Cell

EXTENDED EXPERIMENTAL PROCEDURES

Imaging and Quantification

Dissociated hippocampal cultures were prepared from postnatal day 0 (P0) mice as previously described (Meffert et al., 2003) and were maintained in Neurobasal A medium (GIBCO, 10888) with B27 Supplement (GIBCO 17504-44). Neurons were transiently transfected with Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen) 24 hr before experimentation. For live cell imaging, confocal images of hippocampal pyramidal neurons (excitatory, determined by morphology) in 0.24 – 0.3 μm Z sections were acquired using a 40x, 1.3 NA, EC Plan Neofluoar (whole cells) or a 100x, 1.4 NA Plan Apochromat oil immersion objective (dendrites) on a Yokogawa spinning disk system (Cell Observer, Carl Zeiss) at 37°C in Tyrodes buffer (in mM: 119 NaCl, 5 KCl, 2 $CaCl_2$, 0.2 $MgCl_2$, 30 Glucose, 25 HEPES, .01 Glycine, pH 7.4). EBFP2 was excited at 405 nm and emissions collected at 425–475 nm. GFP was excited at 488 nm and emissions collected at 500 – 550 nm; mCherry was excited at 561 nm and emissions collected at 598–660 nm. Laser power and exposure time were adjusted to minimize phototoxicity and avoid saturation. All experiments were from a minimum of three independent cultures, and no more than three neurons per dish; the experimenter was blinded to conditions during analysis.

For fixed cells, confocal images were acquired in 0.3 μm (dendrites) or 1.7 μm (whole cells for arborization) Z sections on an LSM5 Pascal system (Carl Zeiss) using a 63x, 1.4 NA Plan Apochromat oil immersion objective and 2X optical zoom (dendrites) or using a 25x, 0.80 NA Plan-Neofluor multi-immersion objective and 0.7x–1x optical zoom (for whole cells). GFP was excited at 488 nm and emissions collected at 505–530 nm; mCherry was excited at 543 nm and emissions collected above 560 nm. Laser power and exposure time were adjusted to minimize photobleaching and avoid saturation. All experiments were from a minimum of 3 independent cultures and no more than 3 neurons per dish.

Z-stacks containing the entire neuron or process of interest were analyzed using Imaris 7.0.0 (Bitplane) and ImageJ software. Automated analysis of P body numbers was conducted using Spots detection in Imaris. A quality filter and intensity median filter for the red channel were used to restrict detection of puncta within dendrites only. Colocalization analysis was performed using the Colocalize Spots function. The percent colocalization of P body components was calculated by subtracting the number of colocalized BFP-Dcp1a or YFP-Pat1b puncta from the total number of BFP-Dcp1a or YFP-Pat1b puncta and multiplied by 100. The percent of colocalized fluorescence was calculated for each P body component by first summing the aggregate fluorescence values that colocalized with the other P body marker in 'spots', then dividing this quantity by the value of the total fluorescence intensity within the dendrite for that channel, and multiplying by 100. The Surfaces tool in Imaris was used to create a representation of the dendrite in order to determine total fluorescence intensity corresponding to the dendrite region alone. The red channel (soluble mCherry expression) was used as the source channel to compute the Surfaces. Total fluorescence of a dendritic segment was calculated by summing the intensity fluorescence values of all of the Surfaces representing a single dendrite. Sholl analysis was performed using the Sholl analysis plugin in ImageJ (A. Ghosh lab) from Z-compressed projections traced semi-automatically in NeuronJ. For analysis, dendritic intersections were counted using a circle of 15 μm diameter centered on the cell soma and subsequent circles of increasing 5 μm diameter increments.

Immunoblotting

Primary cultures of mouse hippocampal neurons (DIV 14∼15) were incubated in serum-reduced medium (0.5% B27 supplement) for 2 hr, followed by 0.5 mg/ml Actinomycin-D for 10-20 min. Bath application of BDNF (100 ng/ml) was for designated periods (5 min–2 hr). The cultures were washed 3 times and harvested on ice with lysis buffer (50 mM HEPES, 150 mM NaCl, 10% Glycerol, 1 mM EDTA, 1% Triton X-100, 0.2% SDS) plus freshly added protease inhibitor cocktail (Roche) and PMSF. Protein concentration was determined by Bradford Assay. If required, lysates were treated with Lambda Protein Phosphatase according to manufacturer's instructions (New England Biolabs P0753S). Equal amounts of lysate protein were resolved on SDS-PAGE gels, and electrotransferred to PVDF membrane. Membrane was blocked with 5% milk in Tris-buffered saline tween 20 (TBST) and probed with primary antibodies in 5% milk or BSA in TBST: GluA1 (Millipore AB1504), CaMKIIα (Zymed 13-7300), Homer2 (gift of P.Worley), KCC2 (Upstate 07-432), Kv1.1 (NeuroMab 73-007), βtubulin (U.Iowa DSHB, clone E-7), GAPDH (gift of S. Snyder), GW182 (18033 gift of M. Fritzler or Abcam ab84403), Dcp1a (gift of J. Lykke-Andersen or NeuroMab clone3G4), phospho-ERK ½ (Sigma M 7802), Dicer (NeuroMab clone N167/7), TRBP (Abcam ab72110), Lin28a (Cell Signaling A177), Lin28b (gift of E. Moss or Cell Signaling 5422), Arc (SantaCruz 17839), mCherry (Clontech 632496), GFP (NeuroMab N86/8).

^{35}S Labeling

Cultured neurons were pre-incubated in media containing reduced-serum and Actinomycin-D as previously described, followed by washing and incubation for 10 min with methionine- and cysteine-free DMEM (Mediatech, Inc.), and ^{35}S labeling in the same DMEM with the addition of ^{35}S-methionine/cysteine (^{35}S Met/Cys EasyTag Mix, Perkin Elmer) to a final concentration of 100 μCi/ml. Mock or BDNF stimulation was for 2 hr. Cells were washed and lysed with lysis buffer (see immunoblotting). Lysates were centrifuged and collected supernatants subjected to Bradford assay. To asses newly synthesized proteins, 200-500 μg of lysates proteins were precipitated with 10% trichloroacetic acid (TCA) for 1 hr on ice in the presence of 0.5% deoxycholate (DOC) to remove interfering phenol red. After centrifugation, protein pellets were washed with ice-cold 95% ethanol, solubilized in denaturing buffer (50 mN

Tris pH 8.3, 5 mM EDTA, 0.05% SDS, 6M urea), and subjected to liquid scintillation counting (Econofluor, New England Nuclear, Inc.). ^{35}S Disintegration per minute (DPM) was used to quantitate protein synthesis after subtraction of background readings.

Immunopurification of GW182

Proteins and mRNAs associated with P body component GW182 were isolated through immunoprecipitation of GW182 by modification of previously published protocols (Keene et al., 2006; Moser et al., 2009). Primary cultures of mouse hippocampal neurons (DIV 14-15) were incubated in serum-reduced medium (0.5% B27 supplement) for 2 hr, followed by 0.5 mg/ml Actinomycin-D for 10∼30 min and mock or BDNF-stimulation for 30∼60 min. Cell lysates were harvested in polysomal lysis buffer (100 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES pH 7.0, 0.5% NP-40) with protease inhibitor cocktail and freshly added 20 mM EDTA, 1 mM DTT, 100 U/ml RNase inhibitor (RNaseOut, Invitrogen) and 400 μM Vanadyl ribonucleoside complexes (SIGMA). Lysates were centrifuged and the supernatants pre-cleared by one-hour incubation with recombinant protein G beads pre-washed in NT2 buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM MgCl2, 0.05% NP-40). Antibody coating of protein G beads was carried out with antiserum against GW182 (18033, gift of M. Fritzler) or control isotype-specific serum in NT2 buffer plus protease inhibitor for 4 hr after blocking with NT2 buffer plus 5% BSA and 1 mg/ml heparin for 1 hr. For immunoprecipitation (IP), equal amounts of lysate protein (2–5 mg) was incubated with antibody-coated beads and tumbled for 4 hr at 4°C, followed by washing. RNAs were recovered from GW182 immunoprecipitates by Tri-Reagent as described below.

Recovered RNAs were resuspended in nuclease-free water, measured for RNA concentration, and immediately reverse transcribed into cDNA using a combination of random decamers and oligo(dT) primers. qRT-PCR was carried out as described below. To examine proteins coimmunoprecipitated with GW182, the washed IP beads were incubated in sample buffer at 95°C for 5 min and subjected to SDS-PAGE electrophoresis and immunoblotting.

RNA Extraction, Northern Blots, Quantitative PCR for Individual mRNA and miRNA Species

Total RNA from primary cultures of mouse hippocampal neurons was isolated by Tri-Reagent (Molecular Research Center, Inc.) according to the manufacturer's protocol. Cultures were either homogenized in Tri-Reagent directly, or were first lysed in lysis buffer plus RNase inhibitor (if protein from the same sample was required) followed by Tri-Reagent addition to a portion of lysate. RNA pellets were air-dried and resuspended in nuclease-free water. RNA concentration and quality were assayed by spectrophotometric measurements at an optical density (OD) 260/280/230 nm.

Northern blots were carried out as described (Hwang et al., 2007), using the following LNA probes from Exiqon: Let-7a (5′ AAC TATACAACCTACTACCTCA 3′), pre-Let-7a (5′ GTGGGTGTGACCCTA 3′), miR-17 (5′ CTACCTGCACTGTAAGCACTTTG 3′) and quantified by phosphoimaging.

For analysis of mRNA abundance: 1 μg of RNA was immediately reverse-transcribed into cDNA with a TaqMan reverse transcription kit (Applied Biosystems) and a mixture of random decamer and oligo(dT) primers in a final volume of 30 μl and subjected to TaqMan Gene Expression Assays (Applied Biosystems) for GluA1 (*Gria1*; assay ID: Mm00433753_m1), CaMKIIα (*Camk2a*; Mm00437967_m1), Homer2 (*Homer2*; Mm01314936_m1), KCC2 (*Slc12a5;* Mm00803929_m1), Kv1.1 (*Kcna1*; Mm00439977_s1),GW182 (*Tnrc6a*; Mm00523487_m1), GAPDH (*Gapdh*; Mm99999915_g1), and β-tubulin-III (*Tubb3*; Mm00727586_s1). qRT-PCR was performed using a Stratagene Mx3000P machine and software in 20 μl reactions on a 96-well optical plate at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 55°C for 1 min and 72°C for 30 s. The threshold and threshold cycle (Ct) values were determined using default settings. Standard curves were constructed and used for quantitation of target transcript abundance. In this method, 1:5 dilution series of an independent Standard sample are amplified to generate a curve that relates the initial quantity of the specific target in the Standard samples to the Ct. The standard curve is then used to derive by interpolation the initial sample template quantities based on their Ct values. All derived quantities were further normalized to neuron-specific β−tubulin III, whose translational status is unchanged by BDNF (Schratt et al., 2004). Data were plotted as fold change relative to mock control.

