isoforms encoded from Orb2 genomic locus, includingOrb2A and Orb2B, which are homologous to mammalian CPEB2-3 and ApCPEB proteins (Keleman et al., 2007; Mastushita-Sakai et al., 2010). The two Orb2 protein isoforms Orb2A and Orb2B share a common C-terminal end but they differ in the N-terminal end. While both isoforms have N-

terminal Q-rich domain, Orb2A has 8 amino acids and Orb2B has 162 amino acids preceding the Q-rich domain (Majumdar et al., 2012). The Q-rich domain, present in both isoforms of Orb2, is implicated in Orb2 oligomerization (Majumdar et al., 2012). Biochemical studies found that both Orb2A and Orb2B formed SDS-resistant oligomers. Fluorescence resonance energy transfer (FRET) and fluorescence recovery after photobleaching (FRAP) analysis demonstrated that Orb2A forms higher-ordered and tightly assembled oligomers. The FRAP analysis also revealed that Orb2A puncta are more stable than Orb2B and other *Drosophila* poly-glutamine-rich proteins. Based on this experimental evidence it was suggested that the Orb2A isoform is more efficient than Orb2B in forming self-sustaining SDS-resistant oligomers both *in vitro* and *in vivo*.

In fly head, the abundance of Orb2A and Orb2B isoforms is different. The short Orb2A isoform is rare and is not detectable by western blot analysis. Based on the genomic transgene expression of both Orb2 isoforms, it was observed that the Orb2A isoform is approximately 100-fold less prevalent in fly brain than the Orb2B isoform (Majumdar et al., 2012). The longer Orb2 isoform (Orb2B) is constitutively expressed and readily detectable by conventional western blot analysis in the fly head lysate (Majumdar et al., 2012). In spite of its low abundance the Orb2A isoform is critical for Orb2 oligomerization and the animal's ability to form stable long-lasting memory. When the Orb2A isoform was knocked out the level of Orb2B monomer was unchanged but surprisingly the level of Orb2 oligomer was significantly decreased in the fly head. Based on these findings it was hypothesized that Orb2A might act as a seed and recruit the Orb2B isoform, which results in the formation of self-sustaining Orb2 oligomer in fly brain.

Considering the prion-like conformational states of neuronal CPEB and its role in long-term memory formation, Keleman et al. specifically tested the effect of removing glutamine-rich prion-like domain from Orb2 protein and asked whether the prion-like domain is necessary for the

memory formation (Keleman et al., 2007). The mutant flies without prion domain were defective in forming long-term memory. Importantly the deletion of prion-like domain didn't affect learning, short-term memory, or memory recall (Keleman et al., 2007). This study also narrowed down the set of neurons (*fru*-positive mushroom body γ neurons) in the fly head in which Orb2 function was required for the memory formation. However, this study didn't provide any biochemical evidence for whether *Drosophila* Orb2 is truly a prion, or that the prion property is required for the memory formation.

Majumdar et al. specifically addressed the question of whether the prion-like property of Orb2 is important for memory (Majumdar et al., 2012). They took the advantage of the fact that rare Orb2A plays a critical role in the prion-like conversion of the abundant Orb2B in the fly head. They performed a random mutagenesis screen and identified a number of mutations in the first 8 amino acids that are unique to the Orb2A isoform that prevented Orb2A oligomerization. One of these point mutations in Orb2A, Orb2AF5>Y5, when introduced into the fly, reduced activity-dependent amyloid-like oligomerization of Orb2. Notably, flies carrying this mutation showed a very specific memory deficit. In two different behavioral paradigms, male courtship suppression memory and appetitive associative memory, the memory score of mutant flies were similar to the memory score of wild type a day after training. However unlike wild type flies, in the mutant flies memory begins to decay at 2 days and by 3 days there was no measureable memory. The loss of memory in the Orb2 mutant that can't form Orb2 amyloid is reminiscent of the loss of synaptic facilitation in sensory-motor neuron synapse upon inhibition of the ApCPEB oligomers via antibody.

Goal of this study:

These observations led to a model for long-term memory in which external stimuli recruit a self-sustaining amyloidogenic form of neuronal CPEB in the activated synapse, where CPEB then maintains memory through the sustained and regulated synthesis of a specific set of synaptic proteins. However, the prevailing view is that amyloid formation in general leads to inactivation of proteins. Therefore a central tenet of the hypothesis remains untested: how does neuronal CPEB regulate synaptic protein synthesis and what is the consequence of conversion to the self-sustaining amyloidogenic state?

In an attempt to address this question previously, Sakai et al. found that *Drosophila* Orb2 targets mRNA transcripts of several candidate long-term memory genes (Mastushita-Sakai et al., 2010). These mRNAs are not only targeted by monomeric Orb2 but also oligomeric Orb2 since both conformational states of Orb2 were efficiently pull down by biotin-labeled *in vitro*-synthesized target 3'UTRs (Fig 1.4).

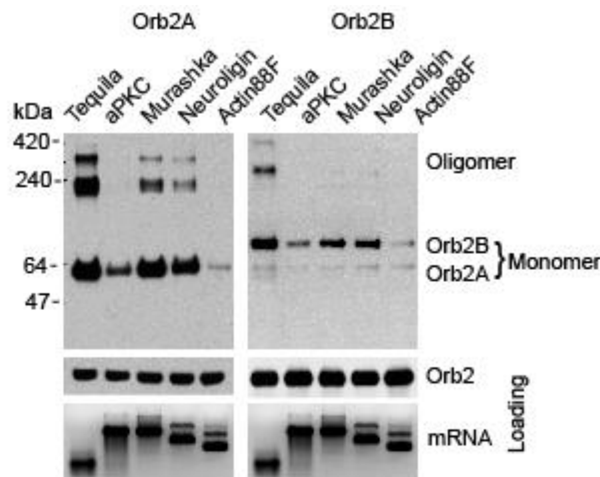


Figure 1.4. Both isoforms (Orb2A and Orb2B) and both conformational states (monomeric and oligomeric) of Orb2 interact with 3'UTRs of target genes (Tequila, aPKC, murashaka and neuroligin). Actin88F 3'UTR serves as a negative control in this RNA binding assay.

Although both Orb2A and Orb2B contain the same conserved RNA recognition motifs (RRM) at the C-terminus and bind to the same mRNA they also vary in various aspects: amino acid sequences leading to the prion domain are different, the Orb2B isoform is constitutively expressed and present all over the neuron, the Orb2A isoform is expressed in an activity dependent manner and present primarily in the synaptic region. Previous studies suggested that Orb2 is a translational repressor (Mastushita-Sakai et al., 2010; Xu et al., 2012; Xu et al., 2014). However, the biochemical functions of the two isoforms of Orb2 and more importantly the functions of monomeric and amyloidogenic oligomeric states in translation are largely unknown. The goal of this study is to address the following issues:

1. Determine the role of Orb2 in protein synthesis

2. Determine the role of Orb2A and Orb2B isoforms and the monomeric and oligomeric Orb2 in protein synthesis

3. Determine the mechanism of Orb2-dependent translation regulation

4. Determine the molecular basis of Orb2-dependent translation regulation

Materials and Methods

Fly strains: The following fly strains were generated in this study: UAS-Luciferase, UAS-FLAGCG13928, UAS-Orb2A320, UAS-Orb2A320Δ8, UAS-Orb2A320Δ80, UAS-FLAG-CG13928. All constructs were inserted in the attp1 site in the second chromosome except UAS-FLAG-CG13928. The CG13928 construct was made by conventional transgenic methods and independent lines in 2nd and 3rd chromosomes were selected. The CG4612RNAi (VDRC Stock#52497), CG13928RNAi (TRiP collection, Bloomington Stock#28646), MzvumGal4 (Bloomington Stock#29031), GeneSwitch-ElavGal4 (Bloomington Stock#43642) were obtained from the Bloomington stock center, Indiana. The 201YGal4 and 17DGal4 were kindly provided by Dr. Troy Zars of University of Missouri. Various genetic combinations were made by standard genetic crosses.

Antibodies: The anti-Orb2 polyclonal rabbit IgG (#273) and anti-Orb2 polyclonal guinea pig IgG (#2233) were raised against full length recombinant Orb2A protein. The anti-Smaug polyclonal (#2442)**,** anti-CG4612 polyclonal (#2444), and anti-CG13928 polyclonal (#2447) antibodies were raised in guinea pigs against 6Xhistidine-tagged purified recombinant full length proteins. All antibodies were affinity purified against the purified recombinant antigens. The following antibodies were obtained from commercial sources; anti-FLAG HRP-linked monoclonal (#A8592, Sigma)**,** anti-Myc 9B11 monoclonal (#2276, Cell signaling Technology)**,** anti-V5 monoclonal (#46-0705, Invitrogen)**,** anti-Oligomer A11 antibody (AHB0052, Invitrogen), anti-mouse HRP linked secondary antibody (#7076, Cell signaling Technology) and anti-rabbit HRP linked secondary antibody (#7074, Cell signaling Technology). The anti-HRP36 rabbit antibody was kindly provided by Dr. Marco Blanchette of the Stowers Institute.

Plasmids and clones: For RNA binding assay the 3'UTR of Tequila was cloned in TOPOII dual vector (Invitrogen) and then linearized with BamHI for *in vitro* transcript synthesis. To make *in vitro* translation construct firefly luciferase was cloned between the 5'UTR and 3'UTR of Tequila, Neuroligin and aPKC in pBSKII vector. For all protein-protein interaction study in S2 cells the open reading frames were cloned into TopoD vector (Invitrogen) and transferred into following vectors obtained from *Drosophila* Genome Research Center (DGRC) using Gateway system; PAC 5.1 C-FLAG, PAC 5.1 C-HA, PAC 5.1C-V5 or PAC 5.1C-6XMyc and pUAST-FLAG vector. For *in vitro* protein purification the genes were cloned in pDEST42-6xHis vector (Invitrogen) using the Gateway cloning system. For expression in the adult fly brain the genes were cloned into pUAST-attB (Kindly provided by Dr. Konrad Basler, University of Zurich) vector. The *Drosophila* Not-CCR4-PAN complex cDNAs were kindly provided by Dr. Elisa Izaurralde of Max Planck Institute for Developmental Biology, Tubingen, Germany.

Orb2-TEV flies: The Orb2 genomic rescue constructs were made as described by Venken et al. (Venken et al., 2006; Venken et al., 2008) . 500 bp fragments from the 5′ and 3′ end of the Orb2 locus were cloned into the pattB vector to generate a capture vector and a 18761 base pair fragment encompassing the Orb2 locus was captured using recombineering. The resulting untagged pattB-Orb2 construct was used to introduce the TEV-protease recognition site ENLYFQG at amino acid position 369 with respect to Orb2B (position 216 with respect to Orb2A) using the counter selection BAC modification kit from Genebridges. Briefly, the Rpls-neo cassette was inserted into the Orb2A/Orb2B specific common exon at the indicated position and replaced with an oligo carrying an in frame TEV-protease recognition site using counter selection. The pattB-Orb2-TEV fragment was inserted in the attP2 site in third chromosome and Δorb2:pattB-Orb2-TEV flies were generated via recombination.

Proteomics: The transcripts of the target 3'UTRs were *in vitro* synthesized using MegaScript T7 transcription kit (Ambion) and labeled with Bio-ATP and Bio-CTP (Enzo) RNA nucleotide analogues. DNase treated RNAs were purified by passing through MicroSpin^{TM} G-50 RNA purification column (GE healthcare). The RNAs were stored at -80°C until used. For RNA-portein pull down heads were isolated from 10ml of flies and lysed in cold lysis buffer containing 250mM Tris pH8.0, 150mM NaCl, 1mM EDTA, 5% glycerol, 1% TritonX-100, 1.5mM fresh DTT, 0.2mg/ml heparin, 0.2mg/ml yeast tRNA, 0.25% BSA, 40u/µL RNase inhibitor and protease inhibitor). The collected heads were homogenized and incubated for 10 mins at 4°C with continuous rotation. Total protein concentration was measured using BCA kit (Pierce) and 10 mg of total protein was incubated on a rotator with 3µg of biotin labeled RNA probe in presence of RNase inhibitor for 40 mins at room temperature. After 40 mins of incubation, pre-equilibrated 500µL streptavidin magnetic beads (Streptavidin M-280 dynabeads, Invitrogen) added and incubated another 40 mins with continuous rotation. The beads with RNA-protein complex were washed with cold lysis buffer for three times for 10 mins on rotation. The bound proteins were then eluted with unlabeled corresponding RNA for overnight at 4ºC

A methanol/chloroform extraction was performed on the eluted protein to decrease lipid content and subsequently precipitated with cold TCA. Precipitated pellets were solubilized in TRIS-HCl pH 8.5 and 8M Urea. TCEP (Tris(2-Carboxylethyl)-Phosphine Hydrochloride, Pierce) and CAM (Chloroacetamide, Sigma) were added to a final concentration of 5mM and 10mM, respectively. Protein suspensions were digested overnight at 37°C using Endoproteinase Lys-C at 1:50 wt/wt (Roche). Samples were brought to a final concentration of 2M urea and 2mM $CaCl_2$ before performing a second overnight digestion at 37°C using Trypsin (Promega) at 1:100 wt/wt. Formic acid (5% final) was added to stop the reactions. Samples were loaded on split-triple-phase fused-

silica micro-capillary columns (McDonald, et al. 2002) and placed in-line with linear ion trap mass spectrometers (LTQ, Thermo Scientific), coupled with quaternary Agilent 1100 HPLCs. Fully automated 10-step chromatography run (for a total of 20 hours) was carried out for each sample, as described in(Florens et al., 2006), enabling dynamic exclusion for 120 sec. The MS/MS datasets were searched using SEQUEST (Link et al., 1999) against a database of 37466 sequences, consisting of 18556 *D. melanogaster* non-redundant proteins (downloaded from NCBI on 2012-03-08), 177 usual contaminants (such as human keratins, IgGs, and proteolytic enzymes), and, to estimate false discovery rates, 18733 randomized amino acid sequences derived from each non-redundant protein entry. Peptide/spectrum matches were sorted, selected and compared using DTASelect/CONTRAST(Tabb et al., 2002). Combining all runs, proteins had to be detected by at least 2 peptides, leading to FDRs at the protein and spectral levels of 0.63 and 0.15, respectively. To estimate relative protein levels, Normalized Spectral Abundance Factors (dNSAFs) were calculated for each detected protein distributing shared spectral counts based on unique peptides, as described in (Zhang, Wen, et al. 2010). PLGEM was used to calculate signal-to-noise (STN) ratios between samples and controls and derive p-values for significant enrichment of proteins in the immunoprecipitates (Pavelka et al., 2008).

Purification of Smaug, CG13928 and CG4612: Protein purification was performed in native condition using auto-induction system(Studier, 2005). All four genes were cloned into pDEST42 vector (Invitrogen) with C-terminal histidine-tag and expressed in BL21 *E.coli* cells (Invitrogen). Bacteria were grown in 1 liter of auto-inducing media (1% N-Z-amine AS, 0.5% yeast extract, 25 mM Na_2HPO_4, 25 mM KH_2PO_4, 50 mM NH_4Cl, 5 mM Na_2SO_4, 2 mM $MgSO_4$, 0.5% or 54 mM glycerol, 0.05% glucose, 0.2% α-lactose and pH adjusted to 7.2 using NaOH) at 25ºC to an OD^{600} of 0.5 to 0.8. Following auto-induction the cells were harvested, washed with cold phosphate

buffer saline (PBS) and lysed in cold native buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole and pH adjusted to 8.0 using NaOH). The 6xhis-tagged protein were purified in Ni-NTA resin (Qiagen), eluted with 300 mM imidazole (Sigma) and dialyzed against PBS+ 5% glycerol at 4°C for subsequent assays.

RNA binding assay: The RNA binding assay was performed as described before by Sakai et al. (Mastushita-Sakai et al., 2010). I*n vitro* synthesized biotin labeled RNAs were used to pull down Orb2. Briefly, *Drosophila* S2 cells were transfected with Orb2A and Orb2B isoforms in 6-well culture plate using Effectene DNA transfection reagent (QAIGEN) and incubated for 48 hr at 25ºC. After 48 hr of incubation 1 mL of transfected S2 cells were washed twice with PBS and lysed in 0.8 mL of RNA binding buffer (20 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1 mM EDTA, pH 8.0; 5% Glycerol, 0.1% Triton X-100; 1.5 mM DTT; 0.2 mg/mL heparin, 0.2 mg/mL yeast tRNA and 0.25% BSA) at 4ºC with continuous rotation. The lysates were centrifuged at 10,000 rpm at 4ºC for 10 mins and supernatant was collected carefully. The 3'UTR was transcribed *in vitro* in presence Bio-ATP and Bio-CTP (Enzo) and ~1 µg of RNA was added to the S2 cell lysates and incubated in presence of RNase inhibitor at room temperature for 40 mins with continuous rotation. Subsequently 100 µL of M-280 Streptavidin conjugated Dynabeads (Invitrogen) were added to each reaction and incubated for another 40 mins at room temperature. The RNA-protein complex was isolated using magnet, washed thoroughly with RNA binding buffer five times, boiled in presence of 1% SDS and run in 4-12% polyacrylamide gel (Invitrogen). The Actin88F 3'UTR was used as a negative control. The association of Orb2 with the target 3'UTR was determined by Western blot using anti-Orb2 polyclonal antibody.

Tequila 5'-3'UTR based translation reporter design: All translation reporters for *in vitro* translation experiments were designed and cloned in pBSKII vector with T7 promoter. The

reporter construct contained firefly luciferase open reading flanked with Tequila 5'UTR and different versions (wild type and mutant) of Tequila 3'UTRs. The linearized (by SalI) vectors were first transcribed with T7 mMESSAGE mMACHINE transcription Kit (Ambion) to make capped mRNA and then in the subsequent reaction the capped mRNAs were tailed by mMESSAGE mMACHINE T7 ULTRA Transcription Kit (Ambion). All transcribed reporter mRNAs were column purified using MicroSpin™ G-50 (GE Healthcare) column. After measuring RNA concentration all reporters were aliquoted in small volume and stored in -80ºC. For expression in adult fly head the reporters were cloned into pUAST-attB vector (kindly provided by Dr. Konrad Basler) and transgenic flies were created by inserting the pUAST-attB constructs in attP1 site in the second chromosome.

***Drosophila* embryo extract preparation:** Embryo extract was prepared according to the protocol described by Jeske et al.(Jeske and Wahle, 2008). Briefly, the flies were housed at 25ºC and 0-to-2hr old embryos were collected on large petri-dish containing apple juice agar medium and yeast paste. After 2-h the embryos were collected into a sieve by scraping them off from agar surface with a soft paint brush and rinsing with a stream of cold tap water. Approximately 1-2 gm of embryos was obtained per 2-h collection. Then embryos were dechorionated at room temperature by submerging them in a beaker containing 1:2 diluted sodium hypochlorite solution with periodic stirring. After hypochlorite treatment for approximately 1 minute, the embryos were washed extensively with cold tap water to remove any chlorine from the preparation. The embryos were dried using blotting paper to remove residual water. Approximately, 1 gm of dried embryos were lysed in 1 ml of freshly prepared embryo lysis buffer (30 mM HEPES-KOH, pH 7.4, 100 mM potassium acetate, 2 mM magnesium acetate, 5 mM dithiothreitol (DTT), and 1 mg/ml Pefabloc SC (Roche). The embryos were homogenized using dounce homogenizer (Kontes™Dounce) in

ice, transferred to 1.5-ml Eppendorf tube and centrifuged at 14,500Xg at 4ºC for 20 minutes. Following centrifugation the soluble cytoplasmic phase was collected and snap frozen in liquid nitrogen as 50-100 µl aliquots. Protein concentration (~30 mg/mL) of the extract was measured using BCA Protein Assay Kit (Pierce).

Generation of Baculovirus and separation of Orb2 monomer and oligomer using gel filtration: Both isoforms of Orb2 were expressed in Sf9 insect cell line using Baculovirus expression system. The viruses were generated using Bac-to-Bac Baculovirus expression system (Life technology). Briefly, full length Orb2A and Orb2B open reading frames were cloned first into TopoD vector and then into pDEST8 vector containing polyhedron promoter. The recombinant bacmids were generated in DH10Bac™ (Life technologies) using blue-white selection in X-gal plates. The high molecular weight recombinant bacmid DNA was prepared from 500ml cultures using CsCl DNA purification methods.

Approximately $1x10^6$ cells were transfected with the viral DNA and incubated for 48 to 72 hrs at 25ºC in 6-well plate. The infected cells were collected, washed with PBS and lysed in 250 µL of lysis buffer containing-150mM NaCl, 10mM Tris-HCl pH7.5, 1% NP-40 (Sigma), 0.1% Triton-X 100(Sigma), 1% SDS and EDTA-free protease inhibitor (Roche). 100ul of total cell lysate was loaded into the 2ml Superose 6 PC column equilibrated with a buffer containing 500mM NaCl and 10mM Tris-HCl pH 7.5 and fractionated using a HPLC (Smart system) system. Altogether 48 fractions (50 µL each) were collected for each lysate and alternative fractions were analyzed in 1.5% Semi-Denaturing Detergent Agarose Gel (SDD-AGE) Electrophoresis. The proteins were transferred to nitrocellulose membrane by capillary methods and probed with anti-Orb2 antibody to detect different size classes of Orb2 proteins. For subsequent use in translation assay the fractions were dialyzed against 1000ml PBS+2.5% glycerol at 4ºC for > 12 hours.

Statistical analysis: For *in vitro* and *in vivo* experiments unpaired two tailed t-test or in case of multiple samples one way ANOVA were performed. Statistical significance was determined compared to control. For the *in vitro* translation assay each time point or each experimental condition has corresponding control. The statistical significance was calculated directly from the ratio of Firefly/Renilla luciferase. For ease of visualization the data is plotted by normalizing the control to 100.

***In vitro* translation assay:** For all *in vitro* translation experiments in this study we used 50ng (~0.75pM) of wild-type and mutant reporter mRNAs. Each translation assay was carried out in 25 µL reaction volume, consisting of 50ng translation reporter, 40% (v/v) embryo extract, 16 mM Hepes-KOH, pH 7.4, 100 µM amino acid mixture (Promega), 250 ng/µl *S. cerevisiae* tRNA (Roche Applied Science), 50 mM potassium acetate, 2.5 mM magnesium acetate, 100 µM spermidine (Sigma), 20 mM creatine phosphate (Roche Applied Science), 80 ng/µl creatine kinase (Roche Applied Science), 800 µM ATP, and 100 µM GTP (Sigma). All the translation reactions contained 20U of RNase inhibitor (Invitrogen) and it was added before adding the translation reporter in the reaction. All reactions were incubated at 26°C for the time points indicated. Luciferase activity was measured using the dual-glo luciferase assay system (Promega). The assay was normalized using renilla luciferase reporter. Luciferase activity was measured in 96-well plate reader (Perkin-Elmer 1420 Multilabel Counter).

To compare the protein expression of Tequila WT and mutant reporters, 25 µl translation reactions were assembled for each reporter in 40% (v/v) embryo extract with different Orb2 background. This assay was performed by using the same amount (50ng or ~75pM) of WT and mutant reporters and maintaining similar assay conditions for all the translation reporters. After 15 min incubation firefly-renilla luciferase activity was measured using dual-glo luciferase assay system.

For *in vitro* assay with separated monomer and oligomer, we used the high molecular weight (HMW) and low molecular weight (LMW) HPLC fractions with corresponding fractions of un-transfected Sf9 cell lysate. The fractions were dialyzed against PBS with 2.5% glycerol overnight at 4°C. Translation reporters were pre-incubated for 30 min with ~100 ng total protein from each dialyzed fractions. Exactly same amount of total protein for different fractions (Orb2 monomer, monomer blank fraction, Orb2 oligomer and oligomer blank fraction) were used. Following pre incubation translation assay was performed in 40% (v/v) embryo extract at 26ºC for 15 mins and luciferase activity was measured.

