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Supplementary Information

Co-Regulation of mRNA Translation by
TDP-43 and Fragile X Syndrome Protein FMRP

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Supplementary materials and methods

Plasmid construction

pEF promoter-based expression plasmids pFlag-TDP-43, pFlag-TDP-43(Δ RRM1), pFlag-TDP-43(Δ RRM2) and pFlag-TDP-43(Δ Gly) encoding Flag-tagged mouse TDP-43 (Tardbp, NM_145556.4) and mutant forms of TDP-43 with deletions of different domains were described before [3]. To construct pSicheck2 vector (Promega, Germany)-based double luciferase reporter plasmids, 5'UTR, 3'UTR, CDS of mouse Rac1 (NM_009007.2) and different sub-parts of these sequences, eg. 3'UTR-1, 3'UTR-2 etc. [Fig 3a] were PCR amplified from total RNA isolated from hippocampal neurons using appropriate primer sets. Also, mutations disrupting specific UG/GU motifs, namely: 2M, 3M, 2M3M in 3'UTR and CDM in CDS were generated using double-PCR method and primers listed in Table S6. The PCR products were then cloned into the vector for use in the luciferase reporter-based translation assay.

The wild type and mutated forms of 3'UTR and CDS of Rac1 were cloned into NotI/AflIII sites of phSYN-myr-dEGFP-BDNF 3'UTR plasmid DNA, a generous gift from Dr. Baoji Xu's lab, Georgetown University, Washington, DC 20057, USA, to replace BDNF-3'UTR in the sites of phSYN-myr-dEGFP-BDNF 3'UTR plasmid and to generate pmyr-dEGFP-3'UTR. The pmyr-dEGFP-based plasmid was used to track the dendritic local translation of the corresponding reporter constructs. All the primers used for cloning are listed in Table S4.

Transfection of plasmid DNAs and RNAi oligos

For RNAi knockdown, the cultured hippocampal neurons at DIV 4 or DIV 12 were transfected with different RNAi oligos: TDP-43 RNAi oligo (TDP-si), FMRP RNAi oligo (FMRP-si) or control oligo (Sc) with the use of lipofectamine 2000 (Invitrogen) following the standard protocol [12]. In one set of experiments, the hippocampal neurons grown on poly-D-lysine coated cover slips. At DIV 12 they were co-transfected with pGFP-FMRP (2 μ g) or pGFP (2 μ g) and TDP-si or Sc oligo. Sc and TDP-si oligos are same as we used in Majumder et al. (2012).

Transfection of HEK293T cells with different plasmid DNAs and FMRP-si oligo (Sigma Aldrich) was performed using the lipofectamine 2000 (Invitrogen) following standard protocol.

Western blotting and RT-PCR analysis

The total proteins were isolated from the cultured hippocampal neurons or HEK293T cells using lysis buffer containing 150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and 1% protease inhibitor cocktail. 20 μ g of the total proteins for each sample were separated by 10% SDS-PAGE and analyzed by Western blotting following the standard procedures. The antibodies used included anti-TDP-43 (GeneTex, 1:4000), anti-FMRP (Millipore: 1:3000), anti-GAPDH (Santa cruz, 1:5000), anti-eIF4E (Thermo scientific: 1:500), anti-CYFIP1(Thermo scientific, 1:6000), anti-eIF3 β (Santa cruz, 1:500), anti-rpL6(Santa cruz, 1:200), anti-actin (Sigma: 1:10,000), anti-Map1b(Genetex: 1:2500), anti-Flag (Sigma: 1:10,000), anti-GFP (1:5000) , anti-Rac1 (Millipore, 1:2000), and anti-GluR1 (Santa Cruz Biotech., 1:500).

For quantitative RT-PCR analysis, the total RNA was isolated using the Trizol method. The first strand cDNA synthesis was done using the Superscript RT (Invitrogen). Real time PCR was performed using the Light Cycler machine (Roche biochemical sciences) and appropriate primers corresponding to mouse Rac1 (NM_009007.2),

Map1b (NM_008634.2), GluR1 (Gria1, NM_001113325.2) and Gapdh (NM_001289726.1) mRNAs. For semi-quantitative RT-PCR analysis, the total RNA was also isolated using the Trizol method. The first strand cDNA was synthesized and PCR was performed using appropriate primers. The full list of RT-PCR primers are given in Table S5.