For individual microRNA abundance assays (Applied Biosystems), 100 ng of total isolated RNA was prepared for reverse transcription with stem-looped primers specific for individual mature miRNAs in a final volume of 15 ul according to manufacturer's protocol; 4°C for 5 min, 16°C for 30 min, 42°C for 30 min, 85°C for 5 min and subjected to TaqMan MicroRNA Assays (Applied Biosystems) for Let-7a (assay ID: 000377), Let-7b (002619), Let-7e (002406), Let-7f1 (000382), miR-107 (000443) and miR-143 (0024). qRT-PCR was performed using a Stratagene Mx3000P machine and software with quantities derived by standard-curve quantitation method. The abundance of noncoding 18 s rRNA in each sample was used as an internal control to normalize all miRNA species.

miRNA Profiling Arrays and Analysis

Murine hippocampal cultures were preincubated for 10–20 min with 0.5 μg / ml Actinomycin D and either mock or BDNF (100 ng/ml) stimulated for 30 min before harvesting.

For miRNA arrays, 1 μg of total RNA for each sample was reverse-transcribed with stem-looped Megaplex RT Primers (Applied Biosystems) in a final volume of 7.5 μl according to manufacturer's instructions: preincubation at 4°C for 5 min; 16°C for 2 min, 42°C for 1 min, 50°C for 1 s, 40 cycles; 85°C for 5 min. The entire cDNA RT product (7.5 μl) was subjected to qRT-PCR on an Applied Biosystems 7900HT Fast Real-Time PCR system using Taqman Rodent MicroRNA Array A. Data were compiled in SDS RQ Manager 1.2.1 and analyzed in Data Assist 2.0 (Applied Biosystems); RQ was calculated as $2^{-\Delta CtBDNF} / 2^{-\Delta Ctmock}$ where ΔCt = (cycle threshold for miRNA of interest) – (cycle threshold for reference control) (Schmittgen and Livak, 2008). Thresholds and cycle threshold (Ct)

values were determined using default settings and the maximum allowable Ct value was set at 35.0 inclusive. Data were normalized by arithmetic mean using MammU6 (4395470) and snoRNA202 (4380914) reference RNAs as controls. miRNA array data are reposited with the NCBI Gene Expression Omnibus (GEO). Initial experiments using an alternative miRNA array platform (Geniom microfluidic miRNA profiling, Febit Inc.) similarly indicated a BDNF-mediated increase in the majority of detectable mature miRNA species.

Lentivirus Preparation and Gene Knockdown by RNAi

Lentiviral stocks were prepared as previously described (Lois et al., 2002). Knockdown was by lentiviral-mediated delivery of nontarget shRNA (Sigma, SHC002), shRNA targeting GW182 (CCTTAGTAATGGAGAGTCAAA), LSM5 (OpenBiosystems TRCN0000109196), Dicer (TRCN0000071320) or Lin28 (TRCN0000102576) to cultures at multiplicity of infection of 5 – 10, 48 hr before imaging, or 4 – 5 days for GW182.

Luciferase Reporter Assays

The siRNA- or miRNA-reporter constructs harbor one perfectly matched or four bulged CXCR4 siRNA target sites, respectively, in the 3′UTR of firefly luciferase mRNA. In the presence of CXCR4 shRNA, perfectly matched sequences are cleaved by siRISC and bulge-containing sequences are targets for translation suppression by miRISC. miRNA and siRNA pathway function were assayed in cells transiently transfected and expressing either the miRNA or siRNA reporters alone (no sh-CXCR4), or coexpressing either of the reporters and CXCR4 shRNA with or without P body disruption (sh-Control-1 or GW182 KD, GFP or GFP-DNGW182, sh-Control-2 or LSm5 KD).

Let-7 luciferase reporters (gift of G.Hannon) harbor a wild-type or a mutant Let-7 miRNA binding site. The Let-7 miRNA binding sites are derived from a short 3′UTR segment of *C. elegans* Lin41 mRNA containing two adjacent proven Let-7 miRNA biding sites and are cloned into 3′UTR of firefly luciferase in a pcDNA backbone. Mutations in seed regions of both Let-7 binding sites were made for a negative control reporter, which was documented not regulated by endogenous Let-7 miRNAs.

Cotransfection of the pCSK-lacZ vector, which constitutively expresses β-galactosidase and is not regulated by shRNA, served to normalize transfection efficiency and extract recovery for each sample in all reporter assays. The DNA amounts used for each well (24 well plate) were 15 ng of any luciferase reporter, 85 ng of β-gal (CSK-LacZ), and/or 75–300 ng of CXCR4 shRNA. Each reporter experiment included extracts from cells transfected with pcDNA3.1 alone as a reference control.

44 hr post-transfection, hippocampal cultures were treated with serum-reduced media (0.5% B27) and Actinomycin-D (0.5 μg/ml) as previously described, followed by BDNF stimulation 100 ng/ml for 4 hr. Cell lysates were collected in 1X lysis buffer (reporter lysis buffer, Promega), and luciferase (Promega) and chemiluminescent β-gal (Roche) reporter assays carried out according to manufacturer instructions using a plate-reading luminometer (Perkin Elmer). Samples were compared by subtracting the background activity of the reference control, and then normalizing the luciferase activity of each sample to its β-gal activity (Shrum et al., 2009). When required, fold change was calculated by dividing normalized stimulated samples by normalized unstimulated samples.

