# Supporting Information

## Poon and Chen 10.1073/pnas.0807740105

### SI Text

**Plasmid Constructs** ***Nuclear export.*** All RARα constructs were generated from rat sequences and cloned into pEGFP-N1 (Clontech). For *RARα-EGFP*, FL rat RARα was PCR-amplified from rat hippocampal cDNA and cloned into pEGFP-N1 using XhoI and NotI. For *RARα (NES-3A)-GFP*, hydrophobic residues were mutated to alanine using site-directed mutagenesis. For *RARα DBD-GFP*, nucleotides 1 to 570 of RARα were cloned into pEGFP-N1 using BglII and EcoRI.

***Recombinant protein expression.*** All RARα constructs were generated using rat sequences and cloned into pGEX-KG. For *GST-RARα*, FL RARα was cloned using BamHI and EcoRI; for *GST-RARαΔF*, nucleotides 1 to 1,200 were cloned with BamHI and EcoRI; for *GST-RARα LBD*, nucleotides 494 to end were cloned using BamHI and EcoRI; for *GST-RARα LBDΔF*, nucleotides 494 to 1,212 were cloned using BamHI and EcoRI; for *GST-RARα H12/F*, nucleotides 1,213 to end were cloned in BamHI and EcoRI; and for *GST-RARβ LBD*, nucleotides 1201 to end were cloned with BamHI and HindIII.

***FL untagged constructs (HEK293 translation assay).*** All constructs were cloned into pciNeo (Promega). FL RARα was cloned in with EcoRI and SalI and RARβ was cloned into pciNeo using NheI/XhoI into NheI/SalI sites of pciNeo.

***In vitro RNA transcription.*** RNAs were generated from constructs cloned into pciNeo or from PCR templates. GFP was amplified from pEGFP-N1 and cloned in using XbaI and NotI; for *R1–5′GFP3′*, 5′UTR was cloned in using NheI and HindIII sites into EGFP-N1. 5′UTR-EGFP was then ligated into pciNEO using NheI and XhoI. GluR1 3′UTR was then cloned in using XhoI and SalI. For *R1–5′GFP*, 5′UTR-EGFP was PCR amplified from 5-GFP-3′ and digested using NheI and XhoI. Fragment was ligated using NheI and SalI, abolishing the XhoI site. For *R1 5′UTR, R1 5′UTR-SELEX, CaMKIIα 5′UTR, CaMKII 5′UTR+R1 SELEX*, appropriate fragments were PCR amplified from plasmid using a T7 adapted forward primer. For CaMKIIα 5′UTR + SELEX, a CaMKIIα reverse primer containing the GluR1 SELEX motif was used.

**Semiquantitative PCR Primer Sequences.** GluR1: forward, CAATCACAGGAACATGCGGC; reverse, TCTCTGCGGCTGTATCCAAG. GluR2: forward, GACTGACACCCCATATCGAC; reverse, GCGATCTGTAGGTCTCATCT. eEF2: forward, GAGCTCTCCGAGAACGACC; reverse, TACAGTGCCCAGGACAGGAT. *CaMKIIα*: forward, CATTGTGGCCCGGGAGTATT; reverse, TGGGAAATCGTAGGCACCAG. *EF1α* forward, CTCCCTGTGGAAGTTTGAGA; reverse, CATATTAGCACTTGGCTCCA. PSD-95: forward, CAGGTTGCAGATTGGAGACA; reverse, CATTGTCCAGGTGCTGAGAATA. GFP: forward, GAGATACGGCAAGCTGACCCTGAA; reverse, AGACGATGTTGTGGCGGATCTTG.

**Antibodies.** Antibodies used were as follows: rabbit polyclonal RARα (1:500 for immunoblotting, 1:250 for Immunocytochemistry [ICC]; Santa Cruz Biotechnology), mouse monoclonal RARα (1:500 for immunoblotting, Chemicon), rabbit polyclonal GFP (1:5000 for immunoblotting, 1:1,000 for ICC; Abcam), mouse monoclonal GFP (1:1,000 for ICC; Abcam), mouse actin (1:5,000 for immunoblotting; Chemicon), MAP2 mouse monoclonal GFP (1:1,000 for ICC; Abcam), MAP2 rabbit polyclonal GFP (1:1,000 for ICC, Chemicon), Cy2-conjugated goat anti-mouse, Cy2 conjugated goat anti-rabbit, Cy3 conjugated goat anti-mouse, and Cy3 conjugated goat anti-rabbit (1:1,000 for ICC; Jackson Immunoresearch).

**Chemicals and Reagents.** Chemicals and Reagents used were as follows: leptomycin B (Sigma); Triton-X100 (Fisher Scientific); pepstatin, chymotrypsin, leupeptin, and PMSF (Calbiochem); minimal essential media (MEM; Invitrogen); RNAsin (Promega); protein A Sepharose (Amersham); proteinase K (Roche); RNase A (Roche); RNase free DNase (Promega); actinomycin D (Sigma); anisomycin (Sigma); and RA (Sigma).

**Immunocytochemistry.** Primary neurons were prepared, transfected, and processed for immunocytochemistry as described (1). Neurons were transfected at 14 days *in vitro* (DIV) with Lipofectamine 2000 (Invitrogen) or treated with leptomycin B (11 ng/ml in methanol), then fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton-X100, and quenched in 50 mM $NH_4Cl$. Normal goat serum (10%) was used to block nonspecific signal for 1 h and cells were incubated in primary antibody for 2 h at room temperature or overnight at 4 °C. Primary antibody was washed off with PBS solution and cells were incubated in secondary antibody for 1 h. Cells were mounted and imaged on an Olympus FV510 confocal microscope. Neurons were analyzed by measuring mean fluorescent intensity along primary dendrites and normalizing to mean nuclear intensity by using ImageJ software.

**FISH.** FISH was performed as described (2). Digoxigenin-labeled ribo-probes were generated from T7-adapted PCR products generated between bases 400 and 800 of the rat RARα coding sequence. Probes were hybridized at 52 °C in 50% formamide and Rapid Hyb buffer (Amersham) and washed at 50 °C in 1× SSC containing 50% formamide. DIG label was detected using an HRP-coupled anti-DIG antibody (Roche) and the signal amplified using a Cy3 TSA kit (Perkin-Elmer). MAP2 was concurrently detected using a mouse MAP2 primary antibody followed by a Cy2 conjugated goat anti-mouse secondary. Images were taken using an Olympus FV510 confocal microscope.

**CLIP.** CLIP was performed as described (3). Briefly, hippocampi from 21-d-old Sprague-Dawley rats were dissected in ice-cold MEM. Tissue was minced with a scalpel, then gently triturated using a fire-polished glass pipet. Dissociated cells were transferred to a Petri dish on ice (volume ≤3 mm depth) and exposed to UV light using a Stratalinker with three 400-mJ pulses with gentle swirling between pulses. Cells were spun down, the MEM was removed, and cells were then lysed in radioimmunoprecipitation buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton-X, 0.1% SDS, 1% sodium deoxycholate) containing aprotinin, leupeptin, pepstatin, chymostatin, PMSF, and RNAsin first by freeze-thawing, followed by overnight lysis at 4 °C on a rotator. Cellular debris was removed by centrifugation and the supernatant pre-cleared with protein A Sepharose beads. Lysate was divided into aliquots in individual tubes depending on sample number. Extreme care was taken to maintain equal volumes. Supernatant was immunoprecipitated with 5 μg of rabbit GFP, RARα, or no antibody. A small sample was treated with RNase A and retained for Western blot analysis. The majority was digested with first with RNase-free DNase I (Promega), then with proteinase K to remove cross-linked protein; RNA was then purified by using TRIzol (Invitrogen). Equal volumes of sample were oligo(dT) primed and reverse-transcribed using Super-

Script II (Invitrogen). Specific targets were amplified by PCR using gene-specific primers and incrementally increasing cycle numbers. (For hippocampal CLIP: GluR1, 22 cycles; GluR2, 25 cycles; eEF2, 28 cycles; and CaMKII$\alpha$, EF1$\alpha$, and PSD-95, 35 cycles each.)

**Western Blotting.** Samples were run on 10% to 15% SDS/PAGE and transferred to PVDF membranes. Membranes were blocked with Blotto (Tris-buffered saline solution containing Tween [TBST] and 5% dry milk). Primary antibodies were diluted into Blotto and incubated 2 h at room temperature or overnight at 4 °C. Primary antibody was washed with TBST and secondary antibody (horseradish peroxidase conjugated goat anti-mouse or rabbit) was added for 1 h in Blotto. Secondary antibody was washed off with TBST and signal detected using ECL (Amersham). Membranes were exposed to film and the signal quantified by densitometry using ImageJ software.

***In Vitro* Domain-Specific RNA Selection.** Purified GST fusion proteins and total RNA from synaptoneurosomes were used for selection. GST fusion proteins were expressed in BL21 cells induced with IPTG. Bacteria was sonicated in lysis buffer (150 mM NaCl, 20 mM sodium phosphate, pH 7.4, 1% Triton X-100, and protease inhibitors) and debris cleared by centrifugation. Protein expression was confirmed by SDS/PAGE followed by Coomassie staining and immunoblotting when possible. GST fused to the various domains of RAR$\alpha$ or RAR$\beta$ were then purified from the bacterial lysate by binding to glutathione-Sepharose beads (Amersham) and equilibrating/washing the protein-bound beads five times in RNA binding buffer (200 mM KOAc, 10 mM TrisOAc, pH 7.7, and 5 mM MgOAc with protease and RNase inhibitors). Total RNA was obtained from synaptoneurosomes (4) obtained from 21-d-old Sprague-Dawley hippocampi using TRIzol. RNA was DNase treated and re-extracted with TRIzol, and the pellet was re-suspended in nuclease-free water and quantified by spectrophotometry. RNA (25 $\mu$g) was added to RNA binding buffer and heated to 95 °C to denature secondary structure, then slowly re-natured. Re-natured RNA was then added to the immobilized GST-fusion protein (100 nmol) in RNA binding buffer and rotated overnight at 4 °C. Beads were then washed several times in RNA binding buffer. RNA was extracted with TRIzol, treated with RNase-free DNase I, then reverse transcribed with oligo(dT) and amplified with PCR using gene-specific primers (GluR1, 18 cycles; GluR2, 21 cycles; eEF2, 27 cycles; CaMKII$\alpha$, 30 cycles; PSD95, 30 cycles; and EF1$\alpha$, 30 cycles).