To test the effect of known translational inhibitors (cyclohexamide and emetine) in our assay system we performed time course using Tequila translation reporter. Regular translation reactions were assembled in absence and presence of 0.01 µM cyclohexamide (CALBIOCHEM) or emetine hydrochloride (Sigma) and translation reactions were carried out for 0, 10, 20, 30 and 40 mins. Luciferase activity was measured using luciferase assay system (Promega).

Polysome profile of *in vitro* translation reaction: The polysome assay was performed according to the protocol describes by Zid et al. (Zid et al., 2009). For each translation reaction of 100 µL volume approximately ~150,000 cpm equivalent translation reporter was added. Translation reaction was carried out for 15 mins at 26ºC and was stopped by adding 0.002 µM of cyclohexamide and the final volume of reaction was adjusted to 200µL by adding buffer. The translation reaction was applied on the top of 7-47% sucrose gradient in resolving buffer (15 mM Tris-Cl, pH 7.4, 140 mM NaCl, 7.8 mM MgOAc-4H_2O) and ribosomal subunits were separated by centrifuging the gradients in a Beckman SW40Ti rotor at 40,000 rpm for 150 min at 4ºC. The centrifuged gradient was fractionated in a Teledyne density gradient fractionator with continuous monitoring of absorbance at 252 nm. For each gradient approximately 23-27 fractions (~ 500µL

each) were collected in 96-well mini-titter plate (Neptune, USA). To measure the radioactivity each fraction was added to 4 mL of scintillation fluid (ScintiSafe Econo 2, Fisher Scientific) and [p32] level was measured in a scintillation counter (LS6500; Beckman Coulter).

Ribosome tagging and polysome immunoprecipitation from adult fly head extract: For ribosome tagging we used the 60S ribosomal subunits Rpl10, Rpl22 and Rpl18 and 40S ribosomal subunit Rps25 because these ribosomal subunits were tagged in other systems. The tagged proteins were first expressed in S2 cells and verified whether they are incorporated into respective ribosomal subunits and if so, whether the tagged ribosomes are part of polysomes. Under our experimental conditions tagged *Drosophila* Rpl10 and Rpl22 although were incorporated in 60S ribosomes but were not in polysomes. Only HA tagged 40S ribosomal subunit Rps25 and FLAG-tagged 60S ribosomal subunit Rpl18 were part of polysomes. The tagged Rps25 and Rpl18 were than used to generate transgenic flies and expressed in all neurons or a subpopulation of neurons and the tagged ribosomes were immunopurified with anti-HA (Rps25) or anti-FLAG (Rpl18) from adult brain. Since 40S ribosomes by itself bind mRNA the FLAG-tagged 60S ribosomal subunit was used in all of our subsequent studies.

The FLAG-Rpl18 tagged ribosomes were efficiently immunoprecipitated from the brain. First, we have compared the complexity of the mRNA recovered from the FLAG-immunoprecipitate (ActinGal4:UAS-FLAGRpl18) and mRNA isolated from polysomes by conventional techniques. The mRNA comparison revealed that the tagged ribosomes indeed co-purifies with translating mRNA. To determine that the tagged ribosome indeed purifies cell type specific mRNA the FLAG-tagged Rpl18 was expressed in subpopulation in neurons, such as neuropeptide F (NPF) or octopaminergic neurons using NPF-Gal4 and Tdc2-Gal4 respectively. These neurons are well characterized allowing for easy identification of cell type specific mRNAs. Ribosomes were

isolated from these neurons and the bound mRNA was compared to that of all cells of the nervous system using RNAseq. Immunoprecipitation of FLAG-Rpl18 revealed not only neuron, but neuronal cell type specific enrichment of the mRNA in the polysomes. These results suggested like other systems, in *Drosophila* also, tagged ribosomes can be used to perform cell type specific polysome analysis.

The FLAG-Rpl18 expressing flies (4-6 days old) were collected in 1.5 mL Eppendorf tube and snap freezed in liquid nitrogen. Fly heads were separated from body by brief pulses of vortex and subsequent sieving. To assess incorporation of Rpl18 in polysomes the separated heads were homogenized in a buffer containing 50 mM Tris pH 8.0, 300 mM KCl, 10 mM $MgCl_2$, 1mg/ml heparin, 1mM DTT, 200 U/ml RNAsin, 1% triton X-100, 0.1% sodium deoxycholate, 100 µg/ml cyclohexamide and one EDTA free protease inhibitor tablet in 10 ml buffer. The lysates were incubated with continuous rotation for 30 mins at 4ºC, centrifuged at 10,000 rpm for 10 minutes at 4ºC and clear supernatant was collected carefully from the top. Total protein concentration was estimated for each fly head lysates using BCA kit (Thermo Scientific). For polysome profile analysis 100 µl of fly head lysates were applied on the top of 7-47% sucrose gradient in resolving buffer (15 mM Tris-Cl, pH 7.4, 140 mM NaCl, 7.8 mM MgOAc-4H_2O) and ribosomal subunits were separated by centrifuging the gradients in a Beckman SW40Ti rotor at 40,000 rpm for 150 min at 4ºC.

For ribosome immunoprecipitation the separated heads were homogenized in a lysis buffer containing 50 mM Tris pH 7.5, 100 mM KCl, 12 mM $MgCl_2$, 1mg/ml heparin, 1mM DTT, 200 U/ml RNAsin, 100 µg/ml cyclohexamide and one EDTA free protease inhibitor tablet in 10 ml buffer. Total 1.5 mg of protein was taken and immunoprecipitated with pre-washed 10 µL anti-FLAG affinity beads (Sigma) for 2 hrs at 4ºC with continuous rotation. After 2 hrs the beads were

washed five times with cold lysis buffer and boiled with 2X SDS-PAGE gel loading dye for 10 mins. The samples were analyzed in 4-12% polyacrylamide gel (Invitrogen). The Western blotting was performed using anti-Orb2 antibody.

Translation assay with Orb2B-TEV: Orb2B monomeric and oligomeric fractions with the corresponding control fractions were dialyzed in PBS and 2.5% glycerol. Approximately 1µg total protein from each fraction was digested with 0.1 µg protease at room temperature for 1 hr. Then translation reporters were pre-incubated with TEV enzyme treated and untreated fractions (150 ng total proteins from each fraction) for 30 mins at room temperature. Translation assay was performed in Δ*orb2* embryo extract for various time points at 26ºC. The firefly and renilla luciferase activities were measured by using Dual-Glo luciferase system.

Orb2B-TEV embryo extract was prepared following the same protocol described before. For digestion of the embryo extract, 30 µg (total protein) of embryo extract was treated with 1 µg of TEV protease for an hour at room temperature. After the enzyme treatment the translation reporters were pre-incubated with the enzyme treated and untreated embryo extract for 15 mins. Translation assay was carried out in Δ*orb2* embryo extract for different time points. To confirm whether any nonspecific degradation takes place due to the protease treatment the wild type embryo extract was also treated in parallel. The specific cleavage of orb2 protein was confirmed by Orb2 western after TEV protease treatment.

Translation with anti-Orb2 antibody treated embryo extract: To immunodeplete Orb2 protein, *Drosophila* embryo extract was treated with anti-Orb2 polyclonal antibody and control IgG. Total 10 µl (approximately 300µg) of embryo extract was pre incubated with 1 µg of anti-Orb2 antibody or control IgG in ice for an hour. The translation reaction was subsequently assembled in ice and

the reaction was carried out at 26ºC for 15 mins. Firefly and renilla luciferase activity was measured with Dual-Glo luciferase assay system.

***In vitro* translation assay with rabbit reticulocyte lysate:** All translation reactions were assembled in ice in 25 µL volumes. The reaction consists of 50% (12.5 µL) TNT lysate, 1 µL TNT buffer, 1 mM complete amino acid mix, 20U RNasin ribonuclease inhibitor (Invitrogen), 50ng (~75pM) of wild-type and mutant translation reporters and 9 µL nuclease free H_2O. The reactions were incubated at 30ºC for different time points and luciferase activity was measured using Dual-Glo luciferase assay system (Promega).

***In vitro* seeding assay.** The seeding assay with Orb2A N-terminal fragments was performed in two steps. In the first step 25 µL translation reactions were assembled in WT or Δ*orb2* embryo extract by adding 100 ng of SF9 cells fractions containing Orb2A320 oligomer or corresponding control fraction for 30 mins at 26ºC. In case of purified recombinant proteins 10 ng of recombinant protein or 10ng BSA as control was used. In the second step Tequila translation reporters were added in the reaction and the luciferase activity was measured following 30 min incubation at 26ºC (Promega).

To perform *in vitro* seeding assay using Orb2A 320mRNA instead of proteins, in the first step, 5ng of Orb2A320 mRNAs were added. For each reaction a corresponding mRNA blank control was carried out. In second step Tequila translation reporters were added in the reaction and the luciferase activity was measured following 30 min incubation at 26ºC (Promega). For longer seeding experiment the mRNAs were translated in WT or Δ*orb2* embryo extract for an hour at 26ºC and then the reactions were incubated at 4ºC for 12-72h. To test the effect of newly formed

oligomer in translation, the translation reporters were pre-incubated for 30 mins with the oligomer and followed by translation in Δ*orb2* embryo extract for 30 mins.

***In vitro* deadenylation assay:** Deadenylation assay was performed according to the protocol described by Jeske et.al (Jeske and Wahle, 2008). To minimize variation the reaction was assembled in ice. The assay consists of 5 to 20 nM [32p]-labeled polyA tailed 3'UTR, 40% (v/v) *Drosophila* embryo extract,16 mM HEPES-KOH, pH 7.4, 50 mM potassium acetate, 2.5 mM magnesium acetate , 100 µM spermidine, 250 mg/ml yeast tRNA (Roche), 80 mg/ml creatine kinase (Roche), 20mM creatine phosphate (Sigma), and 800µM ATP. Every time we used a premix containing HEPES buffer, potassium acetate, magnesium acetate, spermidine, and yeast tRNA to assemble the deadenylation reaction. The uncapped-polyadenylated 3'UTRs were synthesized using mMESSAGE-mMACHINE RNA synthesizing kit (Ambion Inc.). All deadenylation experiments were performed at 26ºC for different time points and at every time point the reaction was stopped by adding 180-190 µL stop solution (25 mM EDTA, pH 8) and making the final volume of 200 µL. Total RNA was extracted from deadenylation reaction using phenol-chloroform (Ambion) extraction method. The RNA was precipitated by adding 50 µL of 7.5 M ammonium acetate, 0.2 mg/ml glycogen (Roche) and 400 µl ethanol. The RNA pellet was air-dried and dissolved in 8 µL formamide loading dye (95% Formamide, 18 mM EDTA and 0.025% each of SDS, Xylene Cyanol, and Bromophenol Blue). The sample was denatured for 3 mins at 90ºC and RNA was separated in 6% polyacrylamide-urea gel according to a standard protocol(Rio et al., 2010). The dried gel was exposed overnight at -80ºC.

***In vitro* polyadenylation assay:** *In vitro* polyadenylation assay in *Drosophila* embryo extract was performed according to the protocol described by Olga et al. (Coll et al., 2014) with minor changes. RNA substrate was prepared by labelling Teq3'UTR with 8 adenine residues at the end

with α[p32]-CTP (3,000 Ci/mmol; 10 µCi/µL) and subsequently a m^7GpppG-cap was added in the 5' end. The labelled 3'UTR was preincubated with Orb2 oligomer and control Sf9 fractions for 30 minutes at room temperature. After pre-incubation regular translation reaction was assembled in Δ*orb2* embryo extract in ice. Reaction was carried out at 26ºC for 30, 60 and 90 minutes. Total RNA was extracted using TRIZOL (Invitrogen) and polyadenylated RNAs were separated in 8M urea-6% polyacrylamide gel.

Measuring PolyA tail by PAT assay: This PCR based method was used to measure the polyA tail length of endogenous Tequila mRNA as well as *in vitro* synthesized Tequila 3'UTR by adding in our *in vitro* cell free translation system. Total RNA was extracted by TRIZOL and phenol-chloroform extraction method. For fly head assay 10 fly heads from each fly line were used to extract total RNA. After extraction the RNA samples were treated with TURBO DNase (Ambion) for 30 mins at 37ºC and subsequently measured the RNA concentration. Total 0.5 µg of RNA was taken for each sample and mixed with 0.5 µL of oligo (dT) (Takara) in 5 µL total volume. Then the mix was incubated for 5 mins at 65ºC. The reaction was mixed with 6.5 µL of pre-warmed master mix (2 µL RT buffer, 1 µL 100 mM DTT, 0.5 µL 10 mM dNTPS, 0.5 µL 10 mM ATP, 0.5 µL T4 DNA ligase (New England Bio-lab) and 1.7 µL H_2O. Then the reaction was incubated for 30 mins at 42ºC. Afterward 0.5 µL of oligo (dT) anchor primer was added to each reaction and incubated overnight at 12ºC. After overnight incubation, 0.75 µL of reverse transcriptase (Takara) was added and incubated for 1h at 42ºC. After RT PCR 15 µL of H_2O was added and incubated for another 30 mins at 70ºC. Then regular PCR reaction was performed using Tequila forward primer as well as oligo (dT) anchor primer for 30 cycles. The PCR products were visualized in 1.5% agarose gel.

mRNA stability assay in translation conditions: To test the effect of Orb2 monomer and oligomer in RNA stability, Tequila 3'UTR was transcribed and labelled with [P32] α-CTP (PerkinElmer) using T7 mMESSAGE mMACHINE transcription Kit (Ambion Inc.). Equal amount of labelled 3'UTR (~20,000 cpm equivalent) was pre incubated in presence of Orb2 monomer, oligomer and corresponding Sf9 control proteins. After pre-incubation regular translation reaction was assembled in ice and incubated at 26ºC for different time points. For each reaction total RNA was extracted using TRIZOL and run in 6% urea-polyacrylamide gel.

ThioflavinT (ThT) binding assay with Orb2 monomer and oligomer: ThT binding assay was performed according to the standard protocol described by Chien et al. (Chien et al., 2003) with minor variation. Different amount of Sf9 lysate cell lysates (0.5, 1.0, 2.0 µg total lysate for Orb2 monomer and oligomer with corresponding blank SF9 cell lysates) were used for the binding assay. Final volume of each sample was made equal using Sf9 cell lysis buffer. Each sample of 20µL volume was added to 180 µL of 25µM thioflavin-T (Sigma) at pH 8.0 in 50mM glycine. The assay was carried out at 25ºC in 96-well fluorescence plate reader (442nm excitation and 485 nm emission) and the reading was taken automatically using SoftMax Pro software (Spectra Max M2 Molecular Devices) in every two min with 1 sec shaking between the measurements. Binding data after 5 minutes are represented in the figure.

Proteinase K digestion: Fractions containing Orb2 monomer and oligomer (0.5 µg of total protein) were digested with 0.1, 1 and 10 ng of proteinase K for two minutes at 37ºC. All reactions were assembled in ice and then incubated at 37ºC water bath for 2 mins. The enzyme activity was stopped by immediately heating the samples to 75ºC. The enzyme treated reaction mixture was passed through nitrocellulose membrane using a dot blot apparatus. The membrane was blocked in 5% milk in TBS buffer and probed with anti-Orb2 antibody.

A11 Western blot assay: For A11 western analysis, different amount of total protein (0.5, 1.0 and 1.5 μg) of corresponding Orb2 monomeric and oligomeric fractions (with corresponding SF9 control blank fractions) were dot blotted on nitrocellulose transfer membrane (Whatman). The membrane was blocked in 5% milk and incubated with rabbit A11 anti-oligomer antibody (Invitrogen) overnight at 4ºC. The same blot was also probed with anti-Orb2 antibody for the detection of Orb2 proteins.

Fourier Transform Infrared-Attenuated Total Reflection (FTIR-ATR) spectroscopy: FTIR-ATR measurements were performed with a Thermo Scientific iS10 FTIR spectrometer equipped with a diamond single-bounce ATR accessory. Measurements were taken with 64 scans integration and 2 cm^{-1} resolution. Control proteins (Ab42, Sup35-NM, Myoglobin, and Concanavalin A) were purchased from Sigma-Aldrich. For FTIR-ATR analysis, control proteins were dissolved at 1 mg/mL in phosphate buffered saline, then diluted 50% in cold methanol for precipitation for 1 hour prior to spectral acquisition. Samples were centrifuged at 16,000 x g for 10 minutes at 4ºC to consolidate the pellet. For infrared spectroscopy analysis of Orb2 monomer and oligomeric fractions total 5μg of monomer, oligomer and corresponding SF9 control fractions were precipitated with 0.5 mL cold acetone for 1 hour in 4ºC and centrifuged at 16,000xg for 10 mins at 4ºC. Pellets were transferred to the ATR accessory and allowed to dry under an ambient air stream before spectral acquisition.

Spectra were corrected for ambient water vapor by subtracting the background collected immediately after each scan scaled to fit the local shape of the spectrum in the 1600-1700 cm-1 region. The liquid water spectrum was subtracted by matching the signal at 2200 cm-1 to a liquid water spectrum via linear least squares. A linear background between 1580 and 1720 cm-1 was subtracted and the spectrum in this region was normalized to obtain the final spectra.

IP and Western blot with S2 cell lysate: To study pair wise protein-protein interaction S2 cells were transfected with 0.1 µg of Orb2 and/or target (HA- , FLAG- or Myc- tagged) DNA constructs using Effectene transfection reagent (Qiagen). After 24 or 48 hrs of transfection cells were harvested, washed with PBS and lysed in PBS based homogenization/lysis buffer (150 mM NaCl, 10 mM Tris pH 7.5, 1% NP-40) for 30 mins. The lysates were centrifuged at 10,000 rpm, 4ºC and clear supernatant was collected in fresh 1.5 mL Eppendorf tube. For immunoprecipitation 1.5-2.0 mg of total S2 cell lysate was incubated in each IP with 10 µL pre washed anti-HA , anti-FLAG, anti-Myc agarose beads (Sigma) for 2 hrs at 4ºC with continuous rotation. After 5 times washing and boiling with 2X SDS proteins loading dye, the immuniprecipitates were run in 4-12% SDS-polyacrylamide gel (Invitrogen). The IPs were western blotted with anti-Orb2 antibody. Total S2 cell lysates were also western blotted for Orb2 and Orb2 partners as loading controls.

***In vivo* single fly head translation assay:** Flies were raised in regular corn meal food at 25°C in a 12h day night cycle. For GeneSwitch-Gal4 inducible expression 3-4 days old adult flies were starved for 18-20 hrs in glass vials containing water soaked Kim wipes. Flies were subsequently transferred to 2% sucrose solution containing 200µM of RU486 (Mifepristone). After 24 hrs of incubation with RU, flies were either immediately processed or transferred to regular fly food for additional 48 hours. The flies were collected in a 1.5 ml Eppendorf tubes and snap-frozen in liquid nitrogen. The heads were separated from body by vortexing for 5-10 sec and individual heads were transferred to the wells of 96-well flat-bottom micro-titter plate (Corning, NY, USA). The heads were then crushed using pipette tips in 50 µl of PBS buffer containing 0.1% NP-40 (Sigma) and 0.1% Triton-X 100 (Sigma). 50 µL of luciferase substrate (Promega) was added in each well, incubated for 10 minutes at room temperature and luciferase activity was measured in using a luminometer.

Male Courtship Suppression Assay. For spaced training, individual males were placed in individual small food tubes (16 X 100 mm culture tubes, VWR) with a mated female for 2 hr. The female was removed and the male was left alone for 30 min. A different mated female was placed in the tube with the male for another 2 hr. The female was removed and the male again rested for another 30 min. A third mated female was introduced in the tube for 2 hr and removed at the end of the trial. Control males were treated exactly the same way except no mated females were introduced into the tube. Memory test was assayed at 5 min, 4, 15, 24, 36, 48, 60 hr after training. All tests were performed in a 1 cm courtship chamber. Fresh mated females were used for all time points. All memory tests were recorded (for 10 min) and analyzed using a customized software. The courtship index of each male was obtained by manual and/or automatic analysis of the movies by an experimenter blind to the genotype and experimental conditions.

Primers used in this study:

Teq3'UTRM1F	5-ctt tac act tta ata cct tct tac atg-3
Teq3'UTR M1R	5-tca tgt aag aag gta tta aag tgt aaa-3
Teq3'UTR M2F	5-cta att tat aag aag tag tga act tgc-3
Teq3'UTR M2R	5-agc aag ttc act act tct tat aaa tta-3
Teq3'UTR M3F	5-gct aat aaa gca gat tag aac gag ca-3
Teq3'UTR M3R	5-ttg ctc gtt cta atc tgc ttt att agc-3
Teq3'UTR M4F	5-agc ata tac tca cac ttt aat atc cac-3
Teq3'UTR M4R	5-gtg gat att aaa gtg tga gta tat gc-3
Teq3'UTR M5F	5-act cac ttt aca cta tcc acc ttc tta-3

Teq3'UTR M5R	5-gta aga agg tgg ata gtg taa agt ga-3
Teq3'UTR M6F	5-ctt taa tct cca cca cat gac tac taa-3
Teq3'UTR M6R	5-aat tag tag tca tgt ggt gga tat taa-3
Teq3'UTR M7F	5-tac atg act act aaa aga att ttg tag-3
Teq3'UTR M7R	5-cac tac aaa att ctt tta gta gtc atg-3
Teq3'UTR M8F	5-gaa ttt tgt agt gaa caa taa agc ag-3
Teq3'UTR M8R	5-tct gct tta ttg ttc act aca aaa ttc-3
Teq3'UTR PM2F	5-cta att tat aag aat gct gta gtg aac-3
Teq3'UTR PM2R	5-aag ttc act aca gca ttc tta taa att-3
Teq3'UTR PM1F	5-ttt aca ctt taa tag taa cct tct tac-3
Teq3'UTR PM1R	5-cat gta aga agg tta cta tta aag tgt-3
Teq3'UTR PolyARev:	5-ttt ttt ttt aat tgc tcg ttc taa aaa agt ctg-3
Teq3'UTRT7:	5-ttg taa tac gac tca cta tag-3
DTAdaptor:	5-gcg agc tcc gcg gcc gcg ttt ttt ttt ttt-3
DTAdaptorR:	5-cgc ggc cgc gga gct cgc-3
Teq3'UTRF:	5-act gcg gct tca aga aca ga-3
aPKC3'UTRF:	5-ctg gat gca ctt ttg gca ta-3
Neuroligin3'UTRF:	5-tag ttt atg tta ctt ttt ggt gta cga-3
Murashka5'UTRF:	5-agg atc ctc agg taa ccc aag ctg tg-3
Murashka5'UTRR:	5-acc atg gct gct gcg cac tgt tgt tg-3
Neuroligin5'UTRF:	5-agg atc cgc gca gaa gac cag agc ct-3
Neuroligin5'UTRR:	5-acc atg gcc ctg ccg agc ttc aat tg-3
aPKC5'UTRF:	5-agg atc cac ttc ggt tct ccg ctt tg-3

aPKC5'UTRR:	5-acc atg gtt gct agt aaa ata ttt tg-3
Pabp2F:	5-cac cat ggc cga tga aga ta-3
Pabp2R:	5-gta-agg agc gta gta att gg-3
Rbp6F:	5-cac cat ggt gac gag aac ga-3
Rbp6R:	5-cca ttt gta aat gcc gca gg-3
Luciferase F:	5-cca ggg att tca gtc gat gt-3
Luciferase R:	5-cac aca gtt cgc ctc ttt ga-3

cDNA Constructs used in this study:

Orb2A	PAC 5.1	no tag
Orb2B	PAC 5.1	no tag
Orb2A160	pDEST42	
Orb2A320	pDEST42	
Orb2A320F5>Y	pDEST42	
Orb2A320Δ8	pDEST42	
Orb2A320Δ8-88	pDEST42	
Orb2A320	pUAST attB ccdb	
Orb2A320Δ8	pUAST attB ccdb	
Orb2A320Δ8-88	pUAST attB ccdb	
Orb2A	pUAST attB	cHA
CG4612	PAC 5.1	cFLAG
Smaug	PAC 5.1	cFLAG and pUAST-cFLAG
Smaug	PAC5.1	cMyc

Smaug	pDEST42	cHis
Pabp2	pUAST	cHA-FLAG
Pabp2	pDEST42	cHis
CG13928	PMT	cFLAG
CG13928	pDEST42	cHis
Syncrip	PMT	cHA-FLAG
Rbp6	PAC 5.1	cMyc
CPSF	PAC 5.1	cMyc
Symplekin	PAC 5.1	cV5
Wispy Gld2	PAC 5.1	cV5
CCR4	PAC 5.1	cHA
NOT1	PAC 5.1	cHA
NOT2	PAC 5.1	cHA
NOT3/5	PAC 5.1	cHA
PAN2	PAC 5.1	cHA
PAN3	PAC 5.1	cHA
Teq5'UTRLucTeq3'UTRWT	pUAST attB	
Teq5'UTRLucTeq3'UTRΔM2	pUAST attB	
Teq5'UTRLucTeq3'UTRM2PM	pUAST attB	
Teq5'UTRLucNO3'UTR	pUAST attB	
Teq5'UTRLucTeq3'UTRWT	pKSII	
Teq5'UTRLucTeq3'UTRΔM2	pKSII	
Teq5'UTRLucTeq3'UTRM2PM	pKSII	

Renilla reporter	pBSK
Actin5'UTRLucactin3'UTR	pKSII
Neuroligin5'UTRLucNL3'UTR	pKSII
Teq3'UTRMutant1	Topo TA
Teq3'UTRMutant2	Topo TA
Teq3'UTRMutant3	Topo TA
Teq3'UTRMutant4	Topo TA
Teq3'UTRMutant5	Topo TA
Teq3'UTRMutant6	Topo TA
Teq3'UTRMutant7	Topo TA
Teq3'UTRMutant8	Topo TA
Teq3'UTR point mutant1	Topo TA
Teq3'UTR point mutant2	Topo TA

Results

Orb2 is a translational repressor:

Drosophila Orb2 has a critical role in memory persistence and it targets a number of neuronal genes involved in formation and growth of synapses as well as synaptic function (Majumdar et al., 2012; Mastushita-Sakai et al., 2010). However, the role of Orb2 in translational regulation of these mRNAs remains largely unknown. Previous studies have suggested several possibilities–including Orb2 directly regulates the translation of these mRNA targets or indirectly by affecting the stability, transport or localization of these mRNAs. Moreover, at the translational level Orb2 can act as a translational activator or repressor or both.