Plasmids and Fluorescently Tagged Constructs

GFP-Dcp1a was a generous gift from J. Lykke-Andersen (UCSD). GFP-Staufen was a generous gift from L. DesGroseillers (U. Montreal). YFP-Pat1b was a generous gift from G. Stoecklin (U. Heidelberg). BFP-Dcp1a was generated by subcloning with EBFP2. GFP-hAgo2 (11590) and GFP-GW182Δ1 (DNGW182, 11592) were purchased from Addgene.

Cloning of let-7a-1 GGAG Mutant

A let-7a-1 precursor miRNA with the conserved Lin28 "GGAG" recognition motif mutated to "GtAt" was generated by two-step PCR site-directed mutagenesis from the pLV-hsa-let-7a-1 vector (Biosettia, mir-p001) using the following primers: TATAGGATCCTCACA CAGGAAACCA (forward, outside; P1); TATAGCTAGCGCTGCACTACATCTC (reverse, outside; P2); CCCACCACTGGTATATAACTA TACAATCTACTG (forward, inside; P3); TGTATAGTTATATACCAGTGGTGGGTGTGA (reverse, inside; P4).

The P1 and P4 primers and the P2 and P3 primers were paired for the first round of PCR reactions. The products of these PCR reactions were then included with the P1 and P2 primers for the second round of PCR reactions to generate the final "GtAt" mutant let-7a-1 fragment. This fragment was then subcloned into the BamHI and NheI sites of the parent plv-hsa-let-7a-1 parent vector.

Statistical and Bioinformatics Analysis

Student's t tests were performed in Excel. Nonparametric unpaired one-way ANOVA with post hoc Bonferroni analysis, and Mann-Whitney U tests were performed in OpenStat 11.9.08 (Softonic) or STATA 10.0 (StataCorp.).

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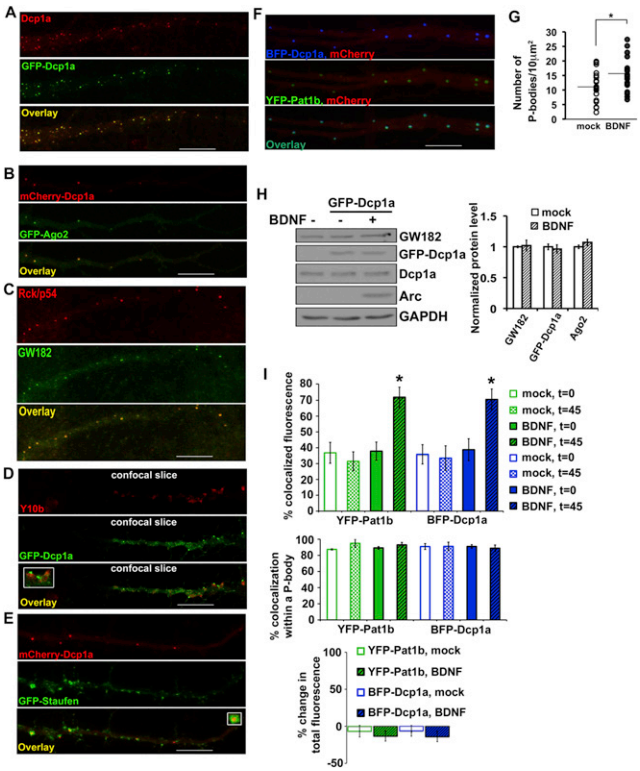

Figure S1. Composition of Neuronal P Bodies, Related to Figure 1

(A) Endogenous Dcp1a (top, red) colocalizes with GFP-Dcp1a (middle, green) in a confocal projection of hippocampal pyramidal neuron dendrites. (Bottom) Overlaid image.

(B) mCherry-tagged Dcp1a (top, red) colocalizes with GFP-tagged Ago2 (middle, green) in a confocal projection. (Bottom) Overlaid image.

(C) Endogenous Rck/p54 (top, red) colocalizes with endogenous GW182 (middle, green) in a confocal projection. (Bottom) Overlaid image.

(D) Consistent with the lack of translation in P bodies, endogenous ribosomal RNA, stained with Y10b (top, red) does not colocalize with GFP-tagged Dcp1a (middle, green) in dendrites of hippocampal pyramidal neurons. (Bottom) Overlaid image. Images shown are a single confocal slice to better appreciate co-localization given the relatively larger volume of Y10b staining. Inset: enlarged view of imaged region in dashed box, showing Y10b puncta closely opposed to but not colocalizing with GFP-Dcp1a puncta.

(E) mCherry-tagged Dcp1a (top, red) does not colocalize with GFP-tagged Staufen (middle, green) in confocal projections from dendrites of hippocampal pyramidal neurons. (Bottom) Overlaid image. Inset: enlarged view of imaged region in dashed box, showing GFP-Staufen surrounding but not colocalizing with a GFP-Dcp1a puncta.

(F) EBFP2-tagged Dcp1a (BFP-Dcp1a; top, blue) colocalizes with YFP-tagged Pat1b (YFP-Pat1b; middle, green). Scale bar represents 10 μm in all images. Images are confocal z-stack projections unless otherwise indicated.

(G) Immunostaining of endogenous P bodies with anti-GW182 antibody shows a high variability in basal dendritic P body number that is collectively and on average shifted to greater numbers of P bodies in BDNF-stimulated neurons. Scatter plot of endogenous P body numbers in individual neuronal dendrites from distinct pyramidal neurons quantified from GW182 immunostaining 75 min after mock (open circles) or BDNF stimulation (filled circle). Bar represents mean. Mean values ± SEM are 11.36 ± 1.21 (mock), 15.48 ± 1.26 (BDNF), $p < 0.05$ by Student's t test.