Immunofluorescence staining

Primary hippocampal neurons grown on poly-L-lysine coated cover slips were washed with PBS and fixed using 4% PFA. After permeabilization with 0.5% Triton X 100 in PBS, the neurons were blocked with 10% FBS (Jackson Immuno Research Laboratories) and then probed with different antibodies: anti-TDP-43 (GeneTex, 1:450), anti-FMRP (Millipore, 1:200), anti-CYFIP1 (Genetex, 1:50), anti-Rac1 (Millipore, 1:100), anti-GluR1 (Santa Cruz Biotech., 1:50), anti-Map1b (Genetex: 1:500), and anti-PSD-95(Sigma, 1:300) diluted in the blocking buffer at 4°C overnight. After wash, the cells were incubated with AlexaFluor 533-, AlexaFluor 564-, AlexaFluor 647-, and/or AlexaFluor 350- conjugated secondary antibodies (Invitrogen) diluted in the blocking buffer for 1 hr. After washing, the cells were mounted with Vectashield (Vector Laboratories) and preserved in the dark at 4 °C. The processed cells on the cover slips were then analyzed under LSM 510 meta two-photon microscope or LSM 780 microscope (Zeiss, Germany).

Fluorescence in situ hybridization (FISH) and combined immunofluorescence (IF) staining

The FISH probes were designed using the program on the website of Biosearch Technology. The reliability of selected probes against 3'UTR of the mouse Rac1 mRNA were tested by OLIGOWALK and mFold software. The sequence specificities of the selected 3'UTR regions for probe design were checked by BLAST on the NCBI website[10]. FISH was performed using Alexa 488-conjugated 5'-TTGACTGGTTCATTGGTTCA-3'(anti-sense probe 1, green, see Figs 5a, 6 and S5) and Alexa 647-conjugated 5'-ACAAAGGTTCCAGGCAGGAC-3' (anti-sense probe 2, red, see Fig S5b) targeting the 3'UTR of Rac1 mRNA, and an Alexa 488-conjugated 5'-TGAACCAATGAACCAGTCAA-3'oligonucleotide sense probe was used as the negative control (Life Technologies, Japan). Cells on poly-D-lysine coated glass coverslips were fixed in 3.7% formaldehyde for 30 min, permeabilized in 70% ethanol at 4 °C for 1 hr, incubated with 10% formamide/2xSSC for 10 min at room temperature, and hybridized overnight at 37 °C with the oligonucleotide probe(s) (12.5nM) in hybridization buffer containing 10% formamide, 2xSSC, and 100mg/mL dextran sulphate. Simultaneous imaging of RNA by FISH and proteins by IF staining was carried out using standard protocols [17]. The FISH probes and antibodies were mixed and incubated with the neurons at 37 °C overnight. The cells were then further incubated with secondary antibodies (1:5000 for each, Invitrogen) in 10% formamide/2xSSC for 30-60 min at 37 °C in the dark. The GLOX imaging buffer was used to decrease the photobleaching of the fluorescent reporters, and the FISH-IF images were acquired by LSM780 (Zeiss, Germany) and analyzed by Zen2010 (Zeiss, Germany) and Metamorph (Molecular devices) software.

Polysome profile analysis

Hippocampal neurons at DIV 6 or HEK293T cells were harvested in PBS containing 100 g/ml cycloheximide at 4°C, and then re-suspended in RSB-150 (10 mM Tris-HCl, pH 7.4, 3mM MgCl₂, and 150 mM NaCl) containing 100 g/ml cycloheximide, 40 g/ml digitonin (Calbiochem), 20 U/ml RNasin (Promega), and

protease inhibitors (Complete; Roche Diagnostics). After incubation on ice for 30 minutes, the lysates were centrifuged at 3,000 g for 2 min at 4°C. The cytoplasmic extracts were further cleared by 30 min centrifugation at 11,000 g and then loaded onto a linear gradient of 15–40% (wt/wt) sucrose in RSB-150 and centrifuged at 40,000 rpm for 2.5 hr at 4°C in a Beckman SW41 rotor. After centrifugation, the gradients were monitored at 254 nm using a fractionation system (GILSON, USA). Proteins and RNAs of different fractions were analyzed by Western blotting and RT-PCR, respectively. The translation status of the mRNAs was assessed by measurement of the relative distributions of the mRNAs in the different polysomal fractions.

RNA-IP

Hippocampal neurons were collected and lysed in 200 µl of lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, and 0.5% NP-40) supplemented with 1× protease inhibitor cocktail (Complete;Roche Diagnostics) and 10 units/ml RNasin (Promega). The total lysate was incubated with 10µl of RNase-free DNase I (Roche Applied Science), at room temperature for 20 min, and then immunoprecipitated with anti-TDP-43 (Genetex), anti-FMRP(Milipore), anti-CYFIP1 (Thermoscientific) or control IgG antibodies (Jackson Lab, USA) at 4 °C for overnight. Agarose beads (GE Healthcare) were used to pull down the antibody-RNP complexes. The RNAs were extracted from the complexes using TRIZOL (Invitrogen) and analyzed by RT-PCR using primers listed in Table S5. Proteins were isolated and analyzed by Western blotting.

Immunoprecipitation

To study the TDP-43/FMRP interaction, lysates of HEK293T cells co-expressing GFP tagged FMRP and Flag-tagged full-length TDP-43 or different mutant forms of TDP-43 were pre-cleaned with 50% protein G-agarose beads (GE Healthcare) for 30 min at 4 °C, and then incubated with anti-Flag (Sigma Aldrich) overnight. Protein G-agarose beads were then added again and the incubation continued for another 4 hr. The bound precipitates were washed 3-4 times and analyzed by Western blotting using anti-GFP (Sigma Aldrich), anti-Flag antibodies.

***In Vitro* RNA-Protein binding assay using biotinylated oligonucleotides**

DNA oligonucleotides corresponding to different regions of Rac1 mRNA were synthesized and annealed with a T7 promoter DNA oligonucleotide (the primers to generate the different oligonucleotides are listed in Table S6). *In vitro* transcription was then performed at 37 °C for 4 hr using the biotinylated rNTP mix (Roche Applied Science) and T7 RNA polymerase (Promega) following the method described before [3]. After DNase digestion of the reaction mixture, the biotin labeled RNA transcripts were gel purified, conjugated to streptavidin beads (Sigma), and then incubated with total protein extracts prepared from transfected HEK293T cells in 10 mM Tris-HCl, pH 7.4, 50 mM KCl, 1.5 mM MgCl₂, and 0.5 mM DTT supplemented with 10 units/ml RNasin for 2 hr at 4 °C with constant shaking. The bead-bound proteins were analyzed by Western blotting against anti-Flag or anti-FMRP. Bead-bound RNAs were isolated by Trizol method and analyzed by RT-PCR to assure similar levels of the RNAs used for pull-down of the proteins.

Dual-luciferase reporter assay of translation

HEK 293T cells were co-transfected with psichcek2 vector-based dual luciferase plasmid(s) with different parts of the Rac1 mRNA sequences cloned at downstream of the firefly luciferase (Fluc) cDNA construct, plus one or more of the following: pFlag-TDP-43(plasmid harboring flag-tagged full length mouse Tardbp cDNA), pFlag-

TDP-43(Δ Gly)[plasmid harboring flag-tagged glycine-rich-domain deletion mutant of mouse Tardbp cDNA], pGFP-FMRP (plasmid harboring GFP-tagged full length mouse Fmr1 cDNA, NM_008031.3), and FMRP-si oligo using Lipofectamine 2000. The cells were harvested at 48 hr post-transfection and the luciferase activities and RNA expression levels were measured using the dual-luciferase reagents (Luciferase Assay Reagent II for Fluc; Stop & Glo Reagent; Promega) and by semi-quantitative RT-PCR, respectively. The relative luciferase activities were calculated after division of the Fluc activity by the Rluc activity.

Local dendritic translation assay

Hippocampal neurons of DIV 12 were transfected with different siRNA oligos to deplete the endogenous TDP-43 or FMRP. 36-42 hr later the neurons were again transfected with phSYNmyr-dEGFP carrying Rac1 3'UTR sequence. 6-12 hr after the second transfection, the neurons were either subjected to live cell imaging to track the GFP reporter expression in dendrites for another 6 hr followed by cell fixation and immunofluorescence staining analysis, or directly fixed and the GFP fluorescence was analyzed along with the immunofluorescence staining. In some cases, the neurons were treated with 5 μ M TTX or vehicle for 6 hr; fixed and the GFP fluorescence was monitored. Quantification of the GFP fluorescent intensities along the longer dendrites of the transfected neurons were analyzed by the Metamorph software (Molecular Devices, USA).

Pulse-labeling assay of translation

Hippocampal neurons at DIV 6 with or without depletion of TDP-43 or FMRP by RNAi were washed and preincubated with methionine- and cysteine-free DMEM (Invitrogen) for 30 min, and then labeled with 100 Ci/ml [³⁵S]methionine/cysteine (³⁵S-ProMix; GE Healthcare) for 1 hr. Subsequently, cells were washed and lysed in a lysis buffer used in immunoprecipitation described before. Total proteins from different samples were subjected to IP against anti-Rac1 (Milipore, Germany). Bead-bound proteins were extracted and resolved by 10% SDS-polyacrylamide gel electrophoresis followed by autoradiography. 10% of the total protein (Input) from each sample was also loaded on gel as a control.

Bioinformatics analysis

To identify the putative common mRNA targets of FMRP and TDP-43, multiple target datasets were selected from literature for analysis. The FMRP target datasets included those from Ascano et al. (2012), Brown et al. (2001), Chen et al. (2003), Darnel et al. (2011), and Myashiro et al. (2003) (see Table S2 for more details of these datasets). A unique gene list consisting of 2717 mRNAs was derived from analysis of these 5 datasets by an in-house Perl script program. Using the same program, this list of FMRP mRNA targets were compared with 5 individual data sets of TDP-43-bound mRNAs compiled in Colombrita et al. (2012), Narayanan et al. (2012), Sephton et al. (2011), Polymenidou et al. (2011) and Xiao et al. (2011)(see Table S2 for more details). Functional annotation of the mRNAs (Table S3) was carried out with the PANTHER classification system.

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Supplementary Figure Legends

Fig S1. Related to Figure 1. **a.** Increased amounts of Rac1 protein in neurons upon depletion of either TDP-43 or FMRP by RNAi knockdown. Total cellular proteins from DIV 6 primary hippocampal neurons transfected with different RNAi oligos (Sc, TDP-si or FMRP-si) for 48 hr were subjected to Western blotting. This experiment was repeated 4-5 times. **b.** Cytoplasmic extracts of DIV 6 hippocampal neurons transfected with different RNAi oligos were separated by sucrose gradient sedimentation. The distribution of Rac1 mRNA (left) and GAPDH mRNA (right) in different fractions were then analyzed by RT-PCR. Error bars on the graphs represent SD from three technical repeats using the same biological samples. Percentage of Rac1 mRNA level was significantly different between Sc oligo- and TDP-si oligo- or FMRP-si oligo-transfected neurons at 2nd - 4th, 7th -9th fractions ($p < 0.01$, pairwise t test). **c.** Analysis of proteins synthesized and pulse labeled by S³⁵ methionine in primary hippocampal neurons transfected with different RNAi oligos. Immunoprecipitates of total cellular lysates using anti-Rac1 (left panel), anti-PSD-95 (middle panel) or control IgG (data not shown) were separated by SDS-PAGE, blotted onto a PVDF membrane, and then autoradiographed. Independent experiments showed similar results. **d.** Cytoplasmic extracts of HEK293T cells transfected with the indicated plasmid and/or oligo were separated by sucrose gradient sedimentation. The distribution of Rac1 mRNA (top) and GAPDH mRNA (bottom) in different fractions were analyzed by RT-PCR. Error bars represent SD from three technical repeats using the same biological samples. Percentage of Rac1 mRNA level was significantly different between pFlag and pFlag-TDP-43 transfected HEK293 cells at 4th, 5th, 8th and 9th fractions and between pFlag-TDP-43 transfected and pFlag-TDP-43/FMRP-si co-transfected cells at 4th, 8th and 9th fractions. ($p < 0.01$, pairwise t test).

Fig S2. Related to Figure 3b. Luciferase reporter assay of translational repression of Rac1 mRNA by TDP-43. HEK293T cells were co-transfected with pFlag-TDP-43 and the dual luciferase vector psicheck2-based constructs containing different portions of the Rac1 mRNA depicted in Fig 3a(i). The relative levels of luciferase RNA (**a**) and protein (**b**) in the extracts of transfected cells were then analyzed by quantitative RT-PCR and dual luciferase assay, respectively, and shown in the histograms. Error bars represent SD (N=3). Significant differences are indicated by *** $p < 0.0001$ (Student's t test).

