Supporting Information

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Constructs, Immunocytochemistry, and Fluorescence Microscopy

The plasmids to express Pum2 shRNA, control mismatch shRNA, and EYFP-tagged Pum2 fusion protein have been previously described (1). Plasmid expressing the cleavage-resistant form of Pum2 tagged with c-myc ($Pum2^R$) was created by mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol and coexpressed with either shPum2 or misPum2. Expression was tested by Western blot analysis as well as immunostaining (Figs. S1 and S2). Rat eIF4E was amplified from cDNA, sequenced, and then cloned into the pEGFP-N1 expression vector (Clontech). For knockdown of eIF4E, primers complementary to rat eIF4E were cloned with *Bgl*II and *Hind*III into the pSuperior vector (OligoEngine). All the primers used in this work are listed in Table S1. Neurons were fixed and immunostained as previously described (2). The fluorescence microscopy setup and image processing have been previously described (3). HeLa cells grown in DMEM supplemented with 10% (v/v) FCS were transfected using the TransIT transfection reagent (Mirus Bio LLC) according to the manufacturer's protocol. EMSAs.

For dual luciferase assay, rat primary cortical neurons (E17) were transfected using Lonza Nucleofector device (Rat Neuron Nucleofector Kit, program 0-003; Lonza). In detail, 5 μg of the reporter plasmid and 20 μg of either overexpression or knockdown plasmids were nucleofected into 6×10^5 cells that were distributed on 3 wells of a 24-well plate. Luciferase assays were performed after 1 (overexpression) to 4 (knock-down) days on transfection using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Raw ratios of Renilla/firefly luciferase activity were calculated and normalized to the mismatch or overexpression control.

Antibodies

The following antibodies were used in this study: mouse monoclonal anti-MAP2 (1:500; Sigma), rat polyclonal anti-Homer 1a/b/c (1:250; Affinity Bioreagents), rabbit polyclonal anti-Synapsin (1:250; Chemicon International), rabbit polyclonal anti-eIF4E (1:250; Santa Cruz Biotechnology), rabbit polyclonal anti-eEF1a (1:500, Abcam), rabbit polyclonal anti-$GABA_B$ Receptor R2 (1:250; Millipore), rabbit polyclonal anti-Gephyrin (1:250, Millipore), goat anti-rat AlexaFluor-546 (1:500; Invitrogen), goat anti-mouse AlexaFluor-546 (1:500; Invitrogen), goat anti-rabbit AlexaFluor-350 (1:500; Invitrogen), and goat anti-rabbit AlexaFluor-488 (1:500; Invitrogen).

Antibody Production and Western Blotting

Encompassing amino acids 1–648 of the Pum2 protein, 6× His-Pum2 was expressed in *Escherichia coli*, purified, and injected into rabbits. Antiserum was immunopurified against GST-Pum2. Western blotting was performed on lysates from nucleoporated neurons (4) and probed with the following antibodies: rabbit anti-Pum2 (1:200; Abcam/Bethyl Laboratories), goat anti-Pum1 (1:200; Bethyl Laboratories), mouse anti-β-Actin (1:2,000; Sigma), mouse anti-eIF4E (1:500; BD Transduction Laboratories), mouse anti-tubulin (1:10,000; Sigma), and rabbit anti-GFP (1:2,000; kindly provided by Werner Sieghart, Medical University of Vienna, Vienna, Austria). Tubulin, β-actin, and GFP served as loading and transfection controls, respectively. Western blots were probed with anti-rabbit Alexa-800 (Li-Cor Biosciences), anti-mouse Alexa-700 (Li-Cor Biosciences), anti-goat Alexa-680 (Molecular Probes), and secondary antibody; scanned; and analyzed with the Odyssey Infrared Imaging System (Li-Cor Biosciences). To determine Pum2 expression during development, rat brains were collected at different stages of development and lysed in lysis buffer: 50 μg of each sample was run on 10% (w/v) SDS/PAGE and processed for quantitative Western blot analysis as described (5).

Sholl Analysis

To determine the length of dendritic protrusions, EGFP-positive dendrites were randomly selected from each condition and the number and length of all protrusions were manually determined using AnalySIS B image analysis software as described (6). All quantifications were tested for significance with a Student's *t* test using SigmaPlot (Systat Software) and were considered significant if $P < 0.05$ and expressed as SEM. At least three independent experiments were performed, and at least five neurons per transfection condition were analyzed.

EMSAs

Both GST-Pum2 full-length and GST-Pum2 lacking the Pumilio homology domain (Pum2-Nt) were cloned into the pGEX-2T expression vector (GE Healthcare), expressed in bacteria, and then purified (1). Gel retardation reactions were carried out using 5 μg of purified Pum2 proteins and 10 ng of labeled pT7-eIF4E-3′-UTR. For competition experiments, labeled and unlabeled RNA (1,000 and 10 ng, respectively) was added simultaneously. For supershift experiments, the anti-Pum2 antibody (1) was added after protein–RNA incubation for an additional 30 min.

Electrophysiology

Standard whole-cell patch clamp recordings were performed under voltage clamp conditions. The neurons were clamped at −70 mV. Bicuculline (20 μM), strychnine (20 μM), and TTX (1 μM) were added to the bath solution to block action potentials and glycinergic synaptic transmission, thus revealing mEPSCs. mEPSCs were determined at room temperature (22–24 °C) on neurons at 18 DIV using an Axopatch 200B amplifier and pClamp 9 hardware and software (Axon Instruments) (7). The bathing solution contained NaCl (140 mM), KCl (6 mM), $CaCl_2$ (3 mM), $MgCl_2$ (2 mM), glucose (20 mM), and Hepes (10 mM) and was adjusted to pH 7.4 with NaOH. Neurons were continuously superfused using a DAD-12 (Adams and List) application system. Electrodes were pulled from borosilicate glass capillaries (Science Products) using a Flaming–Brown puller (Sutter Instruments) to yield tip resistances of 2.5–5.0 MΩ and were filled with a solution containing KCl (140 mM), $CaCl_2$ (1.6 mM), EGTA (10 mM), Hepes (10 mM), Mg-ATP (2 mM), and Li-GTP (2 mM) adjusted to pH 7.3 with KOH. Three days after transfection, mEPSCs were recorded from either transfected or nontransfected cells present within the same culture dish for periods of time sufficiently long to obtain at least 25 consecutive events. Thereafter, 10 μM cyano-2,3-dihydroxy-7-nitro-quinoxaline (CNQX) was applied, which blocks all mEPSCs in the presence of Mg^{2+} (7). mEPSCs were evaluated using the Mini Analysis Program (Synaptosoft, Inc.), and detection thresholds were adjusted for each cell by analysis of traces obtained in the presence of 10 μM CNQX. The interevent intervals of mEPSCs were tested for normal distribution by a Kolmogorov–Smirnov test and then compared by one-way ANOVA. The results show arithmetic means ± SEM, and *P* values below 0.05 were taken as an indication of statistical significance.

1. Vessey JP, et al. (2006) Dendritic localization of the translational repressor Pumilio 2 and its contribution to dendritic stress granules. *J Neurosci* 26:6496–6508.
2. Vessey JP, et al. (2006) Dendritic localization of the translational repressor Pumilio 2 and its contribution to dendritic stress granules. *J Neurosci* 26:6496–6508.
3. Macchi P, et al. (2003) Barentsz, a new component of the Staufen-containing ribonucleoprotein particles in mammalian cells, interacts with Staufen in an RNA-dependent manner. *J Neurosci* 23:5778–5788.
4. Zeitelhofer M, et al. (2007) High-efficiency transfection of mammalian neurons via nucleofection. *Nat Protoc* 2:1692–1704.
5. Xie Y, et al. (2007) The GTP-binding protein Septin 7 is critical for dendrite branching and dendritic-spine morphology. *Curr Biol* 17:1746–1751.
6. Goetze B, et al. (2006) The brain-specific double-stranded RNA-binding protein Staufen2 is required for dendritic spine morphogenesis. *J Cell Biol* 172:221–231.
7. Boehm S, Betz H (1997) Somatostatin inhibits excitatory transmission at rat hippocampal synapses via presynaptic receptors. *J Neurosci* 17:4066–4075.

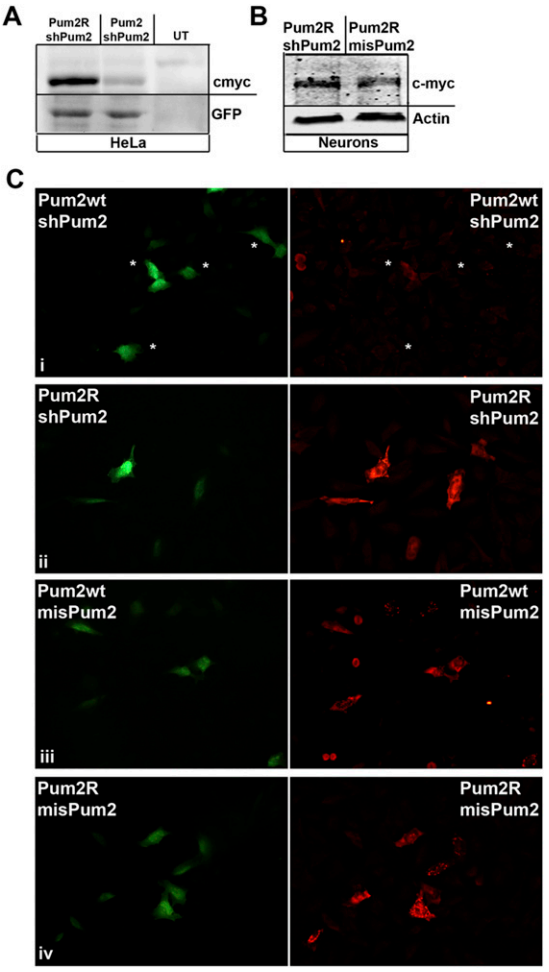

Fig. S1. Western blot analysis of cell lysate prepared from HeLa cells (*A*) and neurons (*B*). HeLa cells coexpressed shPum2, together with either a WT Pum2 tagged with c-myc (Pum2 WT) or an RNAi cleavage-resistant Pum2 tagged with c-myc ($Pum2^R$). Cells were lysed after 1 day of expression and processed for Western blotting. The membrane was probed for GFP expressed by the pSUPERIOR vector that served as an internal transfection and loading control. UT, untransfected. Neurons were transfected with the indicated plasmids and processed for Western blot analysis 3 days later with β-actin as a loading control. (*C*) Pum2 immunofluorescence pictures of HeLa cells expressing Pum2 tagged c-myc, together with either shPum2 (*i*) or mismatch shRNA (misPum2; *iii*) in contrast to cells expressing $Pum2^R$. Asterisks indicate HeLa cells transfected showing silencing of the coexpressed Pum2 WT. (Scale bar: 100 μm.)