(H) Left: Immunoblot showing GFP-Dcp1a and endogenous Dcp1a and GW182 protein levels following 45 min mock (-) or BDNF (+) stimulation. Right: Relative quantification of protein levels normalized to β-tubulin III.

(I) BDNF rapidly enhances the colocalization of P body components by increasing their recruitment to P bodies, rather than by altering their synthesis. (Top) The fraction of total fluorescence of two coexpressed P body markers, YFP-Pat1b and BFP-Dcp1a, that colocalized was quantified from hippocampal dendrites imaged before and after BDNF stimulation using the spots function and colocalize spots tool in Imaris software (Bitplane). The fraction of colocalized fluorescence within a dendritic segment was calculated for each P body component by first summing the aggregate fluorescence values that colocalized with the other P body marker, then dividing this quantity by the value of the total fluorescence intensity within the dendrite for that channel, and multiplying by 100. The percent of the total fluorescence that colocalized was significantly increased for both YFP-Pat1b and BFP-Dcp1a by BDNF (hatched bars) but not mock (checkered bars) stimulation. (Middle) The degree of colocalization within a given P body is not changed by BDNF stimulation. Although the total amount of fluorescence found in P bodies was increased by BDNF for both YFP-Pat1b and BFP-Dcp1a, P bodies always demonstrated a high degree of colocalization for the two P body markers that was not significantly altered by BDNF. Percent colocalization within P bodies was calculated by measuring the amount of fluorescence of YFP-Pat1b or BFP-Dcp1a that colocalized within defined P body punctae, dividing this by the aggregate fluorescence value within all defined P body punctae, and multiplying by 100. (Bottom) Total fluorescence of YFP-Pat1b or BFP-Dcp1a within dendritic segments did not significantly change during mock (checkered bars) or BDNF (hatched bars) stimulation.

Error bars represent SEM. * = $p < 0.05$ by unpaired Student's t test.

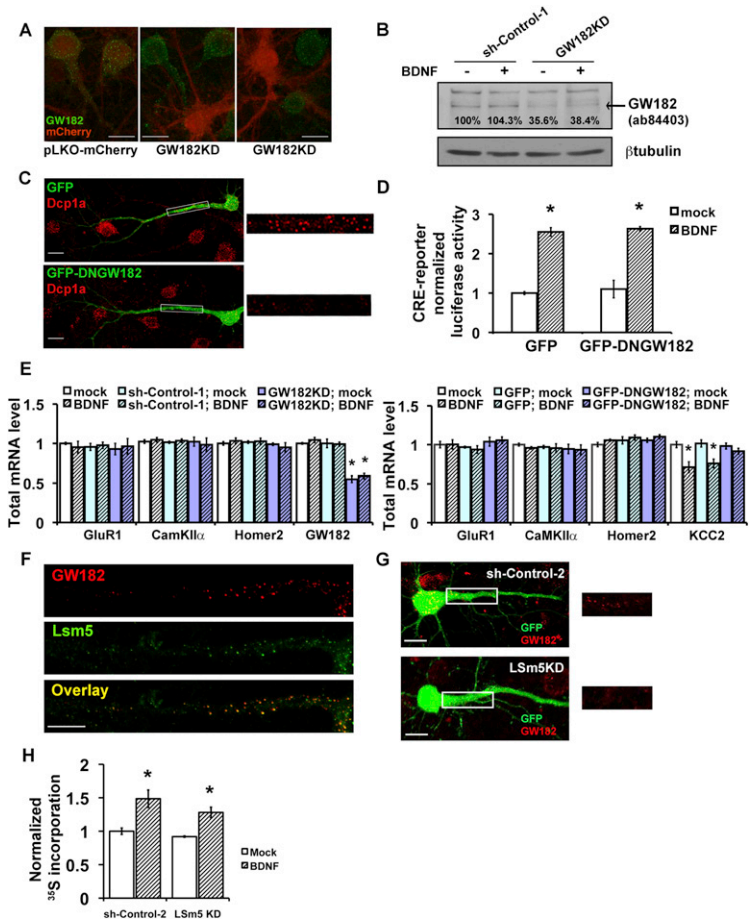

Figure S2. Loss of P Bodies in Neurons Lacking Either GW182 or LSm5 Leaves Other Cellular Responses to BDNF Intact, Related to Figure 2

GW182 was chosen as an initial target for P body disruption because it may perform a scaffolding role in P bodies and does not possess a known enzymatic activity that could be required for general cellular function.

(A) Cultured hippocampal neurons (DIV 14) were infected with replication-incompetent lentivirus expressing mCherry and shRNA targeting GW182, or a control nontarget shRNA and mCherry. Immunohistochemistry for endogenous GW182 indicated effective loss of P bodies in cells expressing GW182 shRNA (GW182KD), but not in control shRNA infected cells.

(B) Immunoblot demonstrating effective knockdown of GW182 using a rabbit polyclonal antibody raised against GW182 (16 amino acid peptide of TNRC6a absent from other TNRC6 isoforms, Abcam ab84403) with no cross-reaction to isoforms TNRC6B and TNRC6C, or Ge-1, another P body component. Neurons expressing either shRNA against GW182 (GW182KD) or scrambled shRNA (sh-control-1) received mock or BDNF stimulation in the presence of Actinomycin-D as previously described (Figure 2). Quantification relative to the control mock condition (set as 100%) of protein levels normalized to β-tubulin is indicated under GW182 bands.

(C) Immunohistochemistry for endogenous Dcp1a showed loss of P bodies in neurons expressing GFP-DNGW182.

(D) BDNF-induced activation of the cAMP response element-binding protein (CREB) transcription factor remains intact in neurons expressing GFP-DNGW182. A luciferase reporter harboring cAMP response-elements (CRE) to monitor CREB activation was expressed in neurons with or without DNGW182. Mock (open bars) and BDNF (hatched bars, 100 ng/ml 3.5 hr) stimulation were performed in the absence of a transcription blocker. Luciferase activity was normalized to coexpressed constitutive β-galactosidase activity and plotted relative to the mock condition.

(E) mRNA abundance in neurons as measured by qRT-PCR with all conditions normalized to mock stimulation (open bars; set as 1.0). mRNA levels of BDNF-upregulated targets were unaltered from basal values (mock) by BDNF stimulation (BDNF; 100 ng/ml, 2 hr), or by GW182 knockdown (GW182KD, left) or GFP-DNGW182 expression (right). The BDNF-induced downregulation of KCC2 transcripts was abolished by loss of GW182 function (right). All qRT-PCRs were normalized to β-tubulin III (neuron-specific isoform) values, which are unchanged by BDNF, within individual cDNA samples to control for consistency between amplification assays. Expression of control shRNA (sh-control-1, left) or GFP (right) serve as controls for GW182KD or GFP-DNGW182, respectively. All experiments done in the presence of Actinomycin-D to isolate changes due only to translation.

(F) Immunohistochemical staining for endogenous P body component LSm5 (middle, green) showed extensive colocalization with another endogenous P body component, GW182 (top, red; overlay, bottom).

(G) Immunohistochemistry for endogenous Dcp1a showed loss of P bodies in neurons expressing shRNA against LSm5 (LSm5KD) but not in control-shRNA-expressing neurons (sh-Control-2). GFP expression served to visualize neuron morphology. Scale bars represent 10 μm in all images.

(H) Increased total protein synthesis in response to BDNF is unaltered after P body disruption by loss of LSm5. Total protein synthesis was assayed by measuring ^{35}S incorporation in sh-Control-2 and LSm5KD neurons, undergoing mock (−BDNF) or BDNF (+BDNF) stimulation 100 ng/ml for 2 hr, in the presence of Actinomycin-D (0.5 ug/ml, pre-incubated 10 min before stimulation). * = $p < 0.05$ by unpaired Student's t test.

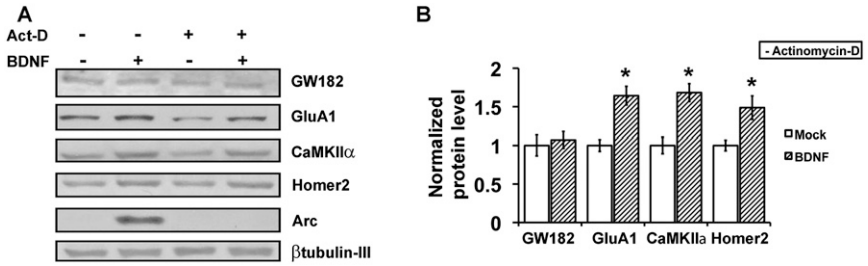

Figure S3. The Transcription Inhibitor Actinomycin-D Does Not Alter BDNF-Induced Protein Synthesis under the Assayed Conditions, Related to Figure 2

(A) Immunoblotting for BDNF targets in cultured neurons treated mock (-) or BDNF (+; 100 ng/ml for 2 hr) stimulation in the absence (−Act-D) or presence of Actinomycin-D (+Act-D; 0.5 ug/ml added 10 min before stimulation). Arc demonstrates strong transcription-dependent upregulation by BDNF and serves as an indicator for Actinomycin-D efficacy.

(B) Protein levels of BDNF targets relative to the mock-stimulated condition (open bars, set as 1.0). Quantification is by densitometry with internal normalization to β-tubulin. Error bars represent SEM. * = $p < 0.05$ by unpaired Student's t test.