**HEK Cell Translation Assay.** HEK293 cells grown in MEM containing 10% FBS were first transfected for 24 h with untagged RAR$\alpha$ or RAR$\beta$. After 24 h, GFP reporter constructs containing or lacking the GluR1 untranslated regions were transfected for 3 h, then treated with 50 $\mu$M actinomycin D (ActD) and/or 40 $\mu$M anisomycin. After 30 min of ActD and/or anisomycin, cells were treated with 1 $\mu$M retinoic acid for 1 h and lysed overnight at 4 °C in radioimmunoprecipitation buffer containing protease inhibitors and analyzed by Western blotting using primary antibodies to GFP or actin. Protein levels were quantified by densitometry using ImageJ software.

**SELEX.** SELEX was performed using GST-RAR$\alpha$ immobilized onto glutathione Sepharose beads. Oligonucleotide and primer sequences used were exactly as described (5, 6). Sequences were aligned and analyzed on the IBM Bioinformatics and Pattern Discovery Group Web site (*http://cbcsrv.watson.ibm.com/Tspd.html*). Briefly, oligonucleotides containing a 30-base random nucleotide stretch flanked by a 5′ T7 promoter adapter and a 3′ adapter sequence were made double-stranded by using PCR. RNA was generated using T7 RNA polymerase and the DNA template digested with DNase I. The resulting RNA pool was then purified with TRIzol and resuspended in RNA binding buffer containing protease and RNase inhibitors. Binding species were selected by incubating the random RNA pool with RAR$\alpha$-GST immobilized to glutathione beads overnight at 4 °C on a rotator. Following RNA selection, the beads were washed several times with RNA binding buffer and the bound RNAs extracted with TRIzol. Extracted RNA was reverse transcribed with SuperScript II (Invitrogen) using a primer to the 3′ adapter sequence, and the cDNA was PCR amplified with Easy-A (Stratagene) using the 5′ and 3′ adapter primers. The PCR product was then transcribed with T7 RNA polymerase and the process repeated for a total of five rounds of selection. After the final round, the product was TA cloned into pGEM-T Easy (Promega). Randomly picked colonies were mini-prepped and sequenced using the SP6 promoter primer. Sequences were aligned and analyzed on the IBM Bioinformatics and Pattern Discovery Group Web site.

**Nitrocellulose Filter Binding Assay.** Filter binding assays were performed as described (7) using recombinant GST fusion proteins. GST fused to the various domains of RAR$\alpha$ or RAR$\beta$ were purified and eluted with glutathione buffer (50 mM Tris pH 8.5, 150 mM NaCl, 50 mM glutathione, 1 mM DTT) and concentrated with a Microcon YM-10, and the amounts were quantified by spectroscopy and densitometry of Coomassie-stained SDS/PAGE gels. RNA probes were generated using PCR templates containing 5′ T7 promoter sites fused to the sequence of interest. Probes were transcribed with T7 RNA polymerase in the presence of $^{32}$P-CTP, then cleaned over a G-25 Microspin column (Amersham). Probes were heat-denatured and slowly re-natured in RNA binding buffer. RNA and recombinant protein were incubated together in the presence or absence of 10 $\mu$M RA at room temperature for 15 min in RNA binding buffer containing 4.5 mM glutathione. For competition assays, increasing amounts of cold probe were added to each sample during the incubation. The protein-RNA complexes were then applied to 0.45 $\mu$m nitrocellulose filter membranes pre-wetted with RNA binding buffer and placed over a vacuum manifold. Samples were washed immediately with equal amounts of RNA binding buffer. The filter was removed and the filter-bound RNA probe was counted using a Beckman LS3801 scintillation counter. Background counts were subtracted and samples were normalized to total probe added (probe added directly to filter with no washing).

***In Vitro* Translation with Rabbit Reticulocyte Lysate.** Nuclease-treated rabbit reticulocyte lysates were obtained from Promega. GST fusion proteins were purified from bacterial lysate by binding to glutathione beads, eluted with glutathione, and concentrated with YM-10 Microcon filters (Millipore). After measuring protein concentrations, 100 nmol of GST-RAR$\alpha$ or RAR$\beta$ LBD proteins were added to amino acid-lacking rabbit reticulocyte lysate containing RNA (denatured and slowly re-natured) encoding either GFP or GluR1 5′-GFP in the presence or absence of 10 $\mu$M retinoic acid (25 $\mu$l reaction volume). After a 15-min incubation, amino acids, including $^{35}$S-Met, were added to the lysate and translation was allowed to proceed for 90 min at 30 °C. Samples were then run on a 15% SDS/PAGE gel and dried on a Bio-Rad 583 gel dryer. Gels were exposed to film overnight at −80 °C and densitometry measured with ImageJ software.