To test the direct effect of Orb2, if any, in translation I intended to utilize a cell free *in vitro* assay system that would allow us to examine the effect of the Orb2 in translation by adding or removing it from the assay system. We used *Drosophila* embryo extract as an *in vitro* assay system to study translational regulation. The main rationale for using embryo extract as an *in vitro* system is the expression of Orb2B isoform in early embryo (Hafer et al., 2011). Moreover many proteins that associate with Orb2 in the adult fly brain are also present in embryo (Hafer et al., 2011; Kruttner et al., 2012; White-Grindley et al., 2014; Xu et al., 2014). The translation in embryo extract is very robust (Jeske et al., 2011), which allow us to test translation of our test reporter in a short time period. I developed a dual reporter system in which firefly luciferase reports Orb2 dependent translation and renilla luciferase serves as an internal control. For *in vivo* translation same translation reporters are driven in adult fly brain using nervous system specific UAS-Gal4 system and measured luciferase enzymatic activity in single fly brain.

To develop Orb2 dependent translation reporter I first determined the sequence elements in the target mRNA that are important for Orb2 recruitment. Among several mRNA targets, I

choose the Tequila gene, the *Drosophila* orthologue of a human neurotrypsin to study Orb2-dependent translation. The rationales of choosing Tequila are- both Orb2 and Tequila are required for long-term memory formation (Didelot et al., 2006; Keleman et al., 2007; Majumdar et al., 2012), it has very short and easily manageable 3'UTR of 113 nucleotides (Fig 2.1A) and most importantly both conformational states of Orb2 efficiently interact with the Tequila 3'UTR. Tequila expression in adult fly brain is regulated by Orb2 (Mastushita-Sakai et al., 2010). A series of small deletions and nucleotide substitutions were made to map the Orb2 binding sites in Tequila 3'UTR (Fig 2.1A) and Orb2 binding was examined using RNA binding assay (Fig 2.1C). In this assay *in vitro* biotin labeled wild type and mutated 3'UTR of tequila mRNA were used to pull down Orb2 protein from S2 cell lysates and allowed us to determine the gain or loss of Orb2 interaction following mutation of various sequences in the UTR. Using this approach we identified two stretches of sequence in the 3'UTR and mutation of these sequences resulted in significant reduction in Orb2-mRNA interaction (Fig 2.1 C&D). One of these sequence is UCCACCUU/G (referred as M1 sequence) which is 90 nucleotides upstream of the 3' end and the other one is UUUUGU (referred as M2 sequence), 48 nucleotides upstream of the 3' end (Fig 2.1 A). The UUUUGU M2 element is analogous to the canonical CPEB-binding element (CPE) UUUUAU (Richter, 2007). Both nucleotide substitution mutation from UUUUGU to GCUUGU (which is denoted as point mutation M2P) and deletion of four U residues (ΔM2) significantly reduced binding of both monomeric and oligomeric Orb2 to the Tequila 3'UTR. However point and deletion mutation of M1 sequence showed ambiguous effect- while deletion mutation had no visible effect but point mutation significantly reduced Orb2-mRNA interaction. Although we do not know the exact reason we wondered whether the deletion of M1 site might have created another site that somehow favored Orb2-mRNA interaction. Nonetheless we concluded that M2P (both

deletion and point mutation) and M1P mutation attenuates Orb2-mRNA interaction and used these mutation to design reporters for *in vitro* and *in vivo* translation assay. All reporter mRNAs carried the same Tequila 5'UTR and the control reporters contained either SV40 3'UTR or Actin 3'UTR.

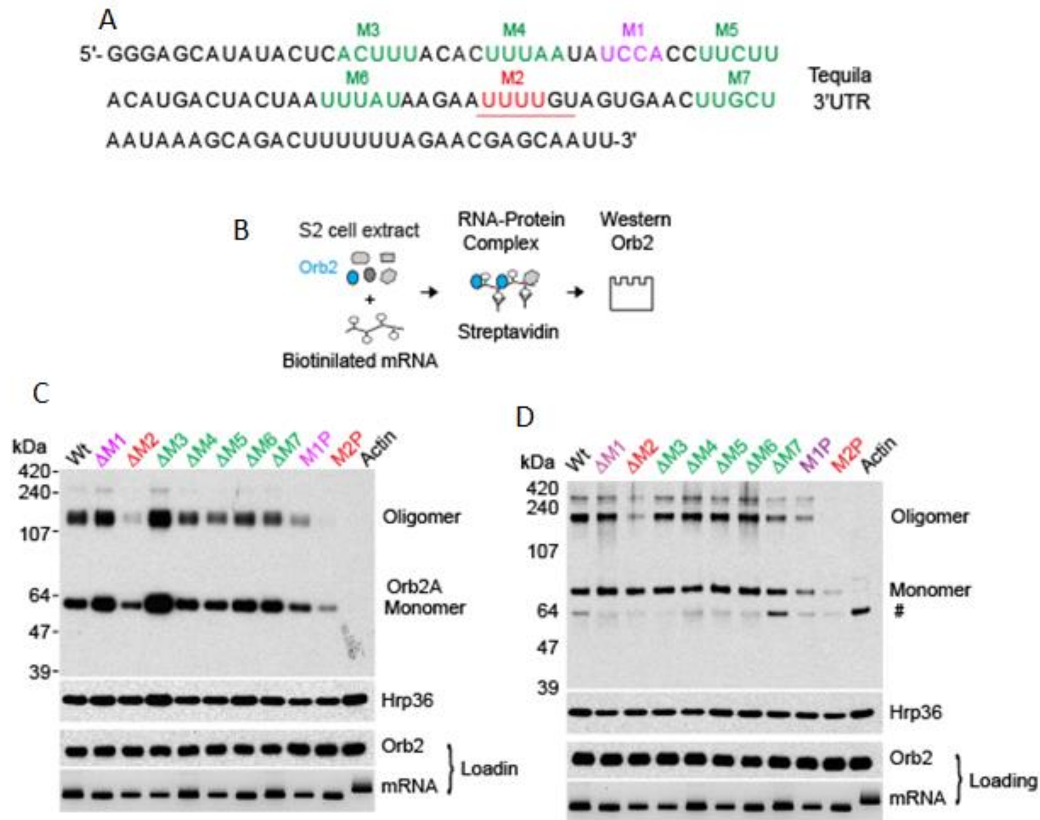


Figure 2.1: Orb2 recruitment is dependent on the conserved elements at the Tequila 3'UTR. (A) Sequence of 113 nucleotides long 3'UTR of Tequila gene. M1-M7 indicates sequences that were mutated. The putative CPE-like element UUUUGU (M2) indicated in red is required for Orb2 recruitment. A sequence (M1) conserved in other *Drosophila* species is indicated in pink. (B) Schematic of the RNA-protein binding assay using S2 cell lysate. (C) Western blot of RNA bound Orb2A protein. Deletion (ΔM2) or mutation (M2P) of M2 elements reduced interaction of both monomer and oligomer. Surprisingly not deletion (ΔM1) but mutation of M1 (M1P) sequence interfered with Orb2 recruitment. The 3'UTR of Actin88F (Actin) controls for non-specific Orb2 binding and Hrp36 serves as a control for general RNA binding. The amount of RNA and Orb2 protein used in the assay are shown below as loading. (D) Western blot of RNA bound Orb2B protein.

In vitro translation showed inverse relationship between reporter expression and the Orb2 recruitment. The reduced Orb2 interaction with the UTR resulted in higher protein expression (Fig 2.2B). The M1P, M2P and ΔM2 reporter all showed higher expression in embryo extract. We assumed that this effect in translation is primarily due to monomeric Orb2B since in early embryo monomeric form of Orb2B isoform is the predominant form of Orb2 (Fig 2.3A). Next we wanted to determine whether the reporter expression is indeed dependent on Orb2 protein present in the embryo extract. To test Orb2 dependence in our *in vitro* assay system we have applied several approaches.

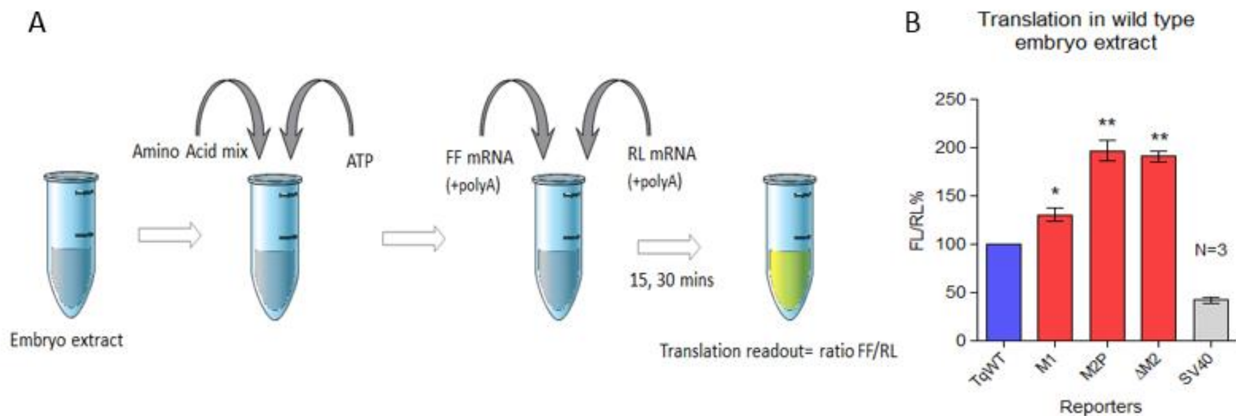


Figure 2.2: Orb2 acts as a translation repressor. (A) Schematic of the *in vitro* translation assay system from 0-2 hour *Drosophila* embryo. (B) Loss of Orb2 recruitment increases protein expression *in vitro*. These data represent normalized ratio of firefly and renilla enzyme activity of 3 independent experiments. Data is expressed as mean ± SEM. The statistical significance was measured by unpaired two-tailed t-test. * indicates p value<0.05, ** indicates p value<0.01 and *** indicates p value<0.001.

First, we prepared cell free embryo extract from flies with different genetic background that either lack Orb2 or provide a way to acutely destabilize Orb2 protein in the extract. When we expressed the wild type reporter in embryo extract prepared from *orb2* null (Δ*orb2*) flies we observed significantly higher expression compared to wild type embryo extract (Fig 2.3B).

Likewise embryo extract made from an Orb2 hypomorphic mutant flies (*Orb2ΔQ*) also showed higher reporter expression than wild type embryo extract (Fig 2.3B). Orb2 has two protein isoforms, Orb2A and Orb2B, but in early embryo primarily the Orb2B isoform is expressed (Fig 2.3A). To test whether Orb2A anyway contribute to the translation we prepared embryo extract from mutant flies which lack only the Orb2A isoform (*Orb2ΔA*) but still expresses the Orb2B isoform. There was no measureable difference in expression of wild type reporter in wild type and *Orb2ΔA* mutant embryo extract. We concluded that since Orb2B isoform is contributing to the Orb2-dependent translation in early embryo extract and can be used to test the role of Orb2A in translation by adding exogenous Orb2A.

The higher expression of the reporters in Orb2 deficient mutant extract could be the secondary consequence of chronic Orb2 protein depletion in the system. To exclude this possibility we immune-deplete Orb2 protein from wild type embryo extract by adding affinity purified anti-Orb2 antibodies. We observed enhanced reporter expression in antibody treated embryo extract (Fig 2.3C). However in this approach Orb2 antibody probably sequester the entire protein complex formed by Orb2. Therefore, we selectively targeted only Orb2 protein and measure the effect in translation. To this end our lab generated a fly line (Δorb2:Orb2-TEV) in which both copies of the Orb2 gene carried a TEV protease recognition (Kapust and Waugh, 2000) sequence at amino acid position 369. It is important to note that the Orb2-TEV fully substituted the function of wild type Orb2 and the embryo extract prepared from Orb2-TEV flies were as active as extract prepared from the unmodified wild type Orb2 flies. We prepared embryo extract from Orb2-TEV transgenic flies and the treated with TEV protease. The Orb2-TEV protein is digested within an hour of addition of protease (Fig 2.3D). The treatment of Orb2-TEV embryo extract significantly enhanced wild type reporter expression than the untreated one (Fig 2.3D). To control this experiment

similarly we treated wild type embryo extract with TEV protease and didn't see any difference in translation performed with treated and untreated embryo extract. This result suggests that acute destabilization of Orb2 protein alone is sufficient to increase reporter expression in our *in vitro* system.

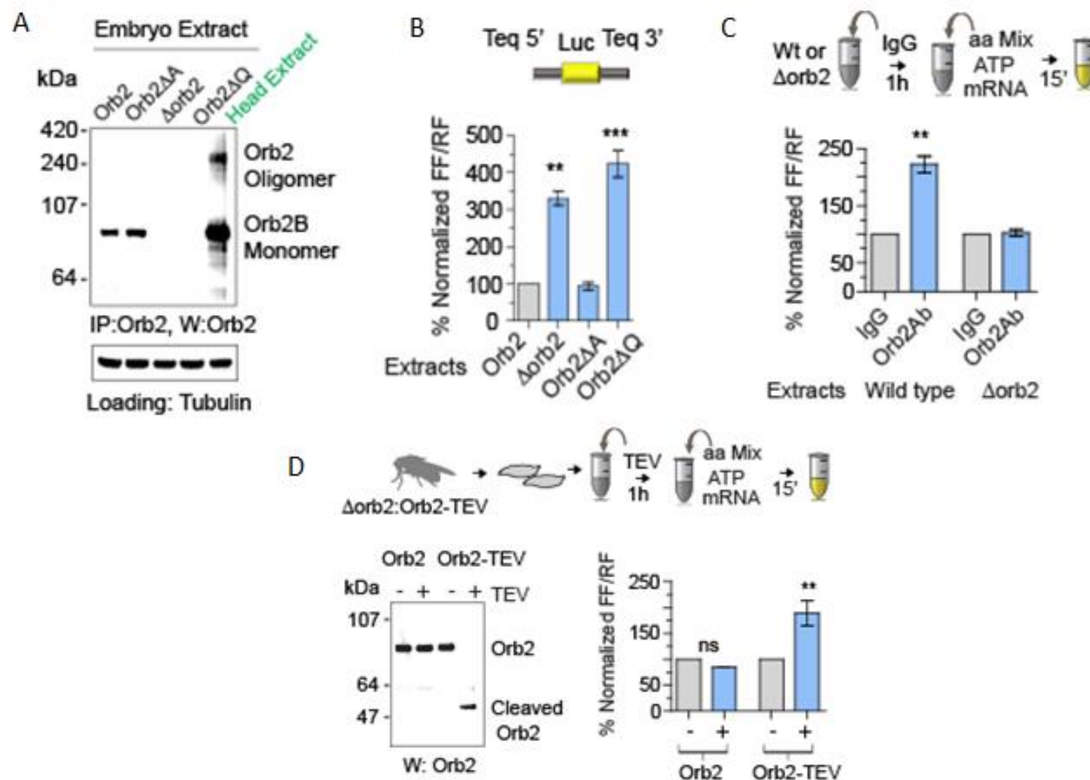


Figure 2.3: Orb2 deficient embryo extract or Orb2 inactivation enhances reporter expression. (A) Embryo extract contains monomeric Orb2B but no oligomeric Orb2. The oligomeric Orb2 is only visible in the head extract. The Δ*orb2* flies lacks the entire Orb2 locus. The *orb2*Δ*Q* lacks the 80 amino acids from Q-rich region common to both Orb2A and Orb2B and is a strong hypomorphic mutant. *orb2*Δ*A* lacks only the Orb2A-specific exon and expression of Orb2B is unaltered. Orb2A protein is not detectable in the embryo extract. Tubulin serves as loading control. (B) The absence of Orb2 enhances wild type Tequila reporter expression. (C) Treatment of embryo extract with Orb2 antibody enhances reporter expression. Addition of Orb2 antibody in extract lacking Orb2 (Δ*orb2*) has no effect. (D) Acute inactivation of Orb2 enhances translation. Top panel: Schematic of the experimental design. A TEV protease site was inserted within the Orb2 gene in a genomic rescue fragment and the genomic fragment was introduced into *orb2* null background. Left panel: Western blot analysis of TEV enzyme treated wild type and Orb2-TEV embryo extract. Right

panel: addition of TEV in Orb2-TEV, but not wild type (Orb2) embryo extract enhances reporter expression. Data is expressed as mean ± SEM. The statistical significance was measured by unpaired two-tailed t-test. * indicates p value<0.05, ** indicates p value<0.01 and *** indicates p value<0.001.

Since Orb2 has a number of mRNA targets and these targets have UUUUGU (similar to canonical CPE) sequence at their 3'UTR, we wanted to examine whether Orb2 shows common translational effect on the reporters designed using 3'UTRs of these target genes. To this end in addition to Tequila, we designed reporter using 5' and 3' UTRs of two other Orb2 target genes aPKC (atypical protein kinase C) and neuroligin (a cell adhesion protein present in synaptic membrane). Previously we found 3'UTRs of Neuroligin and aPKC efficiently interact with Orb2 protein. Both neuroligin and aPKC reporters showed higher expression in Orb2 deficient embryo extract than wild type embryo extract (Fig 2.4 A&B) (Mastushita-Sakai et al., 2010; Xu et al., 2014). These results suggest Orb2 has mRNA element (CPE) specific repression on its target mRNAs.

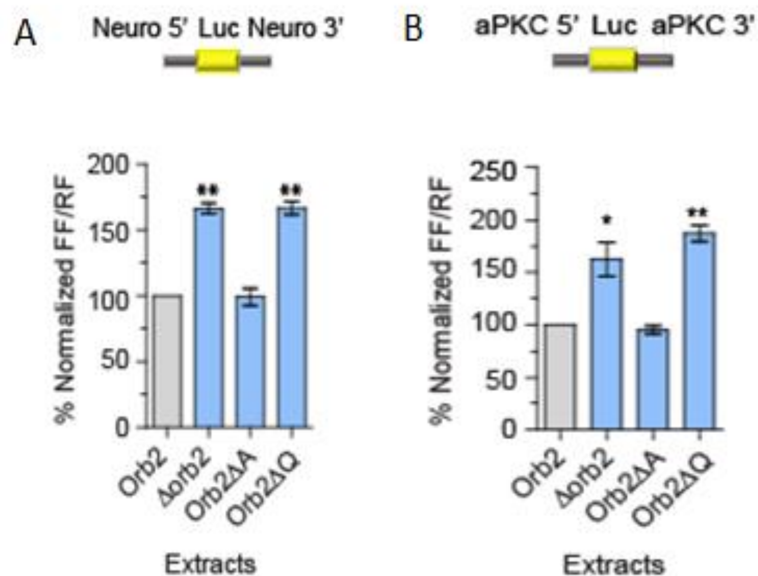


Figure 2-4: Translation of neuroligin and aPKC reporter is Orb2 dependent. (A) Expressing of neuroligin reporter is enhanced in Orb2 deficient or hypomorphic embryo extract. (B) Expressing of aPKC reporter is enhanced in Orb2 deficient or hypomorphic embryo extract. Data is expressed as mean ± SEM. The statistical significance was measured by unpaired two-tailed t-test. * indicates p value<0.05, ** indicates p value<0.01 and *** indicates p value<0.001.

To further confirm that the reporter expression is indeed Orb2 dependent we used the M2-sequence mutant reporters. Expectedly, we didn't see any significant difference in expression of wild type and mutant reporters (M2P and ΔM2) in *orb2 null* (Δ*orb2*) or hypomorphic (*Orb2*Δ*Q*) embryo extract (Fig 2.5A&B). We also expressed both wild type and mutant translation reporters in heterologous mammalian reticulocyte lysate (TnT) where Orb2 protein is not present. In TnT lysate there was no difference in protein expression between wild type and mutant reporters (Fig 2.5C). In another approach we substituted Tequila 3'UTR with the 3'UTR of Actin88F gene in the reporter construct. Actin 88F is not a target of *Drosophila* Orb2. Substitution of Tequila 3'UTR with Actin88F UTR abolished Orb2 dependent translation when the reporter was tested in WT and mutant embryo extract (Fig 2.5D). Taken together these results suggested that the reporter expression in embryo extract is indeed due to the 3'UTR sequence element as well as Orb2 protein.

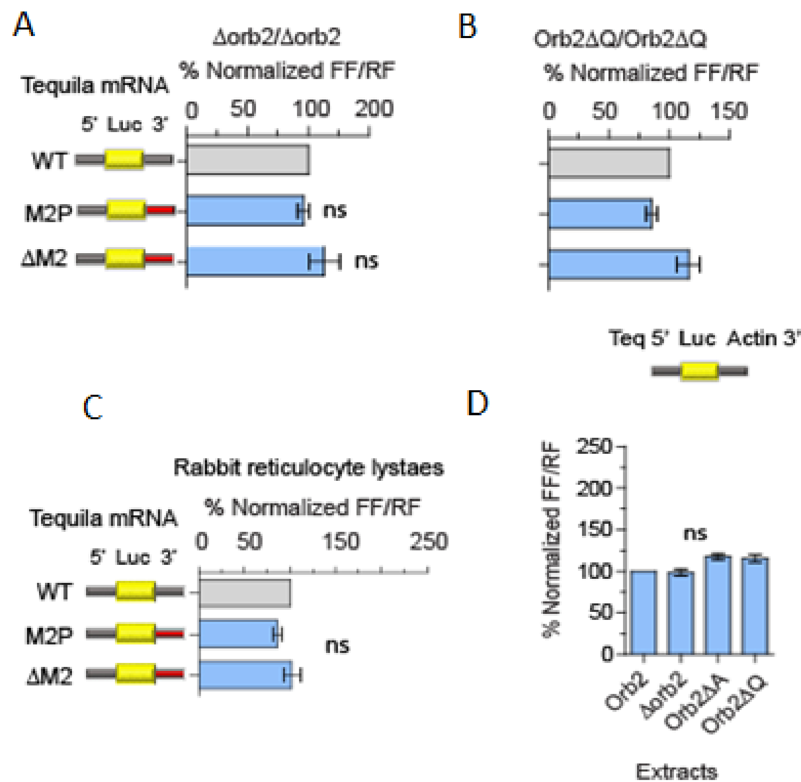


Figure 2.5: Orb2 deficient or hypomorphic embryo extract abolish differences in expression between wild type and mutant reporters. (A) In Orb2 null extract *(Δorb2)* or (B) Orb2 hypomorphic extract (*Orb2ΔQ*) the translation of wild type or M2 mutated reporter is similar indicating M2 sequence is only relevant in presence of Orb2. (C) Mutation of M2 sequence has no effect on reporter translation in rabbit reticulocyte lysates indicating that it does not play a significant role in basal translation. (D) Substitution of Tequila 3'UTR with actin88F 3'UTR abolishes the Orb2-dependent translation. The statistical significance was measured by unpaired two-tailed t-test.

In our *in vitro* assay system we observed significant differences in protein expression between wild type and mutant reporter and also we observed differences in expression between wild type and Orb2 deficient mutant embryo extract. Finally we wanted to examine whether these differences in expression are actually due to the differences in translation of the reporter constructs. To this end we performed polysome profile analysis of *in vitro* translation reaction to examine the association of reporter mRNA with actively translating 80S ribosome. We labelled the reporter

mRNA with radioactive P[32] nucleotide, added in translation reaction and compared the amount of labelled wild type and mutant reporter mRNA in the polysome fractions. Similarly we also compared the reporter mRNAs in polysomes in wild type and Orb2 null embryo extract. Polysome profile analysis of [32P] labelled mRNA revealed that the mutant translation reporter (M2P) that does not recruit Orb2 is more abundant in polysome fractions than the wild type reporter (Fig 2.6B). Likewise the translation reporter is significantly more abundant in the polysome fractions when translation reaction was performed in Orb2 mutant (*Orb2ΔQ)* embryo extract compared to the wild type (Fig 2.6C).

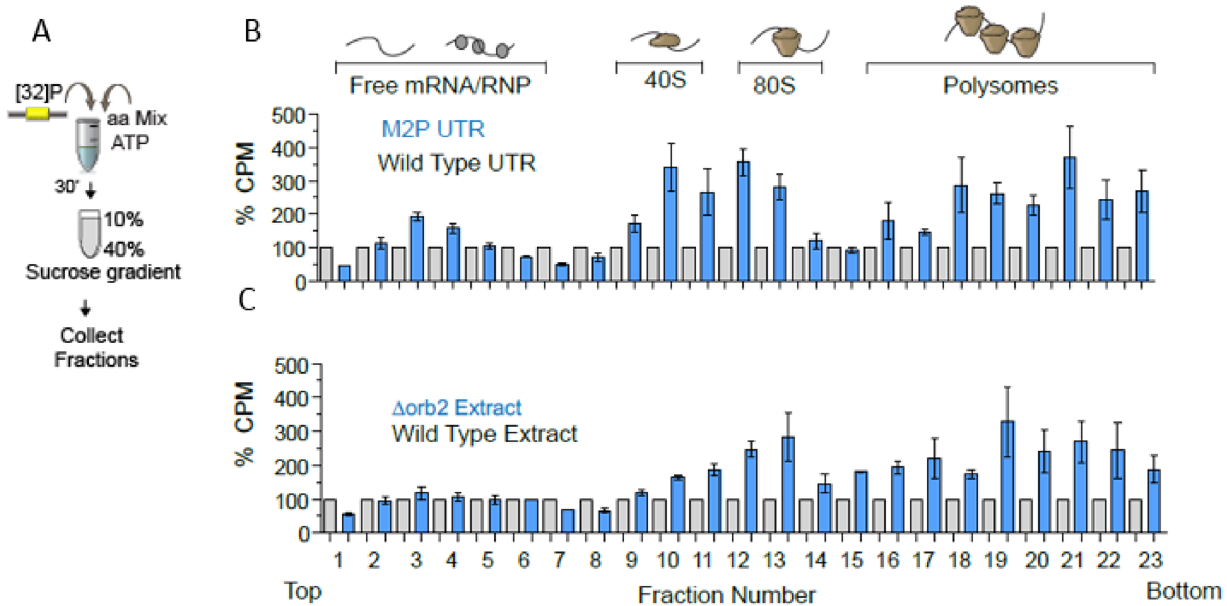


Figure 2.6: Polysome profile analysis of *in vitro* translation reaction shows the relative abundance of translating mRNA. (A) Schematic of polysome assay using [P32]-label mRNA. (B) Mutant reporter (M2P) mRNA is more abundant in polysome fractions compared to wild type. (C) Wild type reporter is more abundant in polysome when translated in Δ*orb2* embryo extract.

Finally we sought to determine whether Orb2 recruitment similarly affects Tequila reporter expression in adult fly brain. For this *in vivo* experiments we generated transgenic flies bearing wild type Tequila 5'UTR along with M1P, M2P, ΔM2 Tequila 3'UTRs and control SV40 3'UTR. All constructs were inserted into the same genomic location in the 2nd chromosome in the attP1 site using attP-attB system to control for the level of expression of the reporter transcript. The reporters were expressed using UAS-Gal4 system in adult mushroom body neurons. The mushroom body neurons are important for long-term memory of *Drosophila* and expression of both Orb2 and Tequila in mushroom body neurons is required for the consolidation of memory (Didelot et al., 2006; Keleman et al., 2007). We expressed these reporters using two mushroom body specific Gal4 drivers, MzVUm-Gal4, which drives expression in all mushroom body lobes (α, β and γ) and 201Y-Gal4 which restrict expression only in the γ-lobe. Expression of the translation reporters in adult mushroom body neurons using both UAS-Gal4 systems revealed same expression pattern, mutations that reduce Orb2 recruitment increase reporter expression (Fig 2.7 B&C).

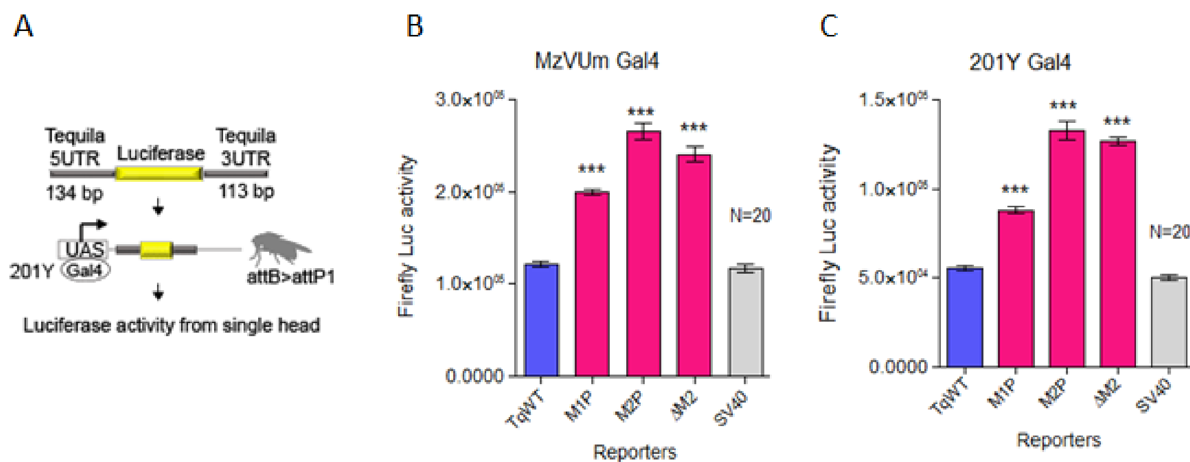


Figure 2.7: Tequila reporter expression in adult fly brain is enhanced upon M1 and M2 sequence mutation. (A) Schematic shows the reporter bearing wild type or mutated 3'UTR was expressed under mushroom body Gal4 driver 201Y. (B) Luciferase activity measured from individual fly heads under mushroom body Gal4 driver MzVum. (C) Luciferase activity measured from individual fly heads under mushroom body Gal4 driver 201Y. The statistical significance was

measured by one way ANOVA. Data is expressed as mean ± SEM. * indicates p value<0.05, ** indicates p value<0.01 and *** indicates p value<0.001.

Orb2 monomer represses and oligomer activates translation:

There are two protein isoforms of *Drosophila* Orb2; Orb2A and Orb2B. The two forms differ in structure- Orb2A has 8 and Orb2B has 162 amino acids at the N-terminal end that are isoform specific, in amount- the Orb2A protein is ~100 times less abundant than Orb2 in the fly head and in biophysical properties- Orb2A is more prone to oligomerize then Orb2B (Keleman et al., 2007; Kruttner et al., 2012; Majumdar et al., 2012; White-Grindley et al., 2014). The abundance, distribution and structure of Orb2A and Orb2B protein indicate they may have different functions. However, when we analyzed the binding of Orb2A and Orb2B with the 3'UTR of target mRNAs we did not observe any difference between the two protein isoforms. This suggests they may be functionally equivalent or the differences between the two forms arise from their differential association with other protein complexes. In addition both isoforms of Orb2, as mentioned before, have two distinct conformational states; a monomeric and a SDS-resistant amyloidogenic oligomeric state (Majumdar et al., 2012). Therefore to determine whether two isoforms and physical states are functionally equivalent or distinct we adopted the experimental strategy of adding back various forms of Orb2 in a translation extract lacking any endogenous Orb2. However, to carry out these experiments we first needed to obtain both Orb2A and Orb2B in monomeric and amyloid states separated from each other.

Isolation of monomeric and oligomeric Orb2:

Recombinant Orb2 is extremely aggregate prone and Orb2A protein rapidly assembles into oligomers even in 6M urea (Majumdar et al., 2012). However, in insect cells, both monomeric and oligomeric Orb2 can be observed suggesting proper cellular milieu is important for the protein

states. Therefore, to obtain monomeric and amyloidogenic Orb2 separated from each other, full length untagged Orb2 proteins were expressed in insect Sf9 cells (which lack endogenous Orb2) using Baculovirus. The whole cell extracts were fractionated in the presence of high salt (500 mM NaCl) and detergents (1% NP-40, 0.1% SDS and 0.1% Triton-X) using a superpose 6 PC size exclusion column. Untransfected Sf9 cells were similarly fractionated to use as a control for subsequent experiments. The Orb2 protein was recovered in fractions consistent with two distinct physical states; a monomeric and an oligomeric Orb2 and the size of the oligomeric form was similar to that of the endogenous Orb2 oligomer (Fig 3.1B). The range of oligomeric species that we have seen in our fractions is similar to that of the endogenous Orb2-oligomer (Majumdar et al., 2012).

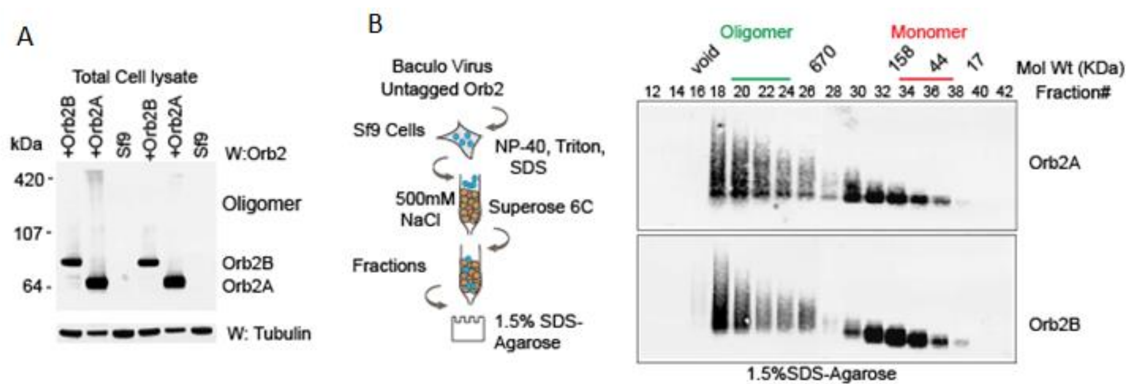


Figure 3.1: Isolation of Orb2 monomer and oligomer using gel filtration. (A) Western blot of total SF9 cell lysates (5 and 10 µg) expressing untagged Orb2A or Orb2B protein. Untransfected Sf9 lysate was used as control. Tubulin serves as a loading control. (B) Separation of untagged wild type Orb2 monomer and oligomer using gel filtration. Left panel: Schematic of the experiment. Right panel: Western blot analysis of various fractions. The relative size of the protein complexes are indicated in the top. Fractions used in *in vitro* assays are indicted with a line.

We examined several biochemical and biophysical properties of Orb2 proteins obtained from gel filtration to address whether the oligomeric Orb2 is amyloid in nature. First, the high

molecular weight Orb2 is resistant to heat and detergents (Fig 3.1 A&B). Second, fractions containing oligomeric Orb2 had significantly higher binding to fluorescence dye Thioflavin T compared to control fractions or fractions containing monomeric Orb2 (Fig 3.2A). Third, the oligomeric but not the monomeric Orb2 reacted with the amyloidogenic anti-oligomeric antibody A11 (Fig 3.2 B). Fourth, the oligomeric Orb2 but not the monomeric Orb2 produced proteinase K resistance fragments (Fig 3.2C). Finally, when analyzed by Fourier Transform Infrared spectroscopy (FTIR) the Orb2 oligomeric fraction showed higher amyloid-β structure compared to the control fraction or fraction containing monomeric Orb2 (Fig 3.2 D&E). Based on these findings we assumed that we have obtained two distinct pools of Orb2 proteins: a monomeric and an amyloidogenic oligomeric Orb2.

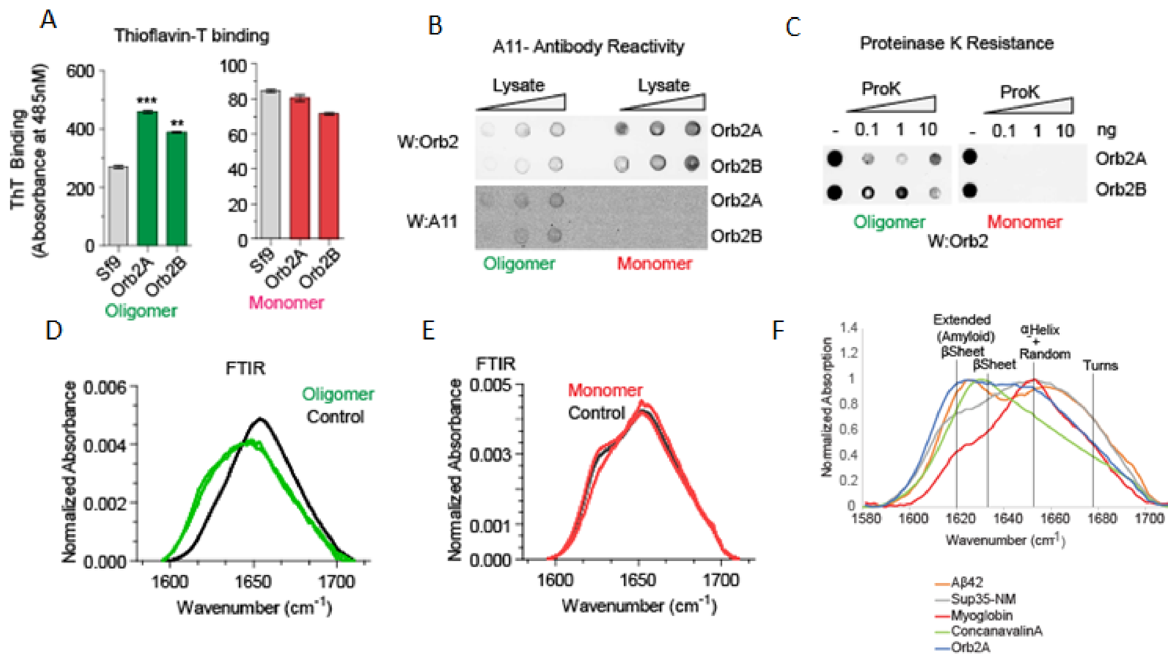


Figure 3.2: Characterization of isolated Orb2 monomer and oligomer. (A) Thioflavin T (ThT) binding of monomeric and oligomeric Orb2. Fractions containing Orb2A and Orb2B show enhanced ThT bonding compared to the control SF9 fractions. On the other hand ThT binding of monomeric fractions is similar to or less than SF9 control fractions. (B) Western-blot analysis of

Orb2 monomer and oligomer fractions using anti-oligomeric A11 antibody. A11 recognizes amyloidogenic oligomers and A11 reactivity was observed only in Orb2 oligomer fractions, but not in monomer fractions. (C) Proteinase K digestion and western blot analysis of different amounts of Orb2 monomer and oligomeric fractions. The monomer is readily digested upon enzyme treatment while the oligomer shows protease resistance. (D & E) Fourier Transform Infrared (FTIR) absorption spectroscopy analysis of Orb2 monomer and oligomeric fractions. The Orb2 oligomeric fractions show more absorption than the control SF9 fractions. (F) Control proteins for peak assignment in the FTIR experiment. The aβ42 peptide is a control for canonical amyloid, yeast prion sup35 for canonical prions, myoglobin for α-helix and concanavalinA for β-sheet. Data is expressed as mean ± SEM. The statistical significance was measured by unpaired two-tailed t-test. * indicates p value<0.05, ** indicates p value<0.01 and *** indicates p value<0.001.

Both monomeric and oligomeric Orb2 fractions were dialyzed in PBS and equivalent amount of proteins were added in null *Orb2* embryo extract. As control untransfected Sf9 cell lysate was fractionated similarly and corresponding fractions of Orb2 monomer and oligomer were used to control the effect of monomeric and oligomeric Orb2 in translation. Addition of monomeric Orb2A or Orb2B protein fractions resulted in significant repression in translation (Fig 3.3A) while addition of oligomeric Orb2 resulted in an increase in translation compared to control fractions (Fig 3.3A). Mutation of the M2 sequence reduced the monomer-dependent repression and abolished the oligomer-dependent increase in translation (Fig 3.3B). We further wanted to verify translational repression or activation by measuring the abundance of reporter mRNA associated in translationally active polyribosme in the *in vitro* translation reaction. Labelled reporter mRNAs with P[32] were added to the translation reaction in presence of Orb2 monomer and oligomer, measured the distribution of labelled mRNA in various ribosomal fractions including polyribosomes. In monomer treated reaction we observed more reporters on the top the sucrose gradient and less in polysome fractions (Fig 3.3C). On the other hand in oligomer treated reaction

more reporters mRNA was found at the bottom of the gradient indicative of mRNA association with polyribosomes (Fig 3.3C).

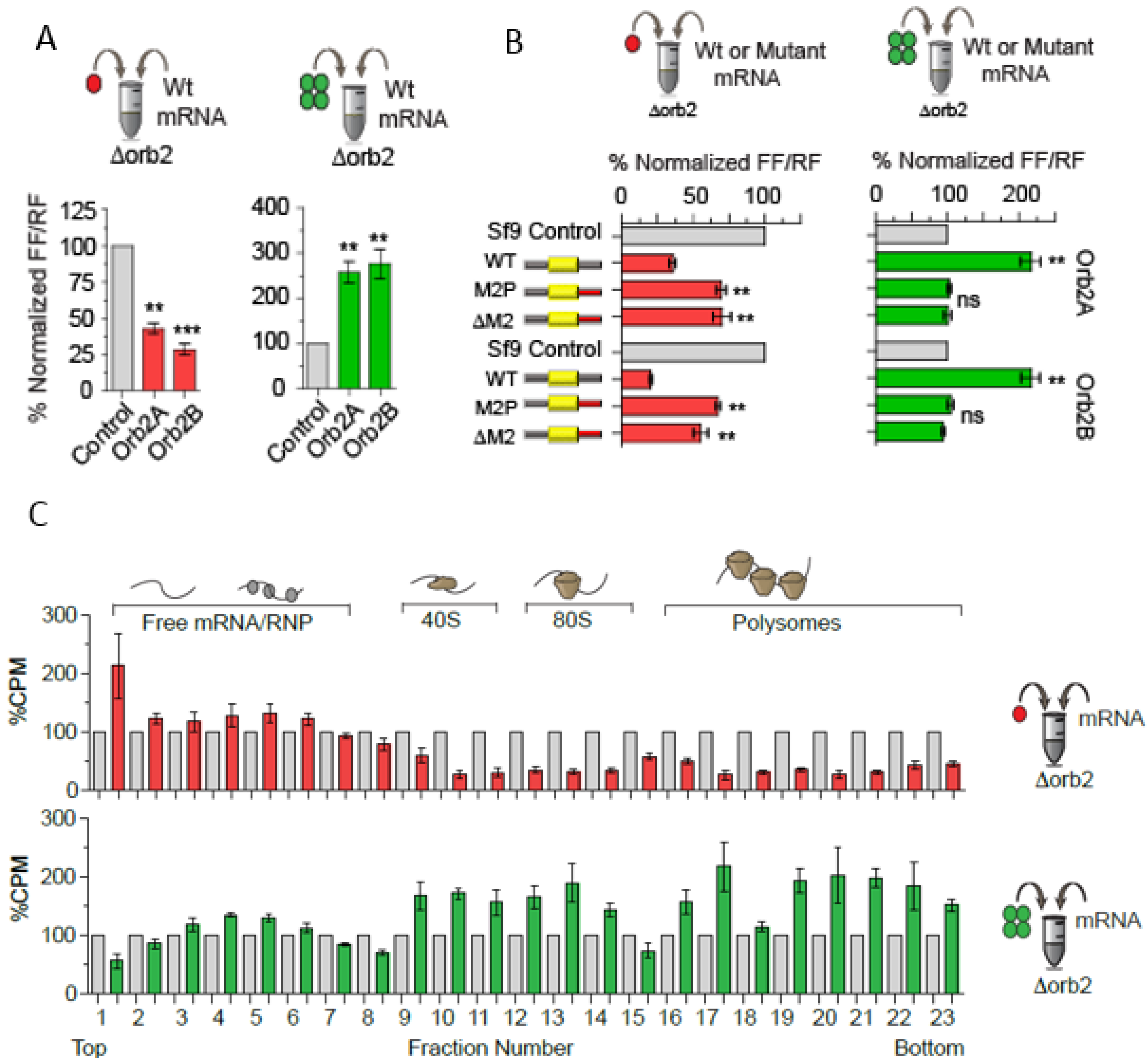


Figure 3.3: Orb2 monomer acts a translation repressor while amyloidogenic oligomer as an activator. (A) Orb2 monomer represses translation and Orb2 oligomer increases translation in Δ*orb2* embryo extract. (B) Mutations of the M2 sequence that reduces Orb2 recruitment also attenuate that translational effect of monomeric and oligomeric Orb2. (C) Polysome profile analysis of *in vitro* translation reactions shows that Orb2 monomer reduces and oligomer increases the abundance of translating mRNA in polysome fractions. Data is expressed as mean ± SEM. The statistical significance was measured by unpaired two-tailed t-test. * indicates p value<0.05, ** indicates p value<0.01 and *** indicates p value<0.001.

To confirm that Orb2 proteins in these fractions are influencing the translation but not some other co-fractionating proteins, we took the advantage of TEV digestion approach. Orb2B isoform carrying TEV recognition site was expressed in Sf9 cell and fractionated using Superose 6 exclusion column (Fig 3.4A). Similar to wild type protein, addition of monomeric Orb2-TEV reduced, whereas the oligomeric form enhanced translation. Both monomeric and oligomeric Orb2-TEV proteins were cleaved upon incubation with TEV protease (Fig 3.4B). Incubation of Orb2-TEV fractions with TEV-protease significantly attenuates the repressive function of the monomer and the activating function of oligomeric Orb2 (Fig 3.4C). Addition of TEV-protease to the wild type Orb2 had no effect. These observations supports that the Orb2 protein in the extracts is largely responsible for the changes in translation activity.

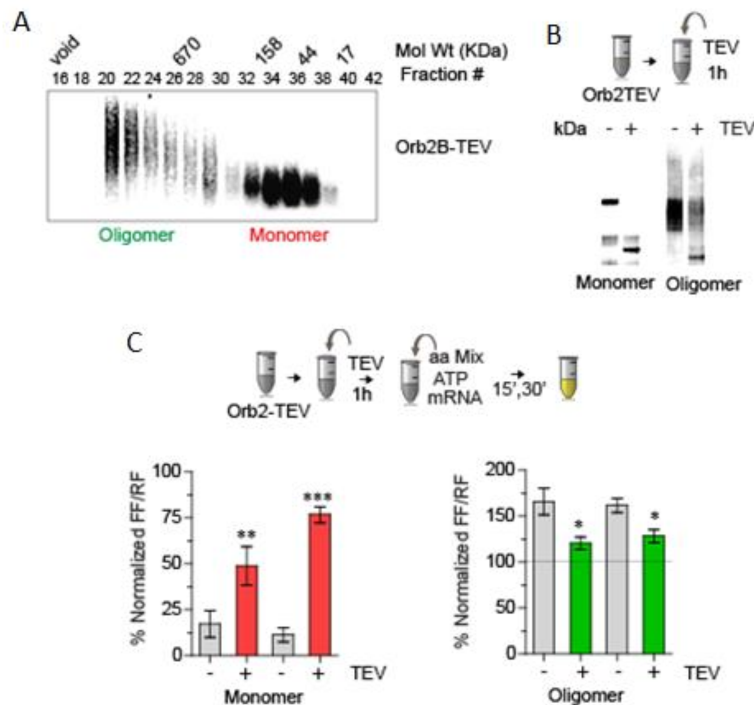


Fig. 3.4: Cleavage of Orb2 protein by TEV protease attenuates monomer dependent repression and oligomer dependent translational activation. (A) Isolation of Orb2B-TEV monomer and oligomer using gel filtration. Alternate fractions were run in SDS-agarose gel and western blotted

with anti-Orb2 antibody. (B) Western blot analysis of TEV enzyme digested Orb2B-TEV monomeric and oligomeric fractions. (C) The translation repression activity of Orb2 monomeric fraction and activating function of oligomeric fractions are mostly due to Orb2. Incubation with TEV protease that cleaves Orb2 significantly reduces (+ lanes compare to - lanes) repressive and activating function of Orb2 monomer and oligomer respectively. Data is expressed as mean ± SEM. The statistical significance was measured by unpaired two-tailed t-test. * indicates p value<0.05, ** indicates p value<0.01 and *** indicates p value<0.001.

Since Orb2 is required for long-term memory (Keleman et al., 2007; Majumdar et al., 2012) we wanted to determine whether Orb2 recruitment is required for experience dependent expression of Tequila reporter in adult fly brain. The reporters were expressed in various lobes of *Drosophila* mushroom body (Fig 3.5A) and luciferase activity was measured following courtship suppression training since this training paradigm, was previously shown, produces Orb2 dependent long-term memory (Keleman et al., 2007; Majumdar et al., 2012). The training dependent significant increase in reporter expression was observed when Tequila wild type reporter was expressed in all mushroom body lobes using MzVum-Gal4 driver (Fig 3.5 B). We also observed similar increase when the wild type reporter was expressed in mushroom body γ-lobe using 201Y-Gal4 (Fig 3.5 C). However, when the mutant reporters were expressed in the same mushroom body neurons there was no significant change in reporter expression (Fig 3.5 B&C). We also noticed that expression of wild type reporter in α-β lobe didn't show any training dependent increase in translation (Fig 3.5D). These several results suggest that Orb2 recruitment is important for training-dependent expression of Tequila in the γ-lobe neurons in mushroom body. These results are also consistent with previous studies that Orb2 activity is important in γ-lobe neurons for long-term memory (Keleman et al., 2007; Kruttner et al., 2012; Majumdar et al., 2012) and that behavioral training increases Tequila expression in the mushroom body (Didelot et al., 2006).

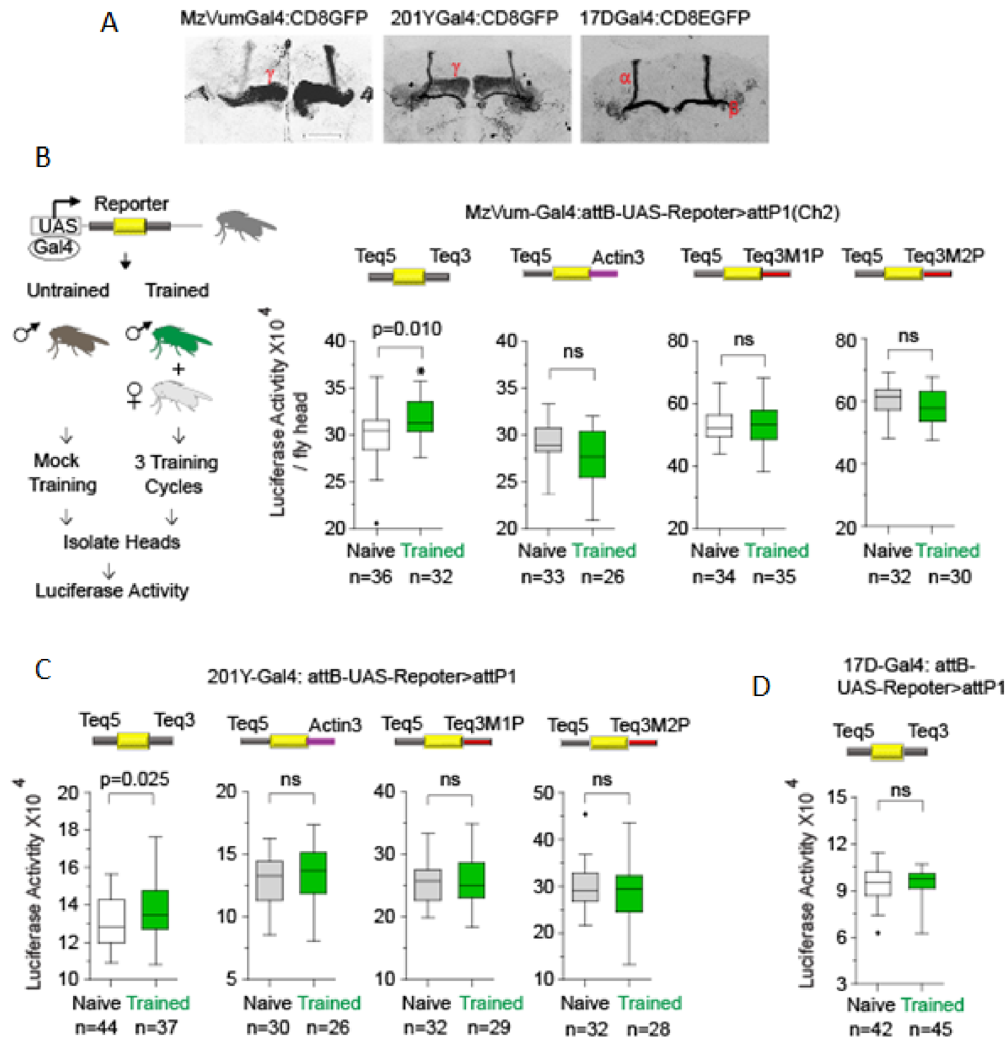


Figure 3.5: Orb2 recruitment is important for training dependent increase in Tequila expression in adult fly brain. (A) The expression patterns of various Gal4 lines. (B) Male courtship conditioning (long-term memory training) enhances wild type Tequila reporter expression. Left panel: Schematic of the experimental design. Right Panel: the training-dependent increase was observed when the reporter was expressed in the mushroom body lobes. (C) Behavioral training-dependent increase in reporter expression is specific to Tequila 3'UTR and mushroom body γ-lobe neurons. (D) Behavioral training doesn't enhance luciferase expression when the reporter expression is restricted to α/β mushroom body lobes. Mean luciferase activity ±sem: Wild type 3'UTR Naive- 300548±4986, Trained-317205± 3693; Actin 3UTR Naive- 293594±3916, Trained-276595±5713; M1P 3UTR Naive- 530199±8630, Trained-533988±9974, M2P 3UTR Naive-607567±8470, Trained- 582807±10358. Data is expressed as mean ± SEM. The statistical significance was measured by unpaired two-tailed t-test. * indicates p value<0.05, ** indicates p value<0.01 and *** indicates p value<0.001. Scale bar, 50μm.

While *in vitro* Orb2 oligomer increases translation there is very little evidence that Orb2 oligomer acts similarly *in vivo*. Therefore we sought to determine whether oligomeric Orb2 associates with actively translating polyribosomes as an indication of its involvement in active translation. To this end we have applied ribosome tagging approach, which entails tagging a protein of either the 40S or 60S ribosome, expression of the tagged ribosomal protein in a specific cell type and immunoprecipitation of ribosomes using antibodies against the tag (Doyle et al., 2008; Mustroph et al., 2009; Sanz et al., 2009) . We tagged several 40S and 60S ribosomal proteins (RPS25, RPL18, RPL22) with FLAG or HA and measured whether tagged ribosomal proteins are incorporated in the active ribosomes. We found one 60S ribosomal protein RPL18 satisfied all the criteria to be expressed *in vivo* and subsequently used for pull down from brain tissues. When N-terminally FLAG tagged Rpl18 was expressed either in S2 cell or in adult fly head the tagged ribosomal subunit was recovered in the active polysomes (Fig 3.6A). To examine the association of Orb2 oligomer with active ribosome we expressed the FLAGRpl18 specifically in the nervous system using the UAS-Gal4 system, immunoprecipitated tagged-ribosomes and probed for Orb2 proteins (Fig 3.6B). The Orb2 proteins immunoprecipitated with ribosomes are primarily heat and SDS-resistant oligomers. Treatment of the extracts with 30mM EDTA or RNaseA that are known to disrupt polysomes also reduced immunoprecipitated oligomeric Orb2.

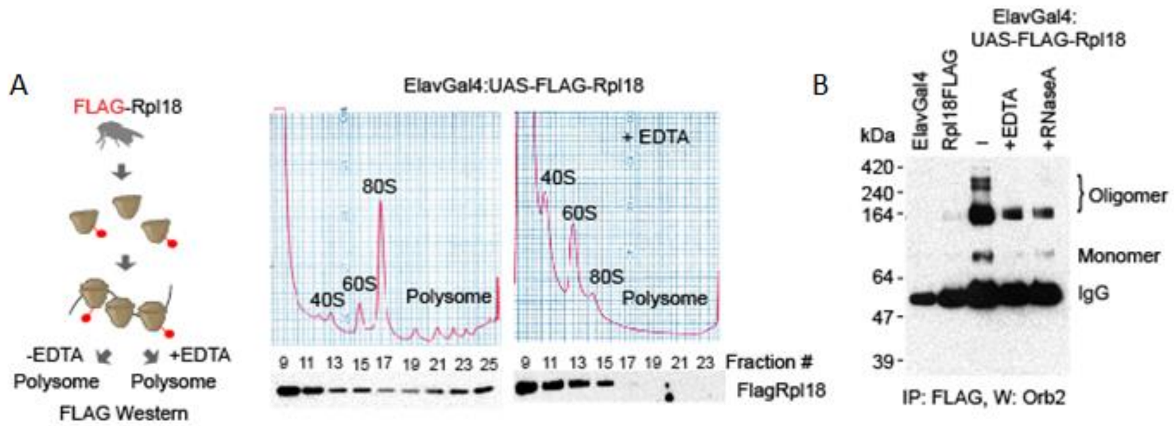


Figure 3.6: Oligomeric Orb2 associates with polyribosomes in adult fly brain. (A) FLAG-tagged 60S ribosomal protein incorporates into the functional polysomes. The left panel shows schematic of the experiment. The polysomes from adult fly brain are shown in the right. Addition of EDTA breakdowns polysomes to monosomes and free the ribosomal subunits. Alternative fraction from the gradient are blotted for the presence of FLAG tagged Rpl18 protein. (B) Primarily oligomeric Orb2 associates with FLAG-tagged 60S ribosomal subunits that are part of polyribosomes. Treatment with 30mM EDTA (pH8.0) or 20unit/ml RNaseA that disrupts polysomes also reduces oligomeric Orb2 association. The RPL18 fly head lysates are immunoprecipitated with anti-FLAG antibodies and probed for Orb2.

Orb2A isoform induces Orb2B oligomerization dependent translation *in vitro* and *in vivo*:

Previous study in our lab demonstrated that the low abundant but highly amyloidogenic Orb2A induces oligomerization of constitutively expressed Orb2B (Kruttner et al., 2012; Kruttner et al., 2015; Majumdar et al., 2012) and only the N-terminal Orb2A that contains the prion-like domain (PLD), but lacks the RRM domain, is sufficient for long-term memory (Keleman et al., 2007). The distinct functional states of the monomeric and amyloidogenic oligomeric forms of Orb2B allow us to examine the functional consequence of interaction of Orb2B with Orb2A prion-like domain. While it is evident that only the prion domain of Orb2A can support long lasting memory, it is not known whether only prion domain of Orb2A is enough to seed Orb2B isoform and cause oligomerization dependent translational enhancement.

In our *in vitro* and *in vivo* translational assay system we examined the role of first half of Orb2A protein (Orb2A320aa which lacks RRM motif) in seeding dependent Orb2B oligomerization and in parallel the role of this oligomerization in reporter translation. Similar to full length Orb2A, both OrbA320 has a monomeric and amyloidogenic oligomeric state. Addition of oligomeric Orb2A320 isolated from insect cells or from bacteria to translation extract containing monomeric Orb2B significantly enhanced translation compared to the control (Fig 4.1B). Addition of oligomeric Orb2A320 to *orb2* null extract had no effect indicating the enhanced translation was not due Orb2A320 and was dependent on Orb2B (Fig 4.1B).

The Orb2A320 protein isolated from bacteria or from insect cell may carry some other proteins or nucleic acids in addition to Orb2 that can influence Orb2B dependent translation. To rule out such a possibility instead of protein, mRNA encoding Orb2A320 was added to extract with (wild type), or without Orb2B *(*Δ*orb2*). Addition of Orb2A320 mRNA resulted in a time-dependent increase in Orb2B oligomerization and translation in wild type.

We also observed a modest increase in translation in Δorb2 extract, but it was significantly lower compared to wild type extracts (Fig 4.1C). The modest increase could result from the maternal contribution of the Orb2B protein in the orb2 null embryos. Nonetheless this experimental approach partially mimicked the *in vivo* conditions in which Orb2A320 mRNAs were expressed and provided a seed and we tested the consequence of seeding in oligomerization and in translation measuring reporter expression.

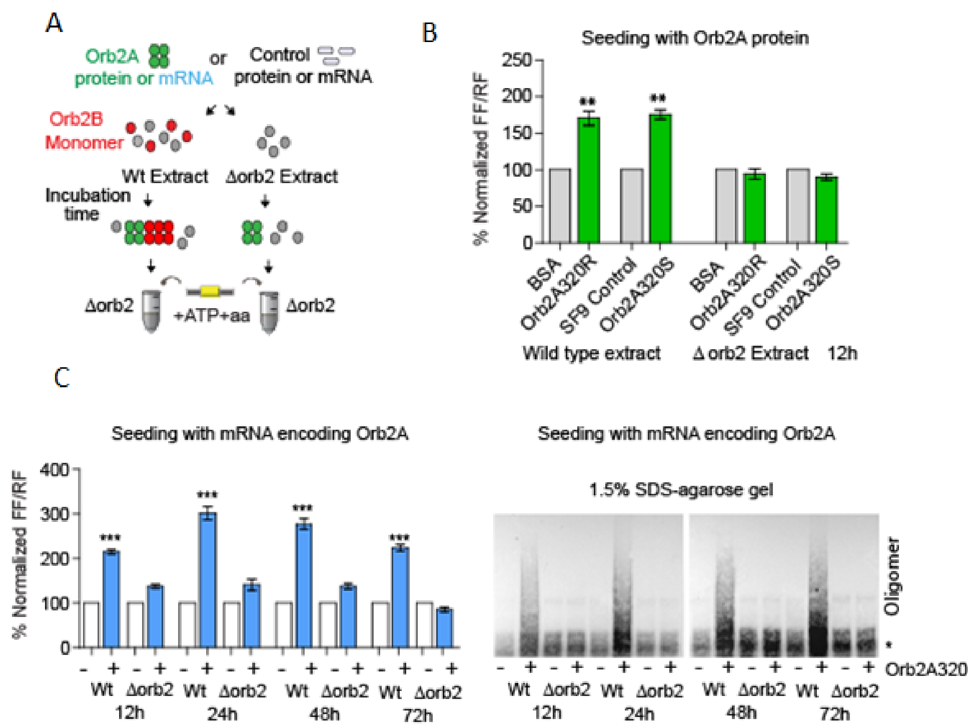


Fig 4.1: Orb2A N-terminal prion-like domain converts monomeric Orb2B to a translational activator. (A) Schematic representation of the experimental design. The wild type embryo extract serves as source for monomeric Orb2B and the Δ*orb2* extract as control. Same amount of purified BSA or similar amount of Sf9 cell extracts serve as control in each reaction. (B) Addition of recombinant (Orb2A320R) or Sf9 cell extracts containing oligomer of N-terminal 320aa of Orb2A (Orb2A320S) enhances translation only in wild type extract. The seeding reaction was carried out for 12hours at 4°C and translation was measured for 30 min. (C) Seeding with mRNA expressing Orb2A320 enhances translation and convert monomeric Orb2B to an oligomer. The seeding reaction was performed for indicated time and subsequently translation (left panel) and oligomeric

state of Orb2 (right panel) were assessed. The translation reactions were analyzed in 1.5% SDS-agarose gel and Western blotted with anti-Orb2 antibody. The * indicates an Orb2-immunoreactive band that is present in wild type and Δorb2 extract. We are unsure about its origin. Data is expressed as mean ± SEM. The statistical significance was measured by unpaired two-tailed t-test. * indicates p value<0.05, ** indicates p value<0.01 and *** indicates p value<0.001.

Next we sought to determine whether Orb2B oligomerization and translational enhancement indeed depends on the ability of Orb2A320 to induce oligomerization. To this end we made several mutated versions of Orb2A320aa mRNA to examine their effects in translation. Previously studies have found that N-terminal 88 amino acids of Orb2A is important for efficient oligomerization and deletion of either first 8 N-terminal amino acids (Orb2AΔ8) or subsequent 80 amino acids (Orb2AΔ80), or the mutation of the F5 to Y (Orb2A F5Y) that significantly reduce Orb2A's ability to form amyloidogenic oligomers (2). Similarly we observed that compared to wild type Orb2A320, mutant Orb2A mRNAs are significantly less effective in enhancing translation (Fig 4.2A), although the expression of mutant Orb2A fragments were similar to that of wild type Orb2A (Fig 4.2B). Therefore mutations that affect Orb2A's ability to form self-sustaining amyloidogenic oligomers also affect its ability to change Orb2B dependent translation. To determine how rapidly the translational enhancement can ensues instead of hours we carried out the seeding reaction for 30 minutes and observed a small but significant increase in translation (Fig 4.2 C&D).

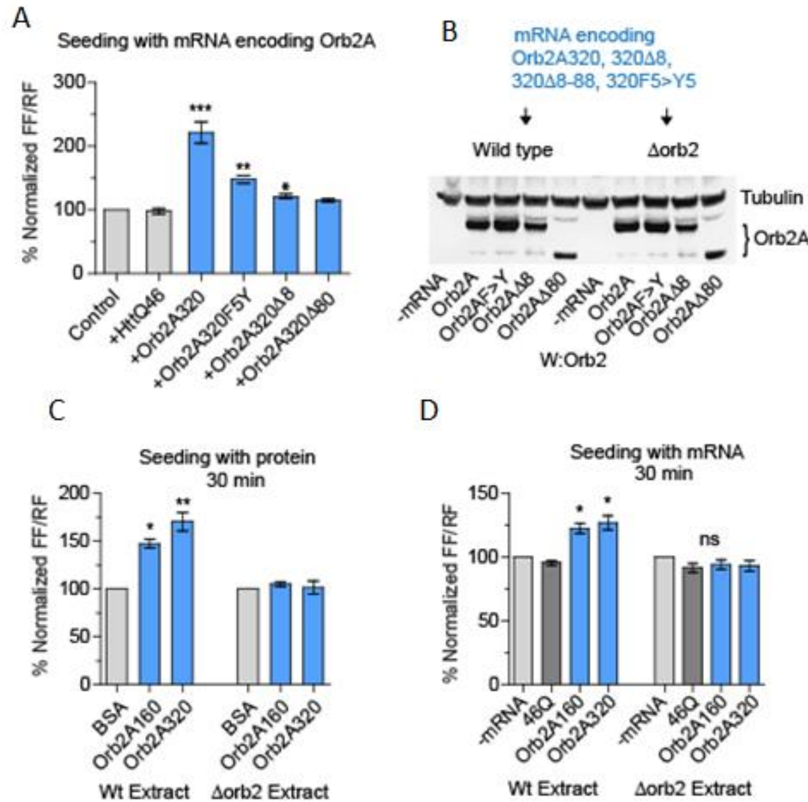


Fig 4.2: Orb2A prion domain (orb2A320aa) enhances translation *in vitro*. (A) Mutation that affects the oligomerization of Orb2A also attenuates its ability to enhance translation. Also Addition of mRNA encoding 46Q residues had no effect indicating the reaction is not influenced by any polyQ-containing protein. (B) The amount of wild type and various mutants of Orb2A320 (Orb2A320, Orb2A320Δ8, Orb2A320Δ80 and Orb2A320F>Y) in wild type or Δorb2 extracts are similar. Tubulin serves as loading control. (C) Recombinant Orb2A160 or Orb2A320 isolated from bacteria also enhances translation only in wild type but not in Δorb2 extract. (D) Transient expression of Orb2A160aa and 320aa in enhances translation. Hn46Q mRNA serves as a control. Data is expressed as mean ± SEM. The statistical significance was measured by unpaired two-tailed t-test. * indicates p value<0.05, ** indicates p value<0.01 and *** indicates p value<0.001.

Next we sought to determine whether these *in vitro* results reflect in *in vivo* fly head translation. We transiently overexpressed either full length Orb2A or Orb2A320 with the Tequila reporter in the adult fly brain (Majumdar et al., 2012). Transient expression of full length Orb2A in the adult brain resulted in a significant increase in the translation from wild type reporter mRNA.

This Oligomerization dependent increase in translation was not observed with mutated M2 reporters (Fig 4.3A). Similar to full length Orb2A protein, expression of Orb2A320 showed significant increase in translation with parallel in detectable Orb2 oligomer (Fig 4.3B). Unlike wild type Orb2A320, expression of Orb2A320Δ80 had less effect in oligomerization as well as enhancing translation (Fig 4.3B). Orb2A320Δ8 in the brain resulted in an increase translation albeit less efficiently than full length Orb2A320 (Fig 4.3B). Consistent with the increase in translation by Orb2A320Δ8 there was also an increase in amount of oligomer suggesting that the seeding may be more efficient in the brain compared to *in vitro*. This indicates seeding is influenced by factors that are either limiting or missing in the embryo extract or modification of Orb2 protein (such as phosphorylation) may influence either the seeding capacity of Orb2A or Orb2B to be a substrate. Nonetheless taken together these results suggest that the Orb2A prion-like domain can induce Orb2B oligomerization and enhance translation.

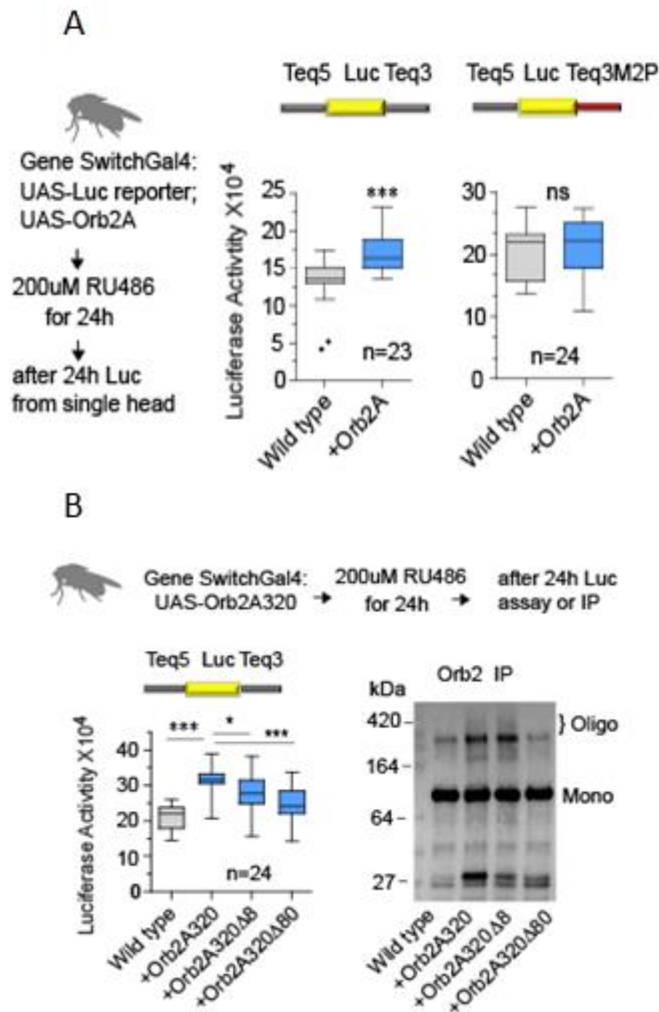


Fig 4.3: Expression of full length Orb2A or Orb2A320aa induces oligomerization dependent translational enhancement. (A) Transient overexpression of full length Orb2A in adult fly brain enhances translation of wild type but not M2 sequence mutated reporter. Schematic of the experiment is shown in the left. (B)Transient expression of Orb2A N-terminal prion-like domain enhances translation in the adult fly brain. Removal of the N-terminal 8 or 80 amino acids significantly reduces the translation enhancing activity of Orb2A (Left panel). Expression of Orb2A320aa protein in fly brain using Gene switch Gal4 enhances Orb2 oligomerization which is detectable by IP Western blot (Right panel). The statistical significance was measured by one way ANOVA. Data is expressed as mean ± SEM and * indicates p value<0.05, ** indicates p value<0.01, *** indicates p value<0.001 and ns indicates not significant.

Orb2 monomer and oligomer have distinct roles in mRNA metabolism:

CPEB is a family of RNA binding protein. In mammals there are four CPEB family members, CPEB1-4. The members of this protein family vary not only in their target but also in their mode of action. Whereas CPEB1 and CPEB4 regulate translation by regulating the length of polyA tail, CPEB2 regulates translation by interacting translation elongation factors (Chen and Huang, 2012). In addition to PolyA tail length, translation regulation by CPEB-proteins is also implicated in mRNA localization (Nagaoka et al., 2012). Our earlier studies revealed that Orb2 does not interact with the component of polyA tail machinery that regulates CPEB1 dependent translation. These observations suggest the following modes of Orb2 dependent translation that are not mutually exclusive: 1) Orb2 may regulate polyA tail length via separate mechanism such as regulating deadenylation of mRNA. Interaction of Orb2 with Smaug dependent deadenylation complex supports this notion. 2) Orb2 may affect the stability of the mRNA and 3) Orb2 may change the accessibility of the mRNA.

How does Orb2 affect the target mRNA translation? Previously we observed that Orb2 interacts with Transducer of Erb2 (Tob)(White-Grindley et al., 2014), a protein that has been implicated in mRNA deadenylation (Hosoda et al., 2011). To determine whether Orb2-regulates deadenylation, [32P] labelled Tequila 3'UTR with ~300 nucleotide long polyA tail was added into the *in vitro* extract. In the wild type 3'UTR, the polyA tail was gradually shortened with increasing time (Fig 5.1A) and mutations of the M2 sequence reduced polyA tail shortening (Fig 5.1A). In another approach we wanted to examine whether wild type and *Orb2* mutant embryo extract differentially deadenylate the Tequila 3'UTR. To test that possibility [32P] labelled Tequila 3'UTR was added both in wild type and mutant embryo extract. Addition of wild type reporter into

extracts lacking Orb2 (Δ*orb2 or Orb2*Δ*Q*) showed less efficient deadenylation compared to the wild type extract suggesting Orb2 is involved in deadenylation of the target mRNA (Fig 5.1B).

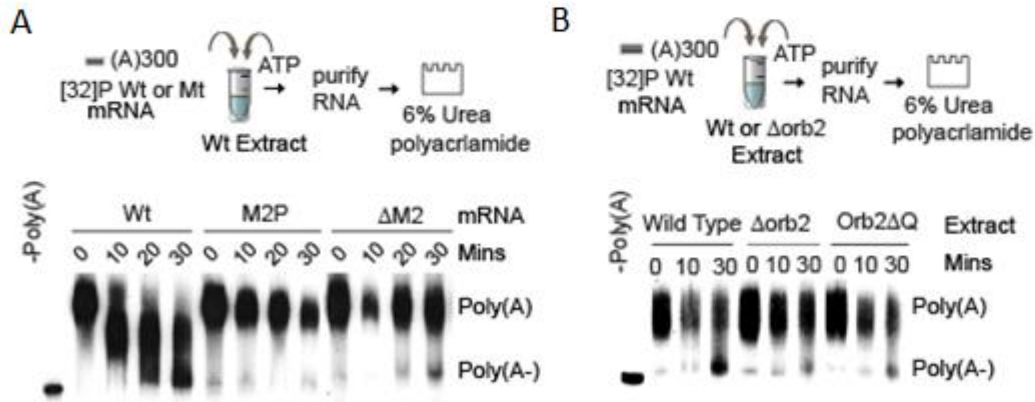


Figure 5.1: Orb2 deadenylates polyA tail. (A) Orb2 promotes deadenylation of wild type Teq3'UTR. Mutation of the M2 sequence reduces deadenylation. **(B)** Deadenylation is reduced in extracts lacking Orb2 (Δ*orb2 or Orb2*Δ*Q*) compared to wild type extract.

Since we had isolated Orb2 monomer and oligomer next we sought to determine whether the functional difference between monomeric and oligomeric Orb2 arises from their differential mRNA deadenylation activity. To this end we carried out the deadenylation assay in *orb2 null* extract in presence of exogenously added monomeric or oligomeric Orb2. Addition of monomeric Orb2 rapidly deadenylates (Fig 5.2A) while the addition of oligomeric Orb2 had no effect on polyA tail (Fig 5.2B). Taken together these results indicate that monomeric Orb2 reduces polyA tail length and eventually destabilizes mRNA.

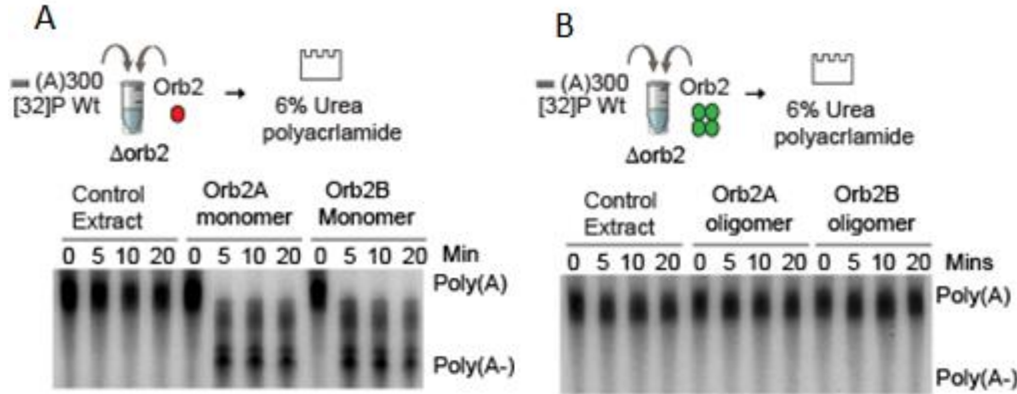


Figure 5.2: Orb2 monomer reduces whereas Orb2 oligomer stabilizes polyA tail. (A) Orb2 monomer deadenylates and destabilize the polyadenylated UTR. (B) Orb2 oligomer doesn't deadenylates the Tequila 3'UTR.

We were interested to study whether the oligomer is simply an inactive version of monomer or it actively participate in polyA tail maintenance either by protecting the existing polyA tail from decay and/or elongation of the polyA tail. Therefore instead of ~300 nucleotides, we used 8-nucleotide long polyA tailed mRNA since 8-10 adenine residues are reported to be sufficient to trigger polyadenylation (Benoit et al., 1999). Unlike long-polyadenylated mRNA the short polyA tailed mRNA was degraded in control extracts or in extract containing Orb2 monomer within 30 minutes. However, in extract containing Orb2 oligomer there was significantly less degradation of the mRNA and there was also low but significant increase in size of the mRNA (Fig 5.3A). PCR-based polyA tail length assay or PAT assay revealed that the incubation with Orb2 oligomer indeed results in longer polyA tail length (Fig 5.3B). Finally, to determine whether Orb2 affects polyA tail length of Tequila mRNA in the adult brain we transiently expressed Orb2A320 and measured polyA tail length using the PAT assay. Consistent with reporter translation and Orb2 oligomerization, expression of Orb2A320 or Orb2A320Δ8 but not Orb2AΔ80

resulted in an increase in polyA tail length (Fig 5.3C). Taken together these results indicate that oligomeric Orb2 protects/elongate polyA tail and stabilizes mRNA.

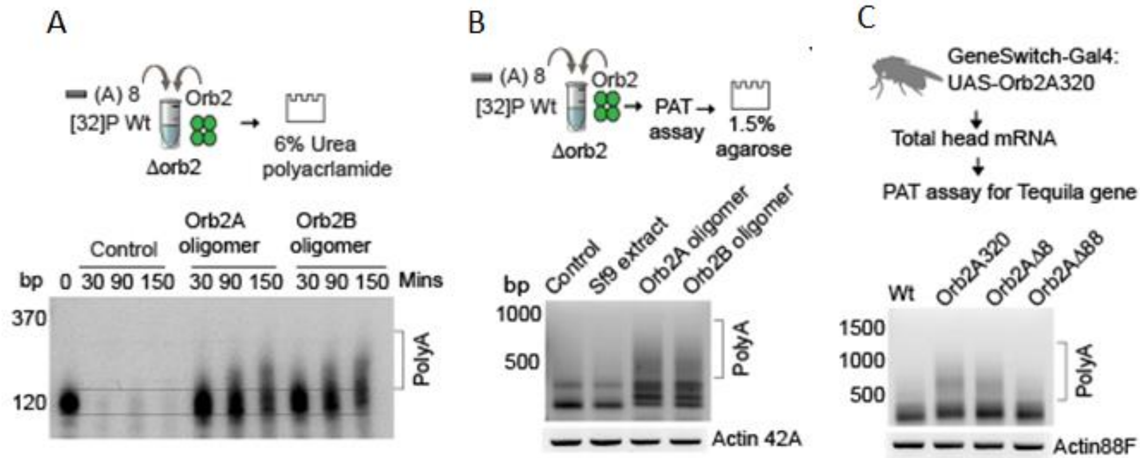


Figure 5.3: Orb2 Oligomer stabilizes and polyadenylates mRNA. (A) Both Orb2A and Orb2B oligomers stabilize and polyadenylate capped and short poly (A) tailed and p32 labeled mRNA *in vitro*. (B) PCR based PolyA tail (PAT) assay shows addition of Orb2 oligomer increases polyA tail *in vitro*. Actin serves as a control for total mRNA used. (C) Expression of Orb2A320 and Orb3A320Δ8 but not Orb2A 320Δ80 increases Tequila mRNA polyA tail length *in vivo*. Actin serves as amount of mRNA used in the reactions. For ease of visualization the position of the starting mRNA has been indicated with two lines. Addition of CG4612 itself has some mRNA stabilizing activity mostly apparent at 30 min time point.

Orb2 monomer and oligomer form distinct translation regulatory complexes:

Molecular basis of Orb2 monomer and oligomer dependent translational regulations is not well understood. Since both states require the same M2 sequence we reasoned that the two forms may form distinct protein complexes assembled on the same mRNA element. Previously we identified Orb2 interacting proteins in the adult fly head by immunopurifying Orb2-protein complex in presence of RNAse (Mastushita-Sakai et al., 2010; White-Grindley et al., 2014). These include proteins that are involved in Orb2-dependent translation as well as stability, localization or modification of Orb2. To determine the mechanism of Orb2 dependent translation we sought to identify protein complexes that are specifically assembled in the Orb2 target Tequila mRNA compared to an Actin88F control in the adult fly head using MudPIT mass spectrometry (Fig 6.1A). Comparing 6 independent controls and experimental groups we identified 29 proteins that were significantly ($p<0.01$) enriched in the Tequila 3'UTR affinity purification. Six of these proteins: CG13928, Rbp6, Pabp2, CG4612, Syncrip, and Imp- were also identified as part of Orb2 protein complex. In pairwise interaction studies, three of these proteins, Pabp2, Rbp6 and CG13928 showed strong interaction with monomeric Orb2 (Fig 6.1B).

We also identified Smaug, a known translational regulator that represses translation by promoting deadenylation (Smibert et al., 1996), as an Orb2 interacting protein. Although initially identified in the early embryo, Smaug is expressed in the adult brain (Fig 6.2A) and forms a complex with Orb2 (Fig 6.2B). We also found in our *in vitro* assay addition of recombinant Smaug reduces wild type reporter translation in presence of Orb2 suggesting that Smaug is one of the accessory factors for Orb2 mediated translation.

To understand the mechanism how Orb2 dependent translation repression is mediated we specifically focused on one of the Orb2 interacting proteins, CG13928 because, i) it interacts with Orb2 monomer both *in vitro* (Fig 6.1B) and *in vivo* (Fig 6.1C). ii) not only Orb2 it also interacts with Smaug- a known factor in translation repression by mRNA deadenylation (Fig 6.2C) and iii) it is enriched in the nervous system (Fig 6.2A)

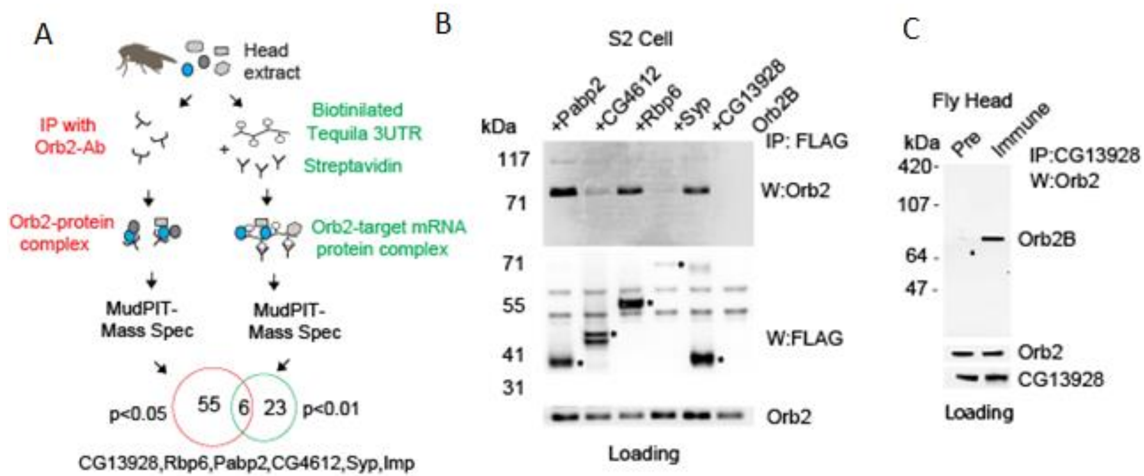


Figure 6.1: Fly head proteomics identified Orb2 interacting protein. **(A)** Schematic of the complementary proteomics analyses from adult fly head. Orb2 interacting proteins from fly head lysate were either immunoprecipitated with Orb2 antibody (previous studies) or RNA affinity purified using an Orb2 target 3'UTR. **(B)** Pairwise interaction study of the common interacting proteins revealed CG13928, Rbp6 and Pabp2 interacts better with monomeric Orb2 compare to CG4612. The low level of interaction between Syncrip (Syp) and Orb2 is due to lower expression of Syp. (C) CG13928 interacts with monomeric Orb2 in the adult fly head. Co-imminoprecipitation of endogenous Orb2 with pre immune or CG13928 serum. Lowers panels show the loading controls.

Since all three proteins (Orb2, CG13928 and Smaug) exist in the *Drosophila* nervous system and these proteins interact with each other, Orb2 interacts with both Smaug and CG13928 *in vivo* and Smaug also interacts with CG13928, we assumed these three proteins could be the components of the same translation repressor complex in fly brain.

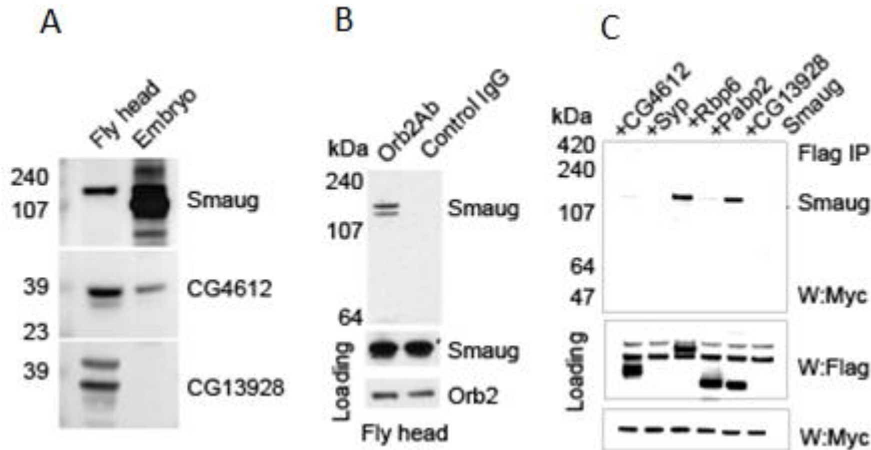


Figure 6.2: Orb2, Smaug and CG4613918 form a protein complex. (A) CG13928 is enriched in the nervous system. Detection of Smaug, CG13928 and CG4612 protein in embryo extract and in adult fly head. (B) Smaug interacts with Orb2 in fly head. The upper panel shows pull down with antio-Orb2 (rabbit Ab) and western with Smaug affinity purified antibody. The lower panels show loading for Orb2 and Smaug proteins. (C) Smaug interacts with some but not all Orb2-interacting proteins. Among the Orb2 partners CG13928 and Rbp6 strongly interact with Smaug. The upper panel shows FLAG IP and Myc western and the lower panels serve as loading controls.

To determine whether CG13928 contributes to Orb2-mediated translation repression we exogenously added purified recombinant CG13928 protein to the translation reaction (Fig 6.3A). Addition of CG13928 reduced translation in a concentration dependent manner in wild type extract but not in extracts lacking Orb2 (Fig 6.3A). When incubated with limiting amount of monomeric or oligomeric Orb2, CG13928 enhanced the translation repressive function of monomeric Orb2 (Fig 6.3B, left panel) but had no significant effect on the oligomeric activity (Fig 6.3B, right panel).

Consistent with translation repression CG13928 also enhanced Orb2 monomer-mediated deadenylation (Fig 6.3C). Similar to the *in vitro* results, overexpression of CG13928 (ElavGeneSwitch: UAS-Teq-Reporter; UAS-FLAG-CG13928) in fly brain suppressed translation, whereas knock down of CG13928 (ElavGeneSwitch: UAS-Teq-Reporter; UAS- CG13928-dsRNA) increased reporter expression (Fig S6C&D). Neither overexpression nor reduction of CG13928 had any effect on the M2 sequence mutated reporter (Fig 6.3D). Taken together these results suggest CG13928 contributes to the repressive function of monomeric Orb2.

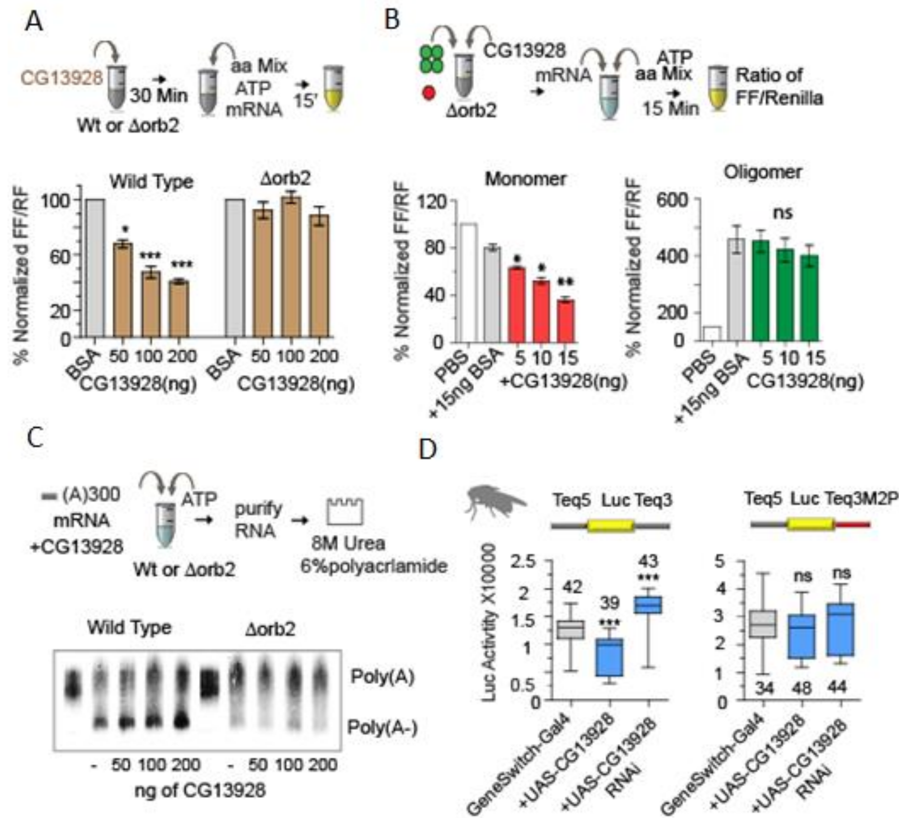


Figure 6.3: CG13928 promotes Orb2 monomer dependent translation repression and deadenylation. (A) CG13928 represses translation in Orb2-dependent manner. Increasing amount purified CG13928 proteins were added and compared with respect to equivalent amount of BSA. (B) CG13928 imparts translation repression through monomeric Orb2. Fixed amount of Orb2 (100 ng of monomer and oligomer fractions) were incubated with increasing amount of CG13928 recombinant protein. CG13928 enhanced repression but had no significant effect on oligomer

mediated translation increase. (C) CG13928 enhances deadenylation in Orb2 dependent manner. Addition of increasing amount of CG13928 protein proportionally enhances deadenylation only in wild type extract but not in Orb2 depleted *(Δorb2)* extract. (D) CG13928 negatively regulates Orb2 dependent reporter translation in the adult fly head. Translation of wild type and M2 mutant reporters were measured from single fly head expressing FLAG-tagged CG13928 or CG13928 RNAi under RU484 inducible Gene-switch Gal4. Overexpression of CG13928 represses while reduction in CG13928 enhances translation from wild type reporter but had no effect on M2 mutant reporters. The statistical significance was measured by one way ANOVA. Data is expressed as mean ± SEM and * indicates p value<0.05, ** indicates p value<0.01 and *** indicates p value<0.001.

What is the underlying biochemical mechanism of monomeric Orb2 mediated translation repression and mRNA deadenylation? Smaug-Caf/Tob-Not-CCR4-PAN is a major deadenylation and translation repression complex and Orb2 interacts with Smaug and Tob (Bawankar et al., 2013; Braun et al., 2011; Hosoda et al., 2011; Jeske et al., 2011; Wahle and Winkler, 2013). Therefore we performed pairwise interaction studies between Orb2 and individual components of Ccr4-Not and Pan2-Pan3 deadenylation complexes. In this experiment individual components (HA-tagged) of Ccr4-Not and Pan2-Pan3 complexes were pair wisely transfected in S2 cell both in absence and presence of CG13928. After 48 hrs of transfection, S2 cell lysates were made and immunoprecipitated with anti-HA beads and probed the western blot with anti-Orb2 antibody. We observed that monomeric Orb2 interacts with Not-CCR4-PAN weakly and CG13928 enhances the association of Orb2 with the deadenylation complexes (Fig 6.4 A&B for Ccr4-Not and Fig 6.4 C&D for Pan2-Pan3 complex). These several results suggest that a monomeric Orb2-Smaug-Not-CCR4-PAN complex mediates deadenylation and translation suppression of Orb2 target mRNA. CG13928 enhances the interaction between monomeric Orb2 and deadenylation complex. The low affinity of the oligomeric Orb2 for CG13928 and deadenylation complex occludes the recruitment of deadenylation complex and thereby protects the polyA tail.

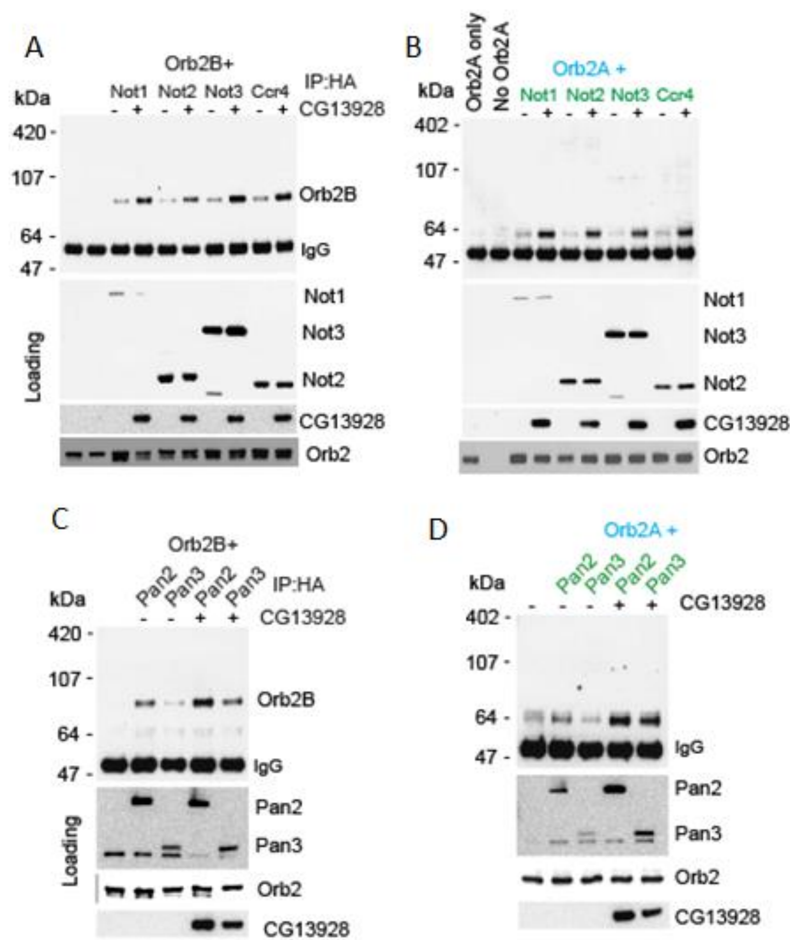


Figure 6.4: CG13928 enhances recruitment of Not-Ccr4 and Pan2-Pan3 deadenylation complex to Orb2. (A &C) CG13928 enhances the recruitment of Ccr4-Not and Pan2-Pan3 deadenylation complex to monomeric Orb2B. HA-tagged Not-CCR4 complex were immunoprecipitated and Western blotted for FLAG-tagged CG13928 and untagged Orb2. (B&D) CG13928 enhances the recruitment of NOT-CCR4 and PAN2-PAN3 deadenylation complexes to monomeric Orb2A.

To understand the molecular basis of oligomeric Orb2 dependent translational activation we focused on the Orb2 interacting protein CG4612 identified from fly head proteomics. We wanted to study CG4612 protein in Orb2 oligomer dependent activation for the following reasons: i) it is a putative polyA binding protein (PABP) and PABP proteins are known to promote polyadenylation and enhance translation (Benoit et al., 1999; Kim and Richter, 2007; Wahle et al.,

1991) and ii), unlike CG13928, CG4612 binds to oligomeric Orb2 (Fig 6.6A). Addition of purified CG4612 resulted in a small but significant increase in translation of the oligomeric Orb2 dependent translation (Fig 6.5A) but no such increase in translation was observed in presence of monomeric Orb2. One plausible explanation of the small increase in translation by exogenously added CG4612 protein is that the translation extract already contained CG4612 (Fig 6.2A). To determine whether CG4612 is required for translation enhancing activity of Orb2 we measured Orb2 oligomer dependent translation in presence of anti-CG4612 antibodies. Addition of affinity purified anti-CG4612 antibody but not control IgG significantly reduced oligomeric Orb2 mediated translation (Fig 6.5B) but had no effect on monomer-mediated translation inhibition. To determine whether CG4612 affects Orb2 oligomer mediated mRNA stability and polyA tail length, Tequila 3'UTR with short polyA tail was incubated with oligomeric Orb2 in presence or absence of CG4612. Consistent with small increase in translation there was also a similar increase in the amount of long polyA tailed mRNA (Fig 6.5C). It is important to notice that CG4612 alone had shown some extent of stabilization and polyadenylation effect when the 3'UTR was pre incubated with CG4612 alone in absence of any Orb2 oligomer (Fig 6.5C).

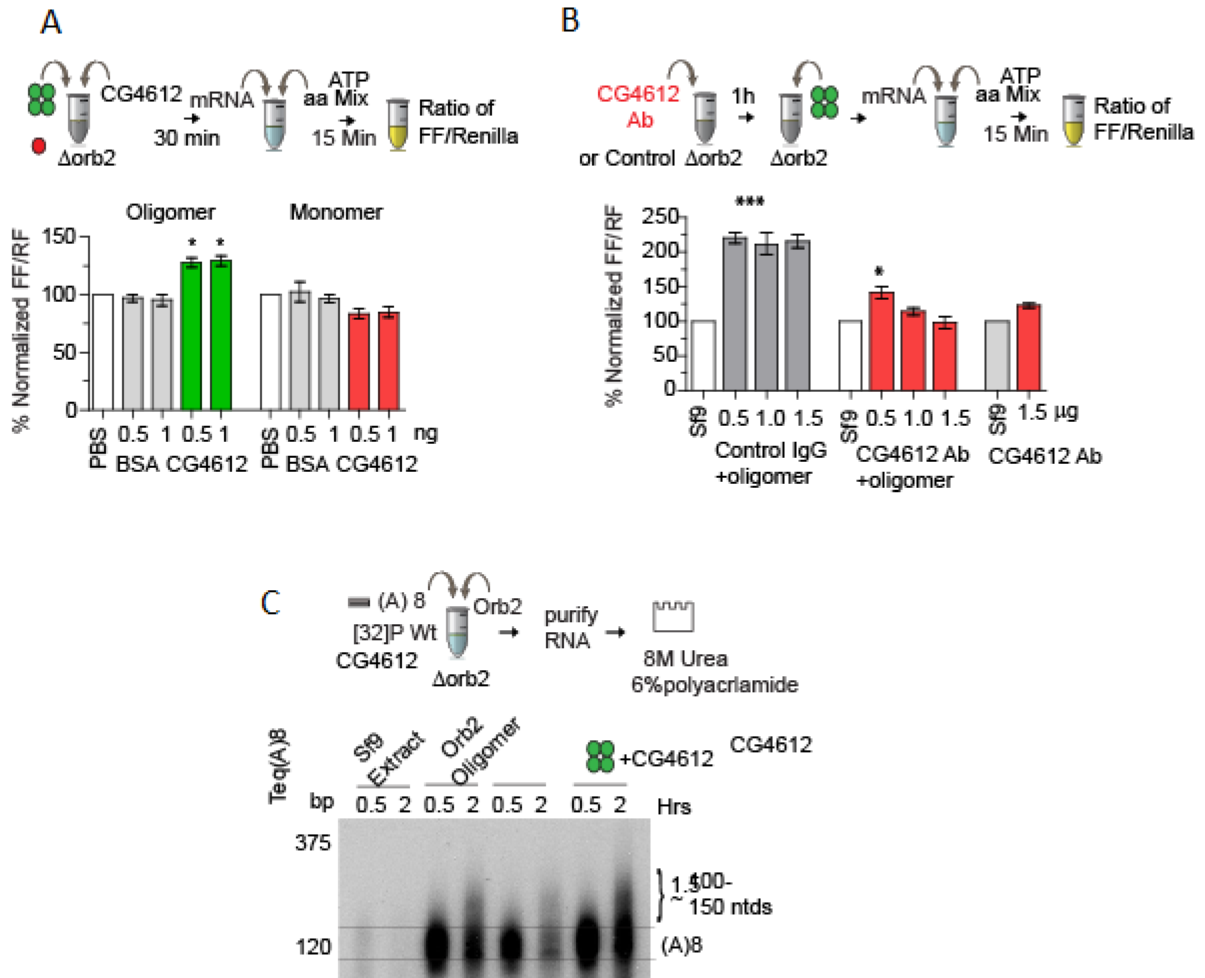


Figure 6.5: CG4612 contributes to Orb2 oligomer mediated translation activation. (A) Recombinant CG4612 enhances translation only when combined with Orb2B oligomer but not monomer. (B) CG4612 activity is required for Orb2 oligomer mediated translation increase. Incubation of the translation extract with anti-CG4612 antibody significantly reduces the translation enhancing activity of oligomeric Orb2. Addition of similar amount of purified control IgG had no effect. Likewise addition of CG4612 antibody did not result in a general translation inhibition. (C) CG4612 enhances Orb2 oligomer-mediated polyadenylation and stabilization of short polyA tailed mRNA. Limiting amount of Orb2 oligomers were used to score the effect of CG4612. For ease of visualization the position of the starting mRNA has been indicated with two lines. Addition of CG4612 itself has some mRNA stabilizing activity mostly apparent at 30 min time point. Data is expressed as mean ± SEM and * indicates p value<0.05, ** indicates p value<0.01 and *** indicates p value<0.001.

The canonical CPEB, CPEB1, upon binding to the UUUUAU element (the canonical CPEB-binding element) recruits a protein complex comprised of cleavage and polyadenylation specificity factor CPSF160, Symplekin and polyA polymerase Gld2 (germ-like development-2) (Kim and Richter, 2008; Kwak et al., 2008; Rouhana et al., 2005; Udagawa et al., 2012). This complex elongates the polyA tail length and enhances translation (Ivshina et al., 2014; Kim and Richter, 2007). Since oligomeric Orb2 binds to a U-rich sequence UUUUGU and controls polyA tail length we asked whether Orb2 associates with CPSF-Symplekin-Gld polyA complex. Orb2 by itself interacted weakly with CPSF160, Symplekin and Glds. Therefore we asked whether CG4612 interacts with polyA components and found both CPSF160 and Symplekin binds to CG4612 (Fig 6.6B). Intriguingly, two polyA polymerases, Wispy (X-Gld) and Gld2 don't interact with Orb2 but strongly associate with Orb2 oligomer in presence of CG4612 (Fig 6.6C). Taken together these results suggest CG4612 facilitates the recruitment of CPSF160-symplekin-Gld2 to the oligomeric Orb2, which elongates and/or maintains the polyA tail to enhance translation.

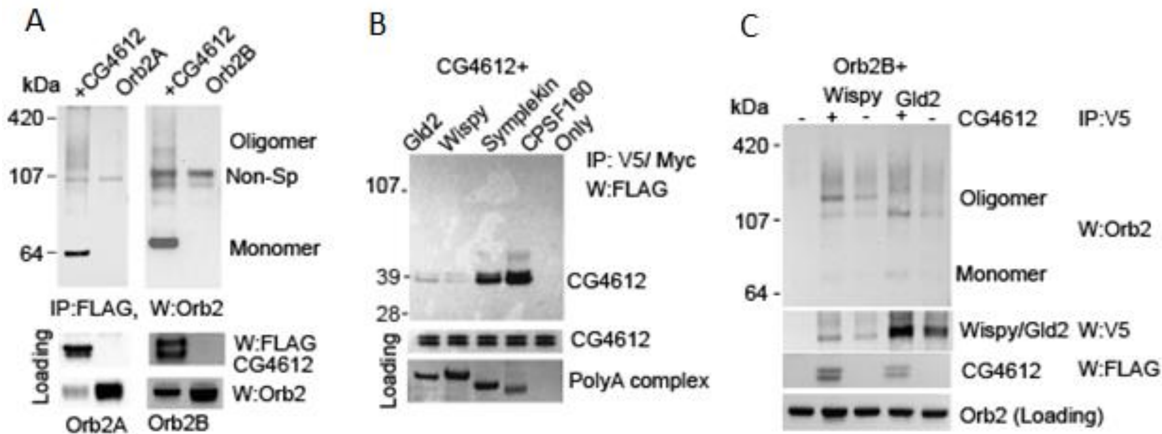


Figure 6.6: CG4612 form complex with Orb2 and cytoplasmic polyadenylation factors. **(A)** CG4612 interacts with Orb2 monomer and oligomers. Untagged Orb2 was co transfected with FLAG-tagged CG4612 and FLAG immunoprecipitate was western blotted for Orb2. **(B)** CPSF160 and Symplekin interact with CG4612. Myc-tagged CPSF160, Symplekin and V5-tagged Gld

proteins were immunoprecipitated and Western blotted for FLAG-tagged CG4612. (**C**) The Gld proteins associate with oligomeric Orb2.

Since Orb2 oligomerization is important for long-term memory we sought to determine whether CG4612, which is involved in Orb2 oligomer dependent translation, is important for long-term memory. To this end we expressed dsRNA against CG4612 in the mushroom body γ-lobe neurons using a 201Y-Gal4 driver and tested these flies using male courtship suppression paradigm to score memory phenotype (Fig 6.7 A&B). In this paradigm male flies learn to suppress their courtship behavior upon repeated exposure to an unreceptive female. The 201Y-Gal4: UAS-CG4612RNAi flies developed normally and when tested they did not display a deficit in short-term memory or memory up to a day. However, when tested 48 hours after training there was significant impairment in learned suppression of male courtship (Fig 6.7A). The memory phenotype due to impairment of CG4612 activity is reminiscent of the phenotype observed with selective perturbation of Orb2 oligomerization (Majumdar et al., 2012).

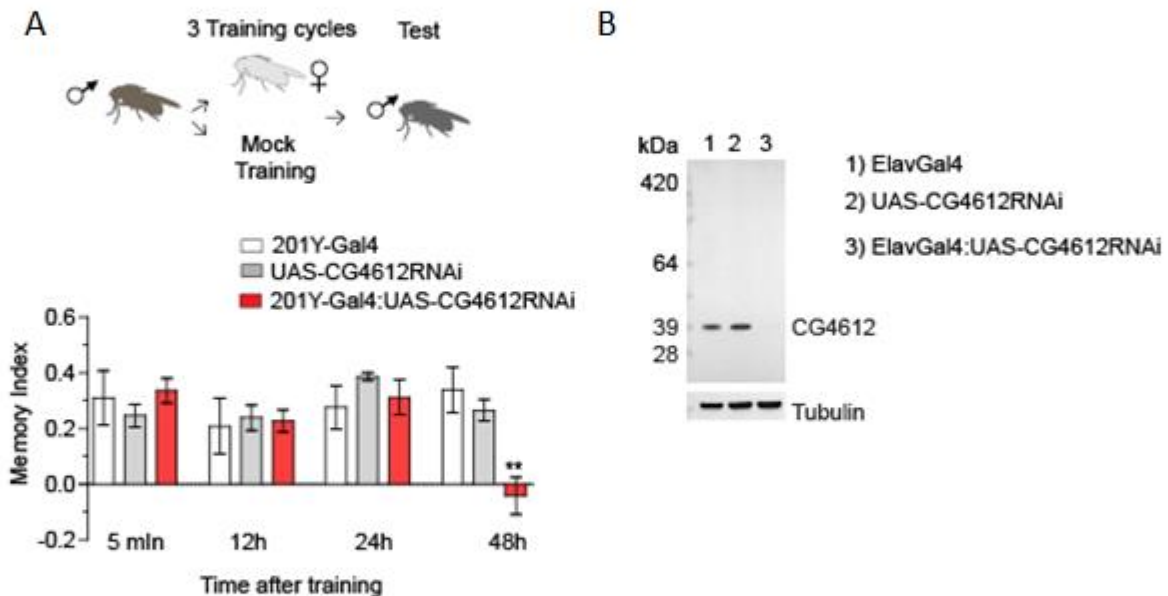


Figure 6.7: CG4612 is required memory persistence beyond 24 hrs. (A) CG4612 is required for long-term male courtship suppression memory. Flies expressing CG4612 RNAi had similar short-term and intermediate term memory but had significantly reduced long-term memory measured at 48h. (B) Expression of CG4612RNAi reduces CG4612 protein level. CG4612RNAi was expressed pan-neuronally and Western blotted for CG4612 protein. Tubulin was used as a loading control. The statistical significance was measured by one way ANOVA. Data is expressed as mean ± SEM and * indicates p value<0.05, ** indicates p value<0.01 and *** indicates p value<0.001.

Similarly we also examined the effect of CG13928 in fly male courtship suppression memory by expressing CG13928 RNAi in the mushroom body γ-lobe neurons using a 201Y-Gal4 driver. The 201Y-Gal4: UAS-CG13928NAi flies showed significant deficit in both short-term and long-term memory (Fig 6.8A). According to the findings in this study CG4612 is not required for early memory but required for the persistence but on the other hand CG13928 is required earlier in consolidation and memory formation. But we are not sure whether this early memory deficit is Orb2 dependent.

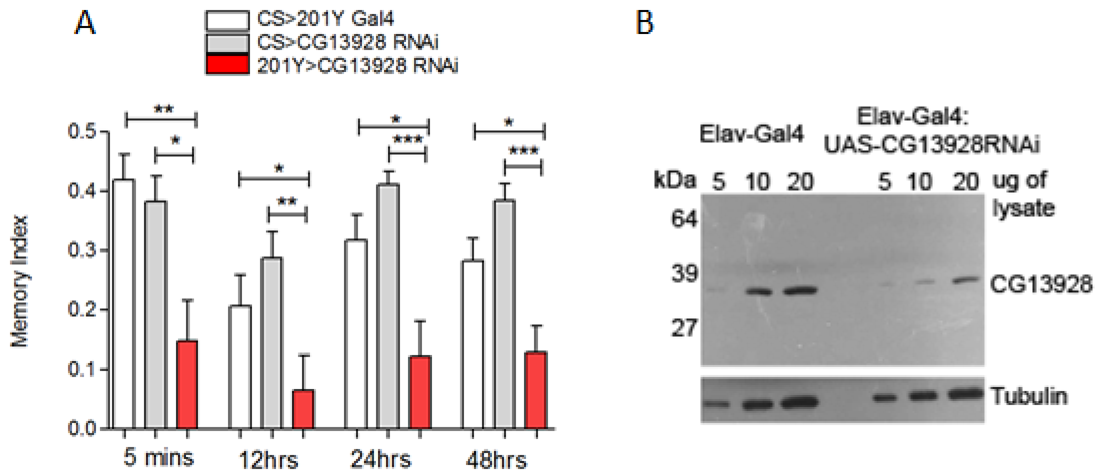


Figure 6.8: CG13928 is required for short-term memory. (A) Flies expressing CG13928 RNAi showed significant deficit in both short and long-term male courtship suppression memory. (B) Expression of CG13928 protein in RNAi and Gal4 flies. Tubulin served as a loading control. The statistical significance was measured by one way ANOVA. Data is expressed as mean ± SEM and * indicates p value<0.05, ** indicates p value<0.01 and *** indicates p value<0.001.

Discussion

Orb2 is a prion-like protein which has a well-established role in the persistence of long-term memory in *Drosophila* (Keleman et al., 2007; Majumdar et al., 2012). However, the biochemical consequences of Orb2's prion-like conversion are largely unknown. This study primarily attempted to understand the translational regulatory activity of Orb2, particularly when the protein exists in two distinct conformational states: a monomeric state and an amyloidogenic prion-like state. In this discussion I have briefly outlined the important observations and tried to place it in the broader context of protein synthesis, memory and the plausible roles of prions and amyloids in the nervous system and beyond.

Orb2-mediated translation: Role of monomeric and oligomeric Orb2

Specific sequences in the 3UTR recruits Orb2 to the target mRNA

Orb2 is an RNA-binding protein that regulates translation of some but not all mRNAs. This suggests like many other RNA-binding proteins there are specific sequences in the mRNA that allow Orb2 to recognize the appropriate target mRNA. For some CPEB family members, such as mammalian CPEB1, the sequence UUUUAU recruits it to the target mRNA, while CPEB3 seems to rely not on specific sequence, but on the secondary structure of the target mRNA (Huang et al., 2006). Based on deletion and point mutation of the 3'UTR of the Tequila gene, we found that recruitment of both Orb2 monomer and oligomeric proteins requires two conserved sequences in the 3´UTR of the target mRNA: UCCACCUU/G (Mutation1 or M1) and UUUUGU (Mutation2 or M2). The UUUUGU sequence is similar to the canonical CPEB-binding element UUUUAU and UUUUGU sequence is present in most of the known Orb2 target mRNA (Mastushita-Sakai et al., 2010). Mutation or deletion of the M2 sequence uniformly reduces Orb2 recruitment to the

Tequila mRNA, suggesting it is a major recognition element for Orb2. That both monomer and oligomer require the same M2 sequence suggest that a given mRNA can be occupied either by the monomer or the oligomer but not by both forms at the same time. Therefore the translational state of the mRNA would be dictated by which form of the protein is bound to the mRNA. Unlike the M2 sequence the function of the UCCACCUU/G or M1 sequence is unclear. While mutation of the M1 sequence reduced Orb2 recruitment, the deletion of it had no significant effect, suggesting some structural aspect of the mRNA may be dependent on the M1 sequence. Since the M1 sequence is conserved in the 3'UTR of Tequila gene in a number of *Drosophila* species, it suggests that this sequence has conserved function, although what that function is remains unclear at this stage. From our analysis it is also unclear whether different conformational states of Orb2 have similar or different affinities for target mRNA, and whether Orb2 proteins by themselves directly bind to these conserved sites or are recruited to these sites by some other mRNA binding protein. Typically direct binding is assayed using purified recombinant protein and labelled mRNA. However, the recombinant Orb2 protein purified from bacteria exists as large aggregates and not in the monomeric form, rendering such experiments impossible. Similarly, the partially purified soluble Orb2 monomer and oligomer from insect cells or fly head also did not address this issue since they contain other proteins in addition to Orb2.

Orb2 monomer acts as a repressor

Earlier studies in S2 cells using reporter mRNA, as well as endogenous translation in male germ cells, suggested that Orb2 is most likely a translational repressor (Mastushita-Sakai et al., 2010; Xu et al., 2012; Xu et al., 2014). However, these cell-based studies failed to delineate exactly how Orb2 acts to regulate protein expression. The repression could be the result of translation inhibition, destabilization of mRNA, destabilization of the newly expressed protein, or all of the

above (Mastushita-Sakai et al., 2010; Xu et al., 2014). This study, combining both *in vitro* and *in vivo* approaches, provides experimental evidence that Orb2 protein in its monomeric form indeed acts as a repressor of protein synthesis. That Orb2 is an mRNA-element specific translational repressor is supported by several lines of evidences: 1) Mutations that attenuate Orb2 recruitment to the target mRNA enhances translation both in embryo extract as well as in fly head. 2) Expression of the reporters in embryo extract lacking Orb2 (null as well as hypomorphic) showed no significant differences between wild type and mutant reporters. 3) Expression of the reporters in heterologous rabbit reticulocyte translation extract, which lacks Orb2, showed no differences in expression between wild type and mutant reporter. 4) Immuno-inactivation by anti-Orb2 antibody or acute inactivation of modified Orb2 by TEV protease showed translation de-repression. 5) Polysome analysis of *in vitro* translation reactions showed that in the absence of Orb2 protein, or in the absence of Orb2 recruitment to the mRNA, the amount of mRNA in the translating ribosomal fractions increases.

That the *in vitro* translation extract prepared from 0-2 hrs embryos can recapitulate some aspects of neuronal translation is likely due to the fact that the predominant Orb2 protein both in embryo and fly head is monomeric Orb2B.That the repressive nature of Orb2B is maintained both in early embryo and neuron suggest the core factors important for Orb2 activity are present in both systems. However there are considerable differences between an embryo and adult neurons and these differences provided an opportunity to further interrogate the Orb2 function. For example, while Orb2 oligomer can be easily detected in fly head lysate, there was no detectable oligomeric Orb2 in embryo extract. Therefore by adding back the oligomer in this system or other neuron-specific factors one can interrogate the functional contribution of these various components in Orb2-dependent translation. Likewise the absence of Orb2B oligomer in the embryo suggest either

Orb2B undergoes different post-translational modifications in embryo and in adult fly brain that favors oligomerization in the brain but not in the embryo. The other possibility is that Orb2A, which is important for Orb2B oligomerization, is not expressed in embryo and therefore Orb2B could not oligomerize (Majumdar et al., 2012). This again provided an opportunity to add back Orb2A or modified forms of Orb2B in the embryo extract and assess the functional consequences.

Orb2 oligomer acts as a translation activator

By adding back Orb2 oligomer into the Orb2 deficient embryo we observed that upon oligomerization Orb2 not only losses its repressive function but it gains a new function. This conclusion is based on the following observations: 1) while isolated monomer represses translation, the isolated oligomer enhances translation. 2) The mutant reporters (M2P and ΔM2) showed significantly less oligomer-dependent translation enhancement, suggesting activation is mRNA element specific and dependent on the recruitment of Orb2 to the mRNA. 3) Inactivation of Orb2 oligomer by TEV protease digestion decreased oligomer-dependent translation activation. 4) Polysome analysis of translation reaction showed significant increase in the amount of reporter mRNA in the translating ribosomal fraction in presence of Orb2 oligomer. These *in vitro* observations are further corroborated by the *in vivo* observation that in the adult neuron the form of Orb2 that associates with translating ribosome is the oligomeric Orb2 although it is less abundant than the monomer. Therefore the oligomerization induced conformational change endows Orb2 with a new activity.

Orb2A triggers Orb2B oligomerization and transform a translation repressor to an activator

Prions and amyloids are distinct form other forms of protein aggregates because once they are formed inside the cell that can induce aggregation of the monomeric state(Prusiner, 1998). The

functional differences between monomeric and oligomeric Orb2 provided an opportunity not only to test induced aggregation but also the functional consequence. Previous studies have shown that just the N-terminus of Orb2A that contains the prion-like domain (PLD) is sufficient for long-term memory (Keleman et al., 2007; Kruttner et al., 2012) provided there is full length Orb2B protein around. These observations are consistent with the model that an important function of the low-abundant Orb2A is to change the activity state of abundant Orb2B protein presumably via inducing oligomerization. We have tested this model by *in vitro* and *in vivo* experiments. Transient expression of the full-length Orb2A isoform or just the N-terminal prion-like domain (Orb2A320) in adult fly head significantly enhanced reporter translation. Likewise, addition of recombinant Orb2A320 oligomer or mRNA encoding Orb2A 320 to the *in vitro* translation reaction induced Orb2B oligomerization with concomitant increase in reporter translation.

The Orb2A-dependent Orb2B oligomerization also suggests that the availability of the Orb2A is an important determinant of when and where Orb2-dependent translation would ensue. Indeed a number of studies indicate that Orb2A expression is tightly regulated. Orb2A protein has a very short half-life (~1h) consistent with its low abundance. Transducer of Erb2 or Tob, a previously known regulator of SMAD-dependent transcription (Yoshida et al., 2000; Yoshida et al., 2003) and CPEB-mediated translation (Hosoda et al., 2011) associates with both Orb2A and Orb2B, but increases the half-life of only Orb2A. Both Orb2 and Tob are phospho-proteins and protein phosphatase 2A (PP2A) dephosphorylates Orb2 (White-Grindley et al., 2014). Stimulation with tyramine or activation of mushroom body neurons enhances Tob-Orb2 association, and Tob promotes Orb2 phosphorylation by recruiting Lim kinase. Phosphorylation destabilizes Orb2-associated Tob, whereas it increases the monomeric Orb2A protein level. PP2A, an autocatalytic phosphatase, is known to act as a bidirectional switch in an activity-dependent changes in synaptic

activity (Belmeguenai and Hansel, 2005; Kikuchi et al., 2003; Mulkey et al., 1993; Pi and Lisman, 2008). Similarly, Lim kinase, which is synthesized locally at the synapse (Schratt et al., 2006) in response to synaptic activation, is also critical for long-term changes in synaptic activity and synaptic growth (Meng et al., 2002). The Orb2A protein level is not only regulated by PP2A-LimK; our unpublished observation suggests that Orb2A expression is also regulated at the mRNA level. The Orb2A transcript is expressed in an un-spliced form in the adult brain. The intron contains multiple stop codons and therefore the transcript does not code full length Orb2A protein. Intriguingly, behavioral training that produces long-term memory also increases the amount of protein coding spliced Orb2A transcript.

These observations suggested a model in which, in the basal state, synaptic PP2A keeps the available Orb2A in a un- or hypo-phosphorylated and thereby unstable state. Upon behavioral training the increase in spliced protein-coding mRNA increases the amount of Orb2A protein. The Tob protein that is constitutively present at the synapse binds to and stabilizes the un- or hypo-phosphorylated Orb2A and recruits the activated LimK to the Tob-Orb2 complex, allowing Orb2 phosphorylation. Concomitant decreases in PP2A activity and phosphorylation by other kinases increases Orb2A half-life. The increase in Orb2A level as well as phosphorylation may induce conformational changes in Orb2A, which allows Orb2A to act as a seed. Alternatively, accumulation and oligomerization of Orb2A could create an environment that is conducive to overall Orb2 oligomerization.

Orb2 monomer deadenylates and Orb2 oligomer stabilizes and polyadenylates target mRNA

What is the mechanistic basis of the opposing functions of Orb2 monomer and Orb2 oligomer? As mentioned before Orb2 associates Tob (White-Grindley et al., 2014), a protein that has been implicated in mRNA deadenylation (Hosoda et al., 2011). In addition, Orb2 associates

with Smaug, a protein involved in mRNA destabilization and deadenylation (Jeske et al., 2006; Jeske et al., 2011). Since deadenylation reduces translation and Orb2 associates with deadenylation factors such as Tob and Smaug, we asked whether the Orb2 repressive function is mediated via mRNA deadenylation. The following observations suggest that Orb2 deadenylates target mRNA: 1) the wild type 3'UTR is more rapidly deadenylated than mutant UTRs (ΔM2 and M2P) that do not recruit Orb2. 2) The deadenylation is more efficient in wild type embryo extract than the *Orb2* mutant embryo extract. 3) Addition of monomeric Orb2 in the *orb2* null extract induces rapid deadenylation of the target mRNA. Unlike monomeric Orb2, oligomeric Orb2 did not show any deadenylating activity. In fact the polyA tail was stabilized in the presence of Orb2 oligomer, suggesting oligomer blocks deadenylation. However, the translation assays suggested that the oligomer is not just devoid of the repressive function of the monomer, but that it also gains new function. Therefore we tested whether oligomeric Orb2 can elongate polyA tail. Several observations suggested oligomeric Orb2 can elongate polyA tail of an mRNA: 1) Both Orb2A and Orb2B oligomer increased polyA tail length by ~50-100 nucleotides compared to control extracts. 2) Transient expression of Orb2A, which induces Orb2 oligomerization, also increases polyA tail length of the Tequila mRNA. Therefore, the net outcome of Orb2 oligomerization is a reduction in deadenylation, an increase in polyA tail length, stabilization of mRNA, and enhanced translation. However, it is unclear whether the polyA tail elongation directly contributes to the increased translation; the relative contributions of inhibition of deadenylation and elongation of the polyA tail in Orb2-oligomer mediated translation also remain unclear.

The molecular basis of Orb2 monomer- and oligomer-mediated translation regulation

Similar to mammalian CPEB1, Orb2 acts as a repressor as well as an activator of translation by regulating the polyA tail length. CPEB1 binds to the CPE-element at the 3'UTR and recruits a

number of proteins including maskin (Stebbins-Boaz et al., 1999), polyA polymerase Gld2, and polyA-specific ribonuclease PARN. The balance between deadenylation and polyadenylation activity of CPEB1 regulates translation (Kim and Richter, 2006; Udagawa et al., 2012).

The Orb2 monomer and oligomer utilizes the same U-rich sequence in the Tequila 3'UTR. However when these two states of the Orb2 occupy the same RNA-element the outcomes are very distinct: repression vs activation, due to deadenylation vs polyadenylation. These observations suggest that these two proteins states are associated with very distinct protein complexes. What are these protein complexes and how are they differentially recruited to the monomeric and oligomeric Orb2? From proteomics-based studies we identified a zinc-finger domain containing protein CG13928 that interacts with monomeric Orb2 but not oligomeric Orb2. The mRNA expression pattern (based on modeENCODE project) and immunostaining indicate that CG13928 is expressed primarily in the nervous system. Embryo extract lacking CG13928 can still support Orb2-dependent translation repression, suggesting that CG13928 is not necessary for Orb2-mediated translation repression but likely facilitates the recruitment of a deadenylation complex to the mRNA. Consistent with this idea, addition of CG13928 enhances Orb2-monomer mediated translation repression as well as deadenylation, and removal of CG13928 from the fly brain enhances Orb2-dependent translation. Intriguingly, there is no obvious structural homologue of CG13928 in mammals. It is unclear why *Drosophila* utilizes a structurally non-conserved protein such as CG13928 for translation regulation. It is worth mentioning that one of the key regulators of mammalian CPEB1-mediated translation is Maskin (Groisman et al., 2000; Groisman et al., 2002; Mendez and Richter, 2001). In *C. elegans*, although CPEB function is conserved, no Maskin homologue has been identified (O'Brien et al., 2005). We speculate that in different organisms, as

long as the core machineries are the same, some regulatory molecules may have different structural features.

Unlike monomeric Orb2, no oligomer-specific interacting protein has been identified, and although CG4612 is required for oligomerization-mediated translation, it binds to both forms of the Orb2 protein. However, oligomer may enhance translation not only by recruitment of a specific protein but also by increasing the affinity for existing interactors. For example, an mRNA bound by oligomeric Orb2 will have more CG4612 compared to a monomer bound mRNA and therefore more of the CG4612-associated proteins. Such a mechanism would be reminiscent of CPEB1-mediated polyadenylation (Mendez et al., 2000b) where unphosphorylated CPEB1 has weak affinity for the CPSF160 complex and phosphorylation increases the affinity of CPEB1 for CPSF160, leading to polyadenylation and translational activation. Although CG4612 is involved in polyadenylation, it may have other functions that are important for translation activation. In mammals, PABPs are multifunctional proteins, and in some cases are known to activate translation by interacting with the translation initiation complex (Bernstein and Ross, 1989; Smith et al., 2014; Vazquez-Pianzola et al., 2011). Curiously, the other PABP, Pabp2, interacts with both forms of Orb2 yet does not have a significant effect on Orb2 function under these experimental conditions, except for translation inhibition at a high concentration. Further studies will be required to dissect the detailed mechanism of translational activation and the role of PABPs and other proteins in Orb2-mediated translation activation.

The molecular composition of Orb2-associated deadenylation and polyadenylation complex

Smaug is a well-known deadenylation factor and is known to cause deadenylation and destabilization of many mRNAs in *Drosophila* (Jeske et al., 2006; Jeske et al., 2011; Smibert et

al., 1996). Smaug has an established role as a local translational regulator in the embryo, where it destabilizes the unlocalized mRNA and serves as a determinant of *Drosophila* posterior body patterning (Smibert et al., 1996). Although initially discovered in *Drosophila* embryo we found that Smaug is expressed in the adult nervous system. Similarly mammalian Smaug1 is abundant in post-synaptic densities, a subcellular region where translation is tightly regulated by synaptic stimulation (Baez and Boccaccio, 2005). These results suggest a conserved role of Smaug in translation regulation in neurons.

Association of Orb2 with Smaug led us to examine whether Orb2 associates with the Smaug-deadenylation complex. Among the two main cytoplasmic deadenylation complexes, Not-CCR4 complex and Pan2-Pan3 complex, Smaug associates with Not-CCR4 complex. Intriguingly, although Smaug interacts with Orb2, the components of Not-CCr4 or Pan2-Pan3 complex do not efficiently interact with Orb2 and CG13928 promotes the Orb2-Smaug-Not-CCr4 complex formation. Based on these observations we conclude that CG13928 acts as an adaptor or stimulator of Orb2 monomer-dependent deadenylation.

On the other hand. to understand how Orb2 oligomer works we focused on two polyA binding proteins Pabp2 and CG4612. Pabp2, is well known for its role in mRNA stability and polyadenylation and CG4612 is a putative polyA binding proteins based on the predicted amino acid sequence. However, although Pabp2 interacts with both Orb2 monomer and oligomer what role it plays in Orb2-dependent translation is unclear since it had no significant effect on Orb2-dependent translation. On the other hand CG4612 had moderate but significant effect on Orb2 oligomer-dependent translation activation. The translation enhancing activity of the CG4612 is partly mediated by recruitment of the two polyA polymerase enzymes X-Gld and Gld2 to the Orb2 oligomer. Both of these interact efficiently with Orb2 oligomer in presence of CG4612, suggesting

CG4612 recruits or tethers the polyadenylation complex to Orb2 oligomer. However, further work is needed to elucidate exactly how CG4612 promotes translation since the effect of CG4612 in polyadenylation is modest. Likewise, the consequence of interaction between CG4612 and Orb2 monomer remains unclear.

A model for Orb2-mediated synapse-specific local protein synthesis

Results from both *in vitro* and *in vivo* assays suggest that monomeric Orb2 protein represses translation of the target mRNAs. We found that monomers of both Orb2A and Orb2B isoform have similar repressive functions. However Orb2A is extremely rare in neurons, while Orb2B is constitutively expressed throughout the neuron including at the synapse. Therefore, it is likely that the repressive function of Orb2 is primarily mediated by the Orb2B protein and since Orb2B is present both in synapse and cell body it most likely suppresses translation in both compartments (Majumdar et al., 2012). Moreover attempts to determine the subcellular distribution of monomeric and oligomeric Orb2 revealed significant enrichment of SDS-resistant oligomeric Orb2 in the synaptic membrane fraction (Majumdar et al., 2012). This membrane associated enrichment of Orb2 oligomer was striking since the monomeric Orb2 is more abundant in the nervous system.

Although direct evidence that the amyloidogenic oligomer regulates translation only in the activated synapse is still lacking, based on these observations we propose a plausible model for synapse-specific persistent translation. We posit that Orb2B binds to the target mRNA and the bound mRNA is transported to the synapses and kept in a repressed state via association with the deadenylation complex. Synaptic activation increases the local concentration of the low abundant Orb2A protein via phosphorylation (White-Grindley et al., 2014) and/or other yet unknown

mechanisms. Increase in the Orb2A protein level triggers self-sustaining amyloidogenic-oligomerization of Orb2A-Orb2B. Binding of the oligomer to the 3'UTR prevents deadenylation and recruits polyadenylation complex and both of these events result in enhanced translation. Because of the self-sustaining and stable nature of the amyloid state, this creates a local and self-sustaining translation activation of Orb2-target mRNA, maintaining the changed state of synaptic activity over time.

Translation regulation by mammalian CPEB

How general is the role of an amyloidogenic aggregated CPEB in the persistence of memory? In mammals there are four CPEB proteins, CPEB1-4 and all of them are expressed in the adult nervous system. Recent studies suggest a functional role of aggregated mammalian CPEB3 similar to *Aplysia* and *Drosophila* (Fioriti et al., 2015; Stephan et al., 2015). CPEB3 forms amyloid-like oligomers in the adult hippocampus and removal of CPEB3 from the hippocampus affects both the consolidation and expression of long-term memory (Fioriti et al., 2015). Functionally, CPEB3 regulates neuronal protein synthesis, and ubiquitination and SUMOylation regulates translation inhibitory function and aggregation of CPEB3 (Drisaldi et al., 2015; Pavlopoulos et al., 2011). Surprisingly, constitutive removal of CPEB3 did not affect one of the cellular correlates of long-term memory, long-term potentiation, but instead enhanced long-term depression and improved some forms of memory (Huang et al., 2014). The mechanistic basis of the consequences of constitutive removal of CPEB3 remains unclear. Among the other CPEB family members, variants of CPEB2 have putative prion-like domains, although it remains to be known whether they confer prion-like properties and whether CPEB2 is required for long-term memory. CPEB1, which is known to regulate synaptic protein synthesis, also impairs some forms of memory when deleted (Alarcon et al., 2004; Udagawa et al., 2012). Interestingly, forcible

dimerization of mammalian CPEB1 via a linker has been shown to act as competitive inhibitor of translation (Lin et al., 2012), although there is no evidence yet that CPEB1 forms such oligomers in the nervous system. CPEB4, similar to CPEB1, seems to be involved in a number of cellular processes and is abundantly expressed including in the nervous system (Novoa et al., 2010; Ortiz-Zapater et al., 2012; Tsai et al., 2013). However the constitutive knock out of CPEB4 has no discernable memory phenotype (Tsai et al., 2013). Nonetheless, the studies with mammalian CPEB3 suggest a self-sustaining amyloidogenic state of CPEBs is involved in stabilization of memory across various species.

Amyloids as a conformation-based protein switch

The amyloidogenic oligomeric Orb2 enhances translation is consistent with its postulated function, but it is nonetheless surprising. It is surprising because in most cases in which proteins formed amyloids the amyloids are either toxic or non-functional. However, that amyloid formation can alter protein activity is not unprecedented. For example Pmel17, a transmembrane glycoprotein, involved in melanosome maturation forms amyloids inside the cell (Fowler et al., 2006). It is postulated that in the amyloid state Pmel17 acts as a scaffold that accelerates melanin synthesis, although the exact mechanism is unknown. Likewise a number of peptide hormones form amyloids in the secretory granules (Maji et al., 2009). However it remains unclear how amyloid formation influence either the function or secretion of these peptide hormones. Recently, MAVS, a mitochondrial membrane protein involved in antiviral response in humans, has been shown to trigger transcription of interferon genes after it adopts an amyloid-like state (Cai et al., 2014; Cai and Chen, 2014).

However, Orb2 is unique in the sense that amyloid formation results in a complete switch in activity states. The differential recruitment of distinct protein complexes to create an altered activity state indicates that prion-like behavior is in essence a protein-conformation based switch, whereby a protein can lose or gain a function that can be maintained over time in the absence of the original stimuli. Although such a possibility has been anticipated there are very few direct biochemical data to support such a possibility.

A putative biochemical "trace" of long-lasting memory

Models for the biochemical basis of memory propose that an external experience creates in specific neurons an enduring biochemical "trace" of the experience, which leads to altered neuronal properties and behavioral output (Crick, 1984; Dudai, 2002; Lynch and Baudry, 1984). Such a biochemical trace of long-lasting memory would need to have several distinct properties: i) be engaged by a temporally defined physiological stimulus; ii) form in response to some but not all experiences; iii) produce a change in the neuronal properties that elicit appropriate behavioral responses; and iv) deal with the natural turnover of individual proteins to enable a persistent change in behavioral output (Crick, 1984; Dudai, 2002; Lynch and Baudry, 1984).

The emerging evidence from *Aplysia*, *Drosophila* and mouse suggests that the self-sustaining aggregates of CPEB may indeed be one of the biochemical substrates of at least some form of long-term memory: first, activity-dependent conversion to the amyloidogenic state suggests it can be engaged by behavioral training; second, phosphorylation or other mechanisms can confer the specificity and selectivity to long-term memory; third, Orb2-dependent activation of synaptic mRNAs alter the protein composition of the synapse, thereby altering synaptic properties and

neuronal output. Finally, once triggered, the stable amyloidogenic and self-sustaining capacity of Orb2 oligomer would outlast the turnover of individual molecules to sustain memory over the long-term. However some questions remain unanswered: how is selective engagement of the self-sustaining state of neuronal CPEB ensured only in response to long-term memory-inducing stimuli? Once engaged, how long does it persist, and is its continued presence necessary for the persistence of memory? Can a transient memory be stabilized by artificial recruitment of the amyloidogenic state?

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