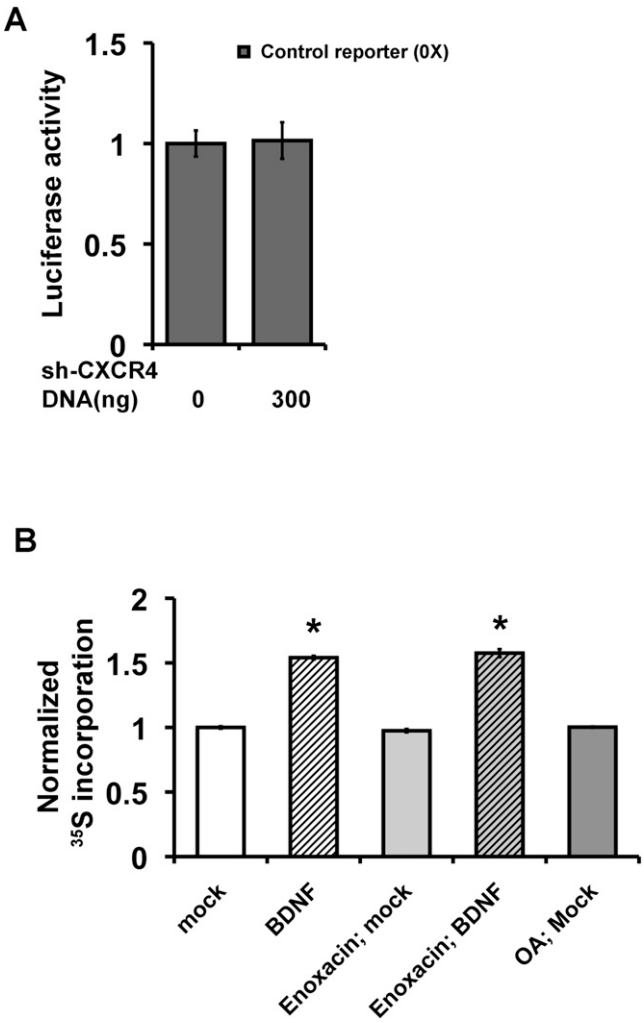

Figure S4. Control Experiments Supporting Involvement of the miRNA Pathway in BDNF Regulation of Protein Synthesis, Related to Figures 2 and 3

(A) Activity of a luciferase reporter lacking binding sites is not affected by expression of shRNA targeting CXCR4. Luciferase reporter assay was carried out (as in Figure 3) in neurons expressing a control luciferase reporter free of any binding site for shRNA against CXCR4 (sh-CXCR4) and treated with mock (−BDNF) or BDNF (+BDNF) stimulation at 100 ng/ml for 2 hr in the presence of actinomycin-D (0.5 μg/ml, added 20 min before stimulation).

(B) Global activation of miRNA biogenesis by Dicer stabilization with enoxacin does not significantly alter the quantity of total cellular protein synthesis. Total protein synthesis was assayed by measuring ^{35}S incorporation in neurons, undergoing mock or BDNF stimulation (100 ng/ml for 2 hr), with or without enoxacin, in the presence of actinomycin-D (0.5 ug/ml, added 20 min before stimulation). Oxalinic acid, a structurally similar control for enoxacin, also does not alter total cellular protein synthesis. Error bars represent SEM. * = $p < 0.05$ by unpaired Student's t test.

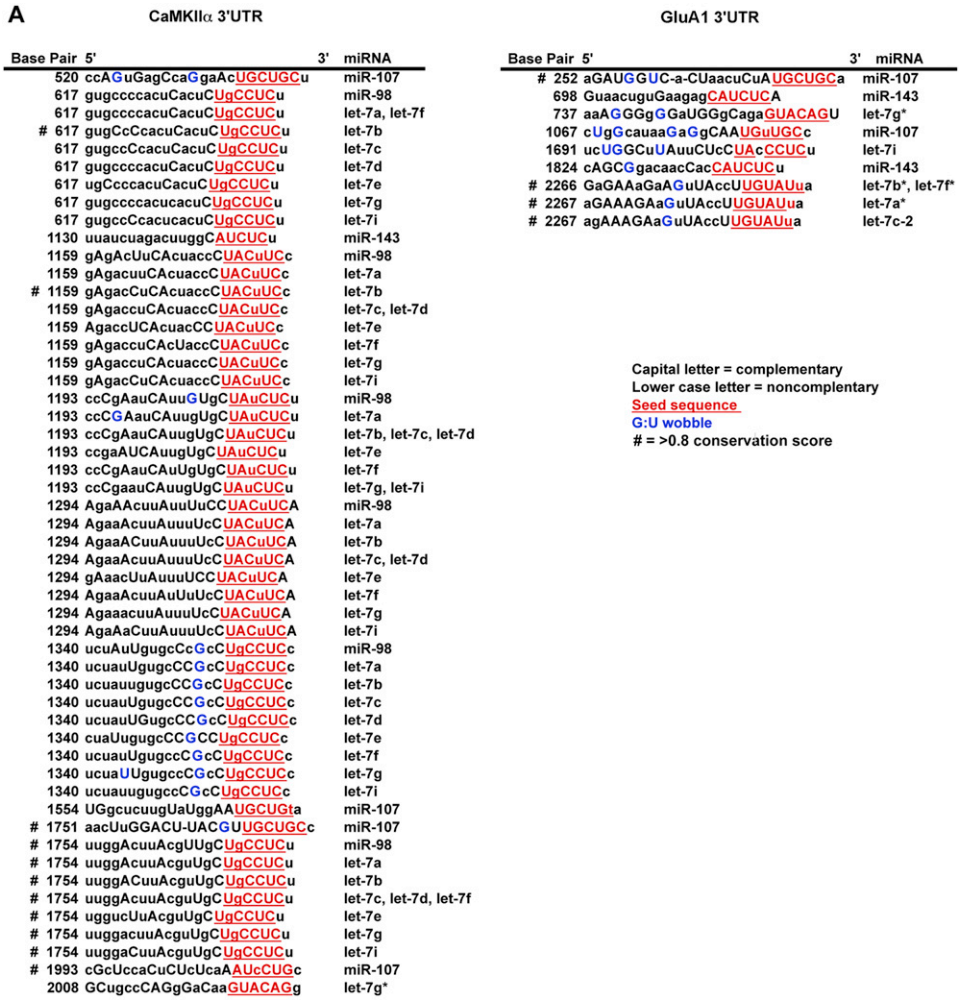

Figure S5. Binding Sites for Lin28-Regulated miRNAs in the 3′UTRs of Example BDNF-Upregulated Targets, CaMKIIα, and GluA1, Related to Figure 4

The binding region for the miRNA seed sequence (nucleotides 2-7 of the miRNA) is underlined and in red. Complementary base pairing is denoted by uppercase letters, noncomplementary base pairing are in lowercase letters, and G:U wobbles are in blue font. Base pair position indicates the base in the 3′UTR of the mRNA where the miRNA 5′ end binds. microRNA seeds having an average phylogenetic conservation score higher than 0.8 are denoted by the pound (#) sign. The conservation score is based on alignment of the longest 3′UTR for 17 vertebrates, including mammalian, amphibian, bird and fish species. Conservation scores range from 0 to 1, with 1 being perfectly conserved and scores over 0.8 predictive of functional relevance (PITA) (Kertesz et al., 2007).

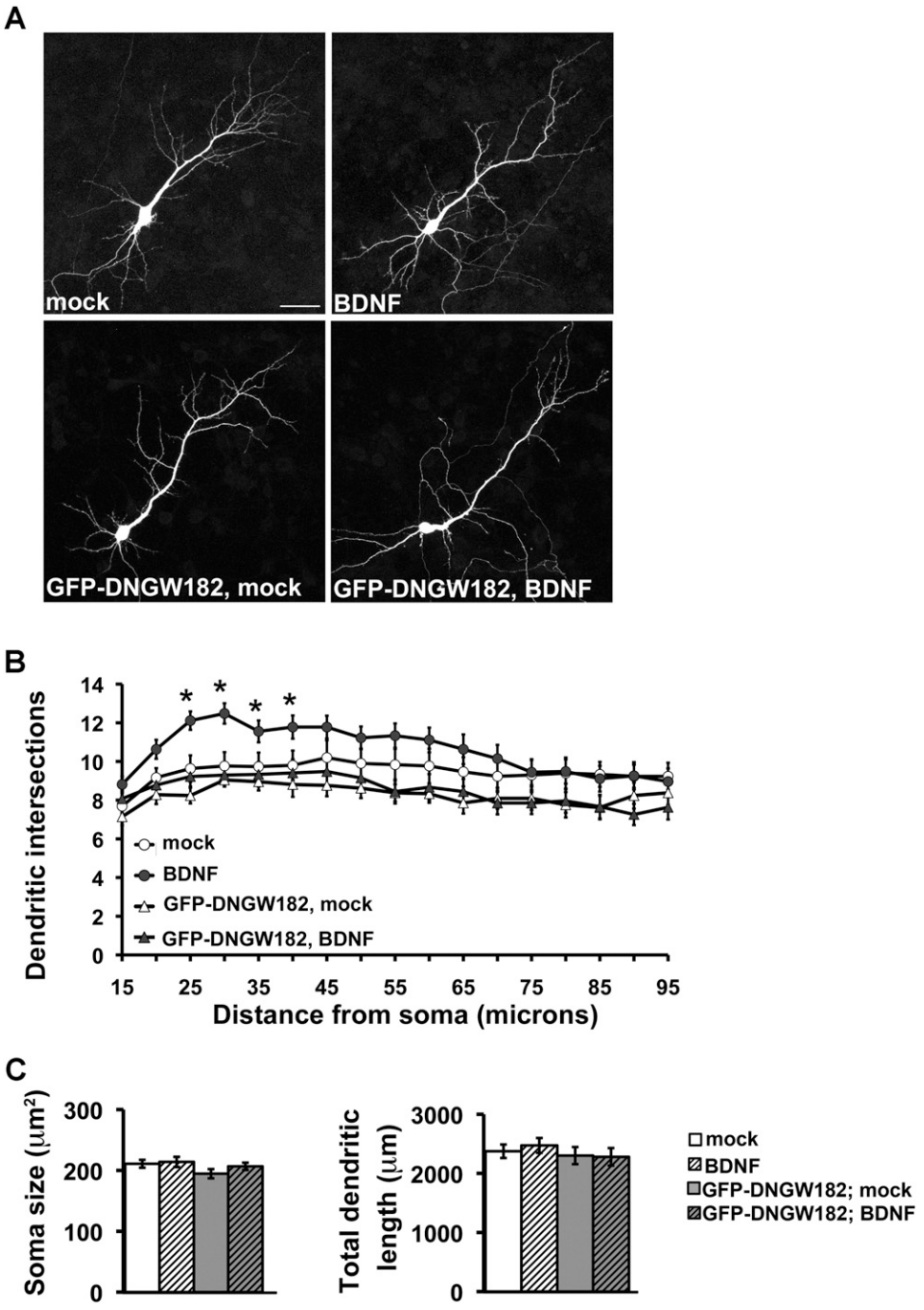

Figure S6. Loss of P Bodies in Neurons Expressing Dominant-Negative Form of GW182 Inhibits BDNF-Induced Dendritic Arborization, Related to Figure 6

(A) Representative images of neurons expressing untagged mCherry and either GFP (top) or GFP-DNGW182 (bottom) following 72 hr of mock or BDNF (25 ng/ml) treatment. Images show red channel (mCherry) only. Scale bar represents 50 μm.

(B) Sholl analysis representing dendritic complexity at increasing distances from the cell body for GFP- (circles) or GFP-DNGW182- (triangles) expressing neurons following mock (open shapes) or BDNF (25 ng/ml, closed shapes) treatment. * = $p < 0.05$ by unpaired one-way ANOVA; Mann-Whitney U test modified Bonferroni correction.

(C) Total dendritic length (right) and soma size (left) are not significantly different between control and GFP-DNGW182-expressing neurons following mock or 25 ng/ml BDNF treatment. Error bars represent SEM mock, n = 30 cells; BDNF, n = 27; GFP-DNGW182, mock, n = 21; GFP-DNGW182, BDNF, n = 27.