1. Nam CI, Chen L (2005) Postsynaptic assembly induced by neurexin-neuroligin interaction and neurotransmitter. *Proc Natl Acad Sci USA* 102:6137–42.
2. Poon MM, Choi SH, Jamieson CA, Geschwind DH, Martin KC (2006) Identification of process-localized mRNAs from cultured rodent hippocampal neurons. *J Neurosci* 26:13390–13399.
3. Ule J, *et al.* (2003) CLIP identifies Nova-regulated RNA networks in the brain. *Science* 302:1212–1215.
4. Aoto J, Nam CI, Poon MM, Ting P, Chen L (2008) Synaptic signaling by all-trans retinoic acid in homeostatic synaptic plasticity. *Neuron* 60:308–320.
5. Buckanovich RJ, Darnell RB (1997) The neuronal RNA binding protein Nova-1 recognizes specific RNA targets in vitro and in vivo. *Mol Cell Biol* 17:3194–201.
6. Darnell JC, *et al.* (2001) Fragile X mental retardation protein targets G quartet mRNAs important for neuronal function. *Cell* 107:489–499.
7. Black DL, Chan RC, Min S, Wang J, Bell, L (1997) The electrophoretic mobility shift assay for RNA binding proteins. *RNA-Protein Interactions: A Practical Approach*, ed Smith CWJ (Oxford Univ Press, Oxford, UK), pp 109–136.

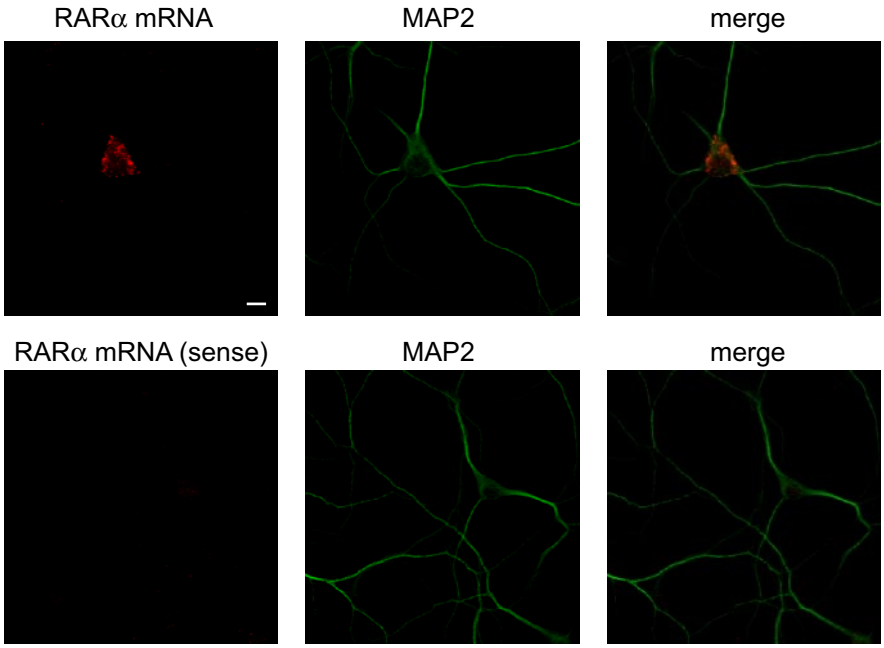


**Fig. S1.** RARα mRNA is present in neurons but is not dendritically localized. FISH of the mRNA encoding RARα in 14-DIV hippocampal neurons (red, mRNA; green, MAP2 protein). *Top*, RARα antisense probe; *Bottom*, RARα sense probe. (Scale bar, 10 μm.)

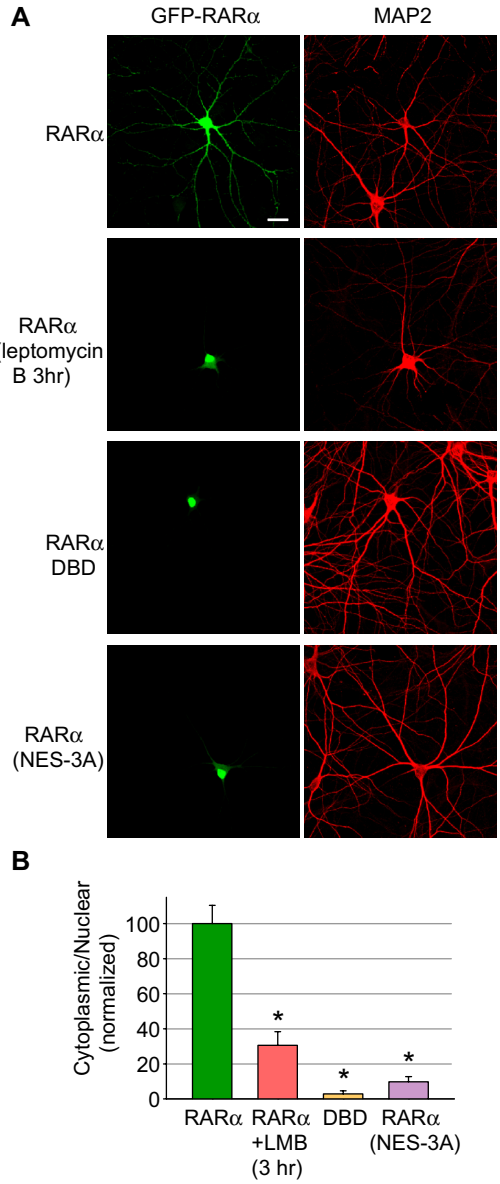


**Fig. S2.** Dendritic localization of RARα utilizes active export from the nucleus. (*A*) Neurons (14 DIV) transfected with RARα-GFP, RARα-GFP followed by LMB treatment, RARα DBD-GFP, or RARα(NES-3A)-GFP. (*B*) Quantification of cytoplasmic/nuclear localization of RARα-GFP ($n = 8$) compared with LMB treatment ($n = 7$), RARα DBD-GFP ($n = 5$), or RARα(NES-3A)-GFP ($n = 7$; *, $P < 1 \times 10^{-4}$, single-factor ANOVA). (Scale bar, 25 μm.)

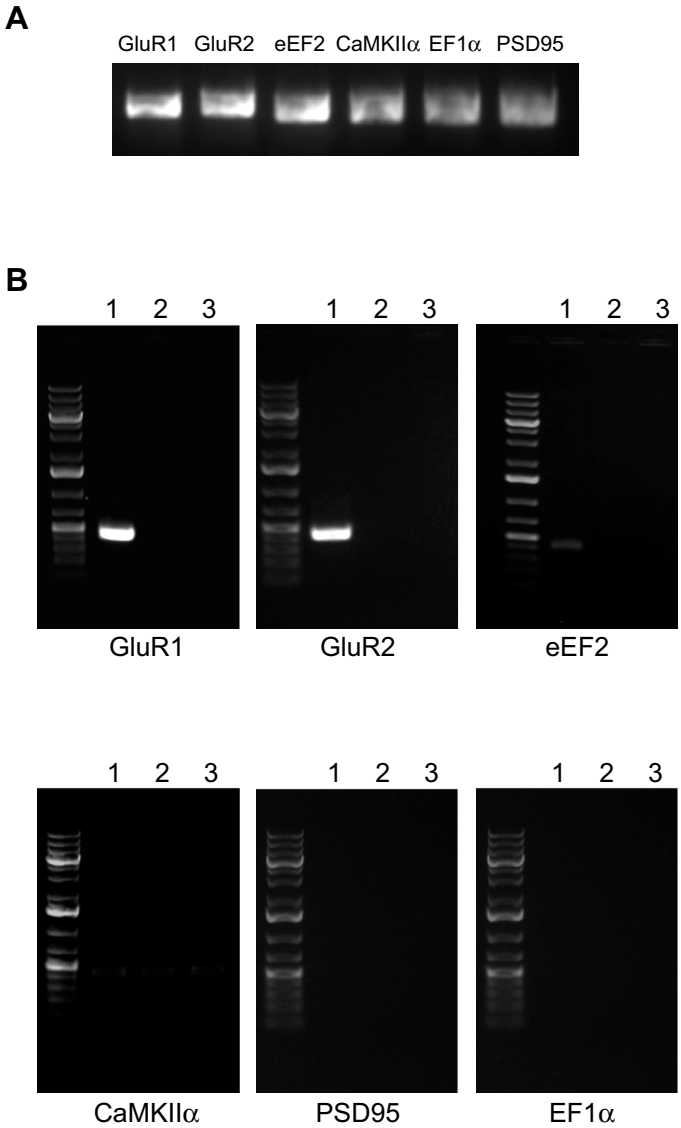


**Fig. S3.** RARα associates with mRNAs in synaptoneurosomes. (*A*) Gene-specific primer set controls. Hippocampal cDNA was used as a template to test semiquantitative PCR primer specificity (22 cycles). (*B*) CLIP in hippocampal synapto-neurosomes. Lane 1, RARα antibody; lane 2, GFP antibody; lane 3, no antibody.

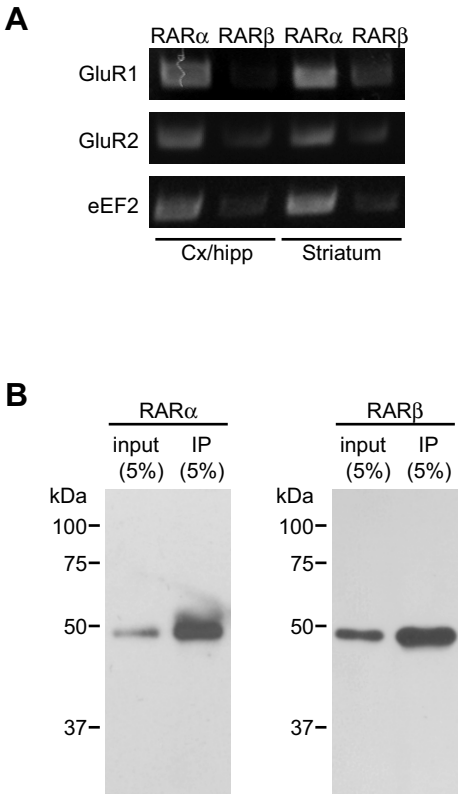


**Fig. S4.** RAR$\beta$ does not associate with RAR$\alpha$-associated mRNAs *in vivo*. (*A*) CLIP of protein-RNA complexes from cortex/hippocampus or striatal tissue using RAR$\alpha$ and RAR$\beta$ antibodies. Protein association with GluR1, GluR2, and eEF2 mRNAs were analyzed by using semiquantitative RT-PCR with equal starting quantities of total RNA (GluR1, 24 cycles; GluR2, 27 cycles; eEF2, 30 cycles). (*B*) Co-immunoprecipitation efficiency of RAR$\alpha$ and RAR$\beta$ antibodies.

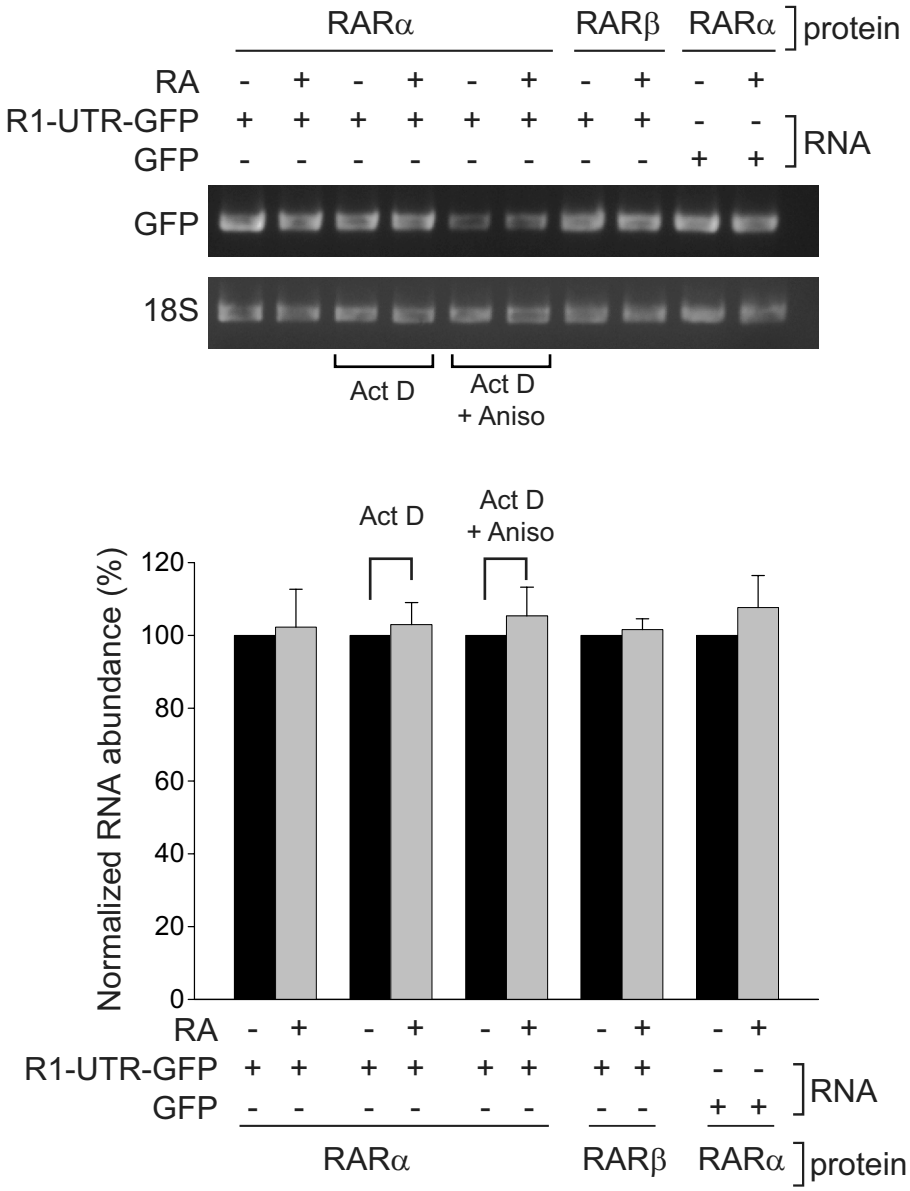


**Fig. S5.** Relative GFP reporter RNA levels are not affected by RA treatment in transfected HEK293 cells. RNA from HEK293 cells after RAR$\alpha$ or RAR$\beta$ transfection followed by GFP reporter transfection and drug treatment (RA, actinomycin D, or anisomycin) was analyzed using RT-PCR and GFP-specific primers ($n = 3$ for each sample, 28 cycles).

**A**

GAGGCCGCGCUGGCAGAUUGUUUUACAAAUCG
GGAGCAAAUCAGUAUAUCCGCGCGGGAUGG
GCGGCCCACCUCCCGUAUACAAUAUUAACGG
GCCAUAUCAAGUUGGUAUUGCAGACAGUUCA
UUCAACGCCAUGGUACACAUCGCGUUUG
GUUCUUCAGUUCUCGGGGGGGAUUUUAAUGC
AGGCUACUCAGGAUUGAUCAUCAAUUCUAG
UAUGGCGGUAGUUGGCACUGGUUCACUUCCUAUG
GACAAAUCUCGCUUUCGGGAUACGGAAGGUUAUG
CGCGAAUGUUAACAGAUCGUUUAAGACUUAUAUG

GGGCGCGGGUUCGAUUGAUCGGUGUGA
GGAUUGAAGUAGGGGCGACAACUGGCCAUUA
GCUGCGGUGGGGGGGUUCGCUUUUUUAGUAGAUU
GGGAAAACGGGAGUGACUGGAGGUCCGGA
GUUAUUCAGUUCUCGGGGGCGAUUUUUAUGA
GGAUUGAAGUAGGGGCGACAACUGGCCAUUA
ACUGCGUUUUUGUUGGGAGGGAGCAACGU
GGGGGGGGGGGAAAAGGCAGAGCUUUUUAUAGG
GGGGGAGAAUUACGGAAAACUCCAGGUGUG
GGGGUGGGGGUUACAAGAACAAGAUCUAGAUAUG

**B**

**GluR1 5'UTR**

AATTCGGCACGAGCTCGGCTCCCCTTCCAAGAGAAACAAGAGAAACCTCACGGAAGGAAGGGAGGAAGGAAAGAA
GCAAGCAAGGAACTGCAGGAAGAAAAGAGCCGGCAGAGCATCAAGAAGAATCGAAGGGAGGGGAGGGGAAAGA
CCAAATCT

**eEF2 5'UTR**

CGCCATCGTCGGCGCGCTTCCCTGTTCACCTCTGTATTTGAGAATCCGACGCCATCTGCCACC

**GluR2 5'UTR**

GAGACTGCCTGCCCCTCTCTGTGACTTTCCTGTGTGTGTGCGTGTGTGTGTATGTGTGTGTATGTGTGTGCGCGCTCG
TGTGAGAGAGGAGAGAGGGAGAAGAGAGCTCGAGAGAGGGTGATTGTGTGAGTGCATGGGAGGGTGCTGATAT
TCCCAGACACCAGGACTACAGCGGCAGCTCAGCTAAAAACTGCATTCAGCCAGTCCTCGGGACTTCGGGAGCAGGG
ACAGGACGCAAGGCATCAACAGCCACCAGCTACAACTGGGAAATAAGGATTCTTCTGCCTTCACTTCGTGTTTTTAG
CAGCTCCTTGCTAAATATCGACCTCACAATGCAGAGGATCTAATTTGCTGAGGAAAACAGTCAAAGAAGGAAGAGG
AAGAAAGGGAAACGAGGGGATATTTTGTGGATGCTCTACTTTTCTTGGAAA

**EF1$\alpha$ 5'UTR**

TTTTTTTTTTTTTCGCAACGGGTTTGCCGTCAGAACGCAGGTGTTGTGAAAGCCACCGCTAATTCAAAGCAAAA

**PSD95 5'UTR**

GCAAAACTCCAATGAAGTCAGAGCCCCCTACTCGCCGCCGCGGCCAGGCCCCCCAAC

**CaMKII$\alpha$ 5'UTR**

AGAAGCCCCAAGCTCGTCAGTCAAGCCGGTTCTCCGTTTGCACTCAGGAGCACGGGCAGGCGAGTGGCCCCCTAGT
TCTGGGGGCAGCGCTTCAGCATCCCAGCCCTAGTTCCCAGCCTAAAGCCTCGCCTGCCTGCCCAGTGCCAGGATG

**Fig. S6.** Both SELEX motifs are present in the 5′UTRs of RAR$\alpha$-associated mRNAs. (*A*) Representative sequences identified by SELEX with motifs highlighted. (*B*) Rat 5′UTR sequences with SELEX motifs highlighted. Motifs are present in GluR1, eEF2, and GluR2 5′UTRs, but are not present in EF1$\alpha$, PSD95, or CaMKII$\alpha$ 5′UTRs.

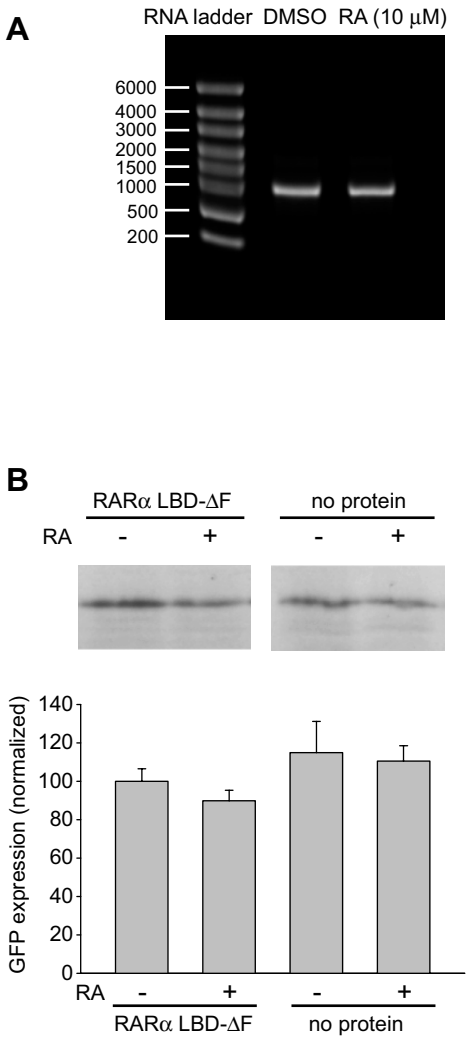


**Fig. S7.** RARα F-domain is required for translational regulation. (*A*) RA treatment does not affect mRNA stability. RA (10 μM) was added to 1 μg GluR1 5′UTR-GFP mRNA and incubated for 105 min. A SYBR green RNA gel was used to detect mRNA integrity. (*B*) *In vitro* translation of GluR1 5′UTR-GFP mRNA in the presence and absence of RARα LBDΔF and/or RA ($n = 3$, $P > 0.5$).

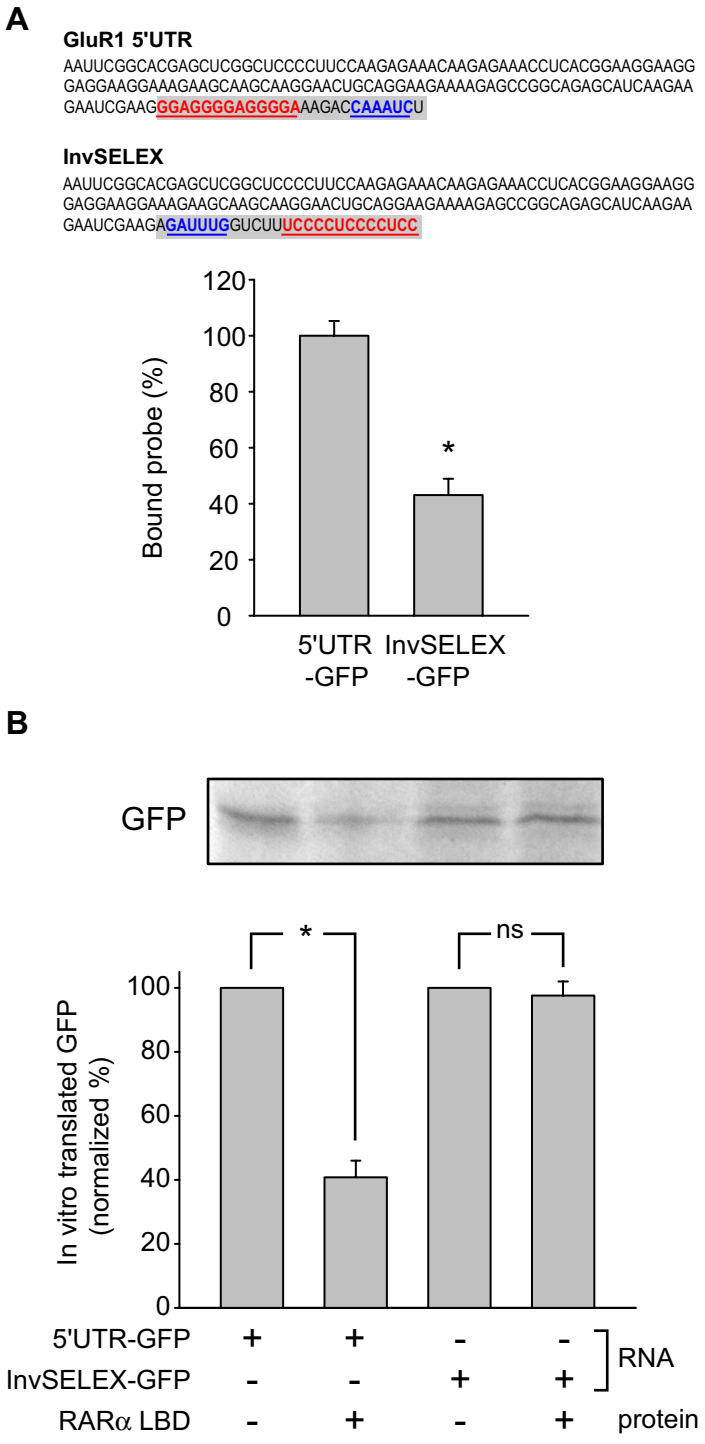


**Fig. S8.** Inverting SELEX motif region within the GluR1 5′UTR reduces RARα LBD binding affinity and removes translation repression by RARα LBD. (*A*) *Upper*, sequence of GluR1 5′UTR and InvSELEX-GFP (GluR1 5′UTR with reverse complemented SELEX motif region, highlighted) RNA probes. Both UTRs were fused to GFP. *Lower*, binding affinity between the RARα LBD and GluR1 5′UTR or InvSELEX RNA probes as assessed by nitrocellulose filter binding (*, $P < 0.0005$, $n = 4$, single-factor ANOVA). (*B*) *Upper*, *in vitro* translation of GluR1 5′UTR and InvSELEX-GFP in the presence or absence of RARα LBD protein (*, $P < 5 \times 10^{-5}$, $n = 5$, single-factor ANOVA).