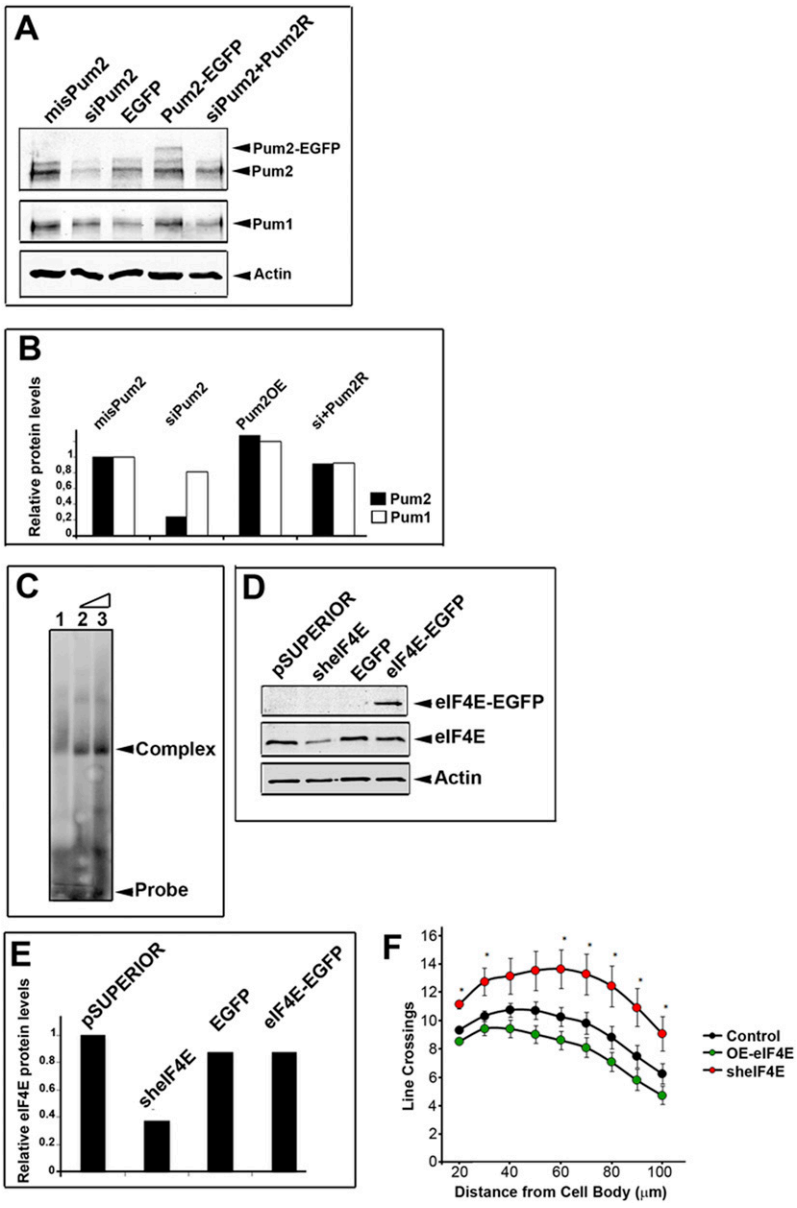

Fig. S2. (*A*) Western blot analysis of cell lysate prepared from cultured neurons expressing mismatch Pum2 shRNA (misPum2), shPum2, EGFP, Pum2 tagged with EGFP, and shPum2, together with an RNAi cleavage-resistant Pum2 tagged with c-myc (siPum2 + Pum2^{R}). Neurons were lysed after 3 days of expression and processed for Western blotting. The membrane was probed for Pum2 as well as Pum1. Actin served as a loading control. (*B*) Quantification of the Western blot shown in *A*. Levels were normalized to β-actin and results for misPum2. (*C*) EMSA analysis. Full-length Pum2 specifically binds to *eIF4E* (lanes 2 and 3, increased concentration of eIF4E) but not to Pum2 lacking the RNA-binding domain (lane 1). (*D*) Representative Western blot of hippocampal neurons transfected with shRNA expression plasmids against eIF4E (sheIF4E), pSUPERIOR, EGFP, and EGFP-tagged rat eIF4E (EGFP-eIF4E). Cells were lysed after 3 days of expression and processed for Western blotting with the indicated antibodies. (*E*) Quantification of the levels of eIF4E protein expression in neurons transfected with the indicated plasmids; levels were normalized to β-actin and results for pSuperior control. (*F*) Sholl analysis of dendritic arbor complexity [Sholl DA (1953) Dendritic organization in the neurons of the visual and motor cortices of the cat. *J Anat* 87:387–406]. Neurons lacking *eIF4E* (sheIF4E, green circles) show significantly more line crossings between 20 and 30 μm and between 60 and 100 μm than control neurons (control, blue circles; $P < 0.05$). Neurons over-expressing GFP-eIF4E (red circles) demonstrate fewer line crossings at all distances measured than control neurons. Three independent experiments were performed, and at least 30 neurons were analyzed for each group.

Table S1. Primers used in this work

Primer	Sequence
eIF4E forward	5′-atggcgactgtggaaccgga
eIF4E reverse	5′-ggagccgctctttgtagctg
scn1a forward	5′-agcttgtccgccctcctacg
scn1a reverse	5′-gtgtttcacgttaaaccccc
scn9a forward	5′-cgaccctgacgccactcagt
scn9a reverse	5′-cataggaaggtggcgagatg
Pum2^{R} forward	5′-ctggatgcaatggaccaagtcggcttggacagcttacagtttgactatcct
Pum2^{R} reverse	5′-aggatagtcaaactgtaagctgtccaagccgacttggtccattgcatccag
eIF4E forward: luciferase assay	5′-gaagacaccttctgagtatc
eIF4E reverse: luciferase assay	5′-ggatctttatcaagttaccag
eIF4E forward: overexpression	5′-atggcgactgtggaaccggaaa
eIF4E reverse: overexpression	5′-acaacaaacctatttttagtggtgg
eIF4E: shRNA	5′-gatccccgctaatccagagcactatattcaagagatatagtgctctggattagcttttta
eIF4E: shRNA	5′-agcttaaaaagctaatccagagcactatatctcttgaatatagtgctctggattagcggg