Supporting Information

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SI Text

Human Staining Results. In hippocampus, Pur α immunoreactivity was similar in cases and controls. In the granule cell layer of the cerebellum, the results were more mixed, with cytoplasmic Pur α inclusions observed in two of four individuals with neuropathologically defined frontotemoral lobar degeneration with TDP-43 inclusions (FTLD-TDP) carrying the *C9orf72* expansion, in two of four FTLD-TDP noncarriers, and in four of six controls. We also found p62-positive TDP-43–negative inclusions in four of four FTLD-TDP expansion carriers, in two of four FTLD-TDP expansion noncarriers, and in two of six controls. Adjacent sections showed molecular layer intranuclear inclusions, and granule layer Pur α inclusions rarely colocalized with p62 immunoreactivity.

Materials and Methods. ***Plasmid construction.*** Full-length Pur α (mouse and *Drosophila*) and hnRNP A2/B1 (mouse) were cloned into pcDNA3.1(+) with an N-terminal FLAG tag. Full-length Pur α (mouse and *Drosophila*) and hnRNP A2/B1 (mouse) cDNAs were also cloned into pGEX-2TK to produce recombinant proteins. The pUAST-EGFP constructs were generated by cloning EGFP cDNA into the pUAST transformation vector. The pUAST-$(GGGGCC)_3$-EGFP and pUAST-$(GGGGCC)_{15}$-CTCGAG-$(GGGGCC)_{15}$-EGFP vectors were created by subcloning the repeat GGGGCC-containing sequence from the pEGFP-N3 constructs. The sequences of the GGGGCC repeats and ORFs of all constructs were confirmed by Sanger sequencