Supplementary Experimental Procedures:

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 the 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 the *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 studies in S2 cells the open reading frames were cloned into TopoD vector (Invitrogen) and transferred into the 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 PAC5.1c-6XMyc and pUAST-n-

FLAG vector. For *in vitro* protein purification the genes were cloned in a 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 (Bischof et al., 2007).

Orb2-TEV flies: The Orb2 genomic rescue constructs were made using methods described by Venken *et al* (Venken et al., 2006; Venken et al., 2008) and the same construct was previously used by Majumdar *et al* (Majumdar et al., 2012). Briefly, 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 an 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 the 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 a MicroSpin^{TM} G-50 RNA purification column (GE healthcare). The RNAs were stored at -80°C until used. For RNA-protein 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 the 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) were 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 protein was 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, ThermoScientific), coupled with quaternary Agilent 1100 HPLCs. A 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, Pabp2,CG13928 and CG4612: Protein purification was performed in native condition using auto-induction system (Studier, 2005). All four genes were cloned into the pDEST42 vector (Invitrogen) with a 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 in 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 a 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-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 magent, 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 a pBSKII vector with a T7 promoter. The reporter construct contained the firefly luciferase open reading frame 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 the T7 mMESSAGE mMACHINE transcription Kit (Ambion) to make capped mRNA and then in the subsequent reaction the capped mRNAs were tailed by the

mMESSAGE mMACHINE T7 ULTRA Transcription Kit (Ambion). All transcribed reporter mRNAs were column purified using MicroSpin^{TM} G-50 (GE Healthcare) column. After measuring RNA concentration, all reporters were aliquoted in a small volume and stored in -80^{O}C. 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 was 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^{TM}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 the SF9 insect cell line using the Baculovirus expression system. The viruses were generated using the 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 a pDEST8 vector containing polyhedron promoter. The recombinant bacmids were generated in DH10Bac^{TM} (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 virus and incubated for 48 to 72 hr at 25ºC in a 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). The total cell lysate was centrifuged at 16,000xg for 20 minutes and supernatant was collected. 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 an 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, the unpaired two-tailed t-test or in the case of multiple samples, a one way ANOVA were performed. Statistical significance was determined compared to controls. For the *in vitro* translation assay each time point or each experimental condition has a 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 a 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 26ºC for the time points indicated. Luciferase activity was measured using the dual-glo luciferase assay system (Promega). The assay was normalized

using renila 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-renila luciferase activity was measured using Dual-Glo luciferase assay system.

For the *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, the 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 a time course using Tequila translation reporter. Regular translation reactions were assembled in the absence and the 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 described by Zid et al (Zid et al., 2009). For each translation reaction of 100 µL volume, approximately ~150,000 cpm of equivalent translation reporter was added. Each translation reaction was carried out for 15 mins at 26ºC and was stopped by addition of 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 a 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-titer 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, polysome analysis and ribosome 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 have been successfully tagged in other systems (Mustroph et al., 2009; Sanz et al., 2009). The tagged proteins were first expressed in S2 cells and it was verified whether they were incorporated into respective ribosomal subunits and if so, whether the tagged ribosomes were part of polysomes. Under our experimental conditions tagged *Drosophila* Rpl10 and Rpl22, although incorporated in 60S ribosomes, 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 were 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 copurified with translating mRNA. To determine that the tagged ribosome indeed purifies cell type specific mRNA, the FLAG-tagged Rpl18 was expressed in a 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 tubes and snap freezed in liquid nitrogen. Fly heads were separated from bodies by brief pulses of vortex and subsequent sieving. To assess incorporation of Rpl18 in polysomes, the separated heads were homozeniged 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 lysate using a BCA kit (Thermo Scientific). For polysomes 100ul of 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 (Gift from Dr. Marco Blanchette) treated and untreated fractions (150 ng total proteins from each fraction) for 30 mins at room temperature. The translation assay was performed in Δ*orb2* embryo extract for various time points at 26ºC. The firefly and renila luciferase activities were measured by using Dual-Glo luciferase system.

Orb2B-TEV embryo extract was prepared following the same protocol already described. 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 extracts for 15 mins. Translation assay was carried out in Δorb2 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 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, the *Drosophila* embryo extract was treated with anti-Orb2 polyclonal antibody and control IgG. Total 10 µl (~300µg total protein) 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 a corresponding control fraction for 12h at 4°C or 30 min at 26ºC. In the case of purified recombinant proteins 10 ng of recombinant protein or 10ng BSA was used as a control. 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 the *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 experiments 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 oligomers 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:** The 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. A premixed buffer containing HEPES buffer, potassium acetate, magnesium acetate, spermidine, and yeast tRNA were used to assemble the deadenylation reaction. The uncapped-polyadeenylated 3'UTRs were synthesized using the 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) to a 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 cold 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:** The *in vitro* polyadenylation assay in *Drosophila* embryo extracts 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 pre-incubated with Orb2 oligomer and control SF9 fractions for 30 minutes at room temperature. After pre-incubation, a regular translation reaction was assembled

in Δ*orb2* embryo extracts and the reaction was carried out at 26ºC for 30,60 and 90 minutes. Total RNA was extracted using TRIZOL (Invtrogen) 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* exogenously added Tequila 3'UTR in *in vitro* cell free translation system (Coll et al., 2014). 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 total RNA samples were treated with TURBO DNase (Ambion) for 30 mins at 37ºC to remove genomic DNA contaminations. A total of 0.5 µg of RNA was mixed with 0.5 µL of oligo (dT) (Takara) in 5 µL total volume and incubated for 5 mins at 65ºC. The RNA-oligodT was then mixed with 6.5 µL of pre-warmed master mix containing 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. The reaction mix was incubated for 30 mins at 42˚C. Afterward 0.5 µL of oligo (dT) anchor primer was added to each reaction and incubated overnight at 12˚C. Following overnight incubation, 0.75 µL of reverse transcriptase (Takara) was added and incubated for 1h at 42 ˚C for cDNA synthesis. After RT reaction 15 µL of H_2O was added to each sample and incubated for additional 30 mins at 70˚C. Then regular PCR reaction was performed using Tequila forward primer as well as oligo (dT) anchor primer for 30 cycles using LA-Taq polymerase (Takara). The PCR products were run in 1.5% agarose gel and stained with ethidium bromide.

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 a T7 mMESSAGE mMACHINE transcription Kit (Ambion Inc.). Equal amount of labelled 3'UTR (~20,000 cpm equivalent) was pre-incubated in the presence of Orb2 monomer, oligomer and corresponding SF9 control proteins. After pre-incubation, a 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: The ThT binding assay was performed according to the standard protocol described by Chien et al (Chien et al., 2003) with minor variation. Different amounts 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. The 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) 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 amounts 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 scan 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, a total of 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 day old adult flies were starved for 18-20 hrs in glass vials containing water soaked Kimwipes. 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 an 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 bodies by vortexing for 5-10 sec and individual heads were transferred to the wells of 96-well flat-bottom micro-titer 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. The male courtship suppression assay was performed as described by McBride et. al (McBride et al., 1999). 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 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
LuciferaseF: 5-cca ggg att tca gtc gat gt-3
LuciferaseR: 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
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	
Renila 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	

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