Fig S3. Related to Figure 3c. **a.** Interaction of FMRP with the glycine-rich domain of TDP-43. HEK293T cell lysates co-expressing GFP-FMRP with different Flag-tagged TDP-43 forms were immunoprecipitated with anti-GFP and then probed with anti-Flag and anti-GFP on Western blots. Note that anti-GFP can pull down full-length Flag-tagged TDP-43 [Flag-TDP-43, compare lanes 1 and 5], Flag-tagged TDP-43 with RRM1 deletion [Flag-TDP-43(Δ RRM1), compare lanes 3 and 7] or Flag-tagged TDP-43 with RRM2 deletion [Flag-TDP-43(Δ RRM2), compare lanes 4 and 8], but not Flag-tagged TDP-43 with the glycine-rich domain deletion [Flag-TDP-43(Δ Gly), compare lanes 2 and 6]. Data were reproducible in 3 different sets of experiments. **b.** Comparison of the expression levels of TDP-43, FMRP and Rac1 proteins in DIV 6 mouse primary hippocampal neurons and human HEK293T cells by Western blotting, as exemplified in the panels on the left. Statistical analysis is shown in the histogram on the right. Significant differences are indicated by *** $p < 0.0001$ and * $p < 0.01$ (Student's t test).

Fig S4. Related to Figure 4a. Effects of 4EGI-1, TDP-si oligo and FMRP-si oligo on the polysomal distributions of Rac1 mRNA. Polysomal distributions of Rac1 mRNA (**a**) and GAPDH mRNA (**b**) in different RNAi oligo-

transfected DIV 6 mouse primary hippocampal neurons with or without 4EGI-1 treatment for 30 min were determined by RT-PCR analysis. Error bars represent SD from three technical repeats using the same biological samples. Percentage of Rac1 mRNA level was significantly different between control 4EGI-1 treated and TDP-si transfected/ 4EGI-1 treated neurons at 2nd and 7th-9th fractions and between control 4EGI-1 treated and FMRP-si transfected/4EGI-1 treated neurons at 6th-9th fractions ($p < 0.01$, pairwise t test).

Fig. S5. Related to Figure 5a and Figure 6. Validations of the specificities of Rac1 FISH probe 1 in primary hippocampal neurons. **a.** Representative microscopic pictures of DIV 14 hippocampal neurons transfected with Sc RNAi oligo. Top panels, FISH carried out on primary neurons with use of Rac1 anti-sense probe 1 (green, see Supplementary materials and methods for details). Bottom panels, FISH carried out on primary neurons with Rac1 sense probe (negative control). The corresponding DIC images are shown to present the morphologies of the neurons. The result was reproducible in three independent experiments. **b.** Representative pictures of DIV 6 primary hippocampal neuron soma regions showing the FISH signals obtained with use of different anti-sense probes against the 3'UTR region of Rac1 mRNA. Hippocampal neurons in culture were transfected at DIV 4 with control Sc oligo (upper panels), or Rac1 RNAi oligo (lower panels). At DIV 6, the level of Rac1 mRNA was examined by FISH using Rac1 probe 1 (green, see Supplementary materials and methods for details) or probe 2 (red, see Supplementary materials and methods for details). Note the decrease of the FISH signals in Rac1-depleted cells (Rac1-si) in comparison to Sc oligo-transfected cells, indicating the specificity of the probes. DAPI staining signals (blue region in merged panels) indicate the locations of the nucleus. The scale bars, 5 μm .

Fig S6. Related to Figure 4c and Figure 7a. Decreased CYFIP1 interaction with GluR1 mRNA, Map1b mRNA and Rac1 mRNA by RNAi knockdown of TDP-43 or FMRP. **a.** RNA-protein complexes in total cell extracts of DIV 6 primary hippocampal neurons transfected with different RNAi oligos were immunoprecipitated by anti-CYFIP1 or control IgG, and analyzed by semi-quantitative (left) and quantitative (right) RT-PCR using primers specific for GluR1 mRNA and Map1b mRNA. GAPDH mRNA signals served as the negative control (shown in Fig 4c). RNA inputs were similar, as shown in Fig 7a(i). **b.** RNA-protein complexes isolated from the total cell extracts of primary hippocampal neurons transfected with different RNAi oligos were analyzed by quantitative RT-PCR using primers specific for Rac1 mRNA. Significant differences are indicated by *** $p < 0.0001$, ** $p < 0.001$ and * $p < 0.01$ (Student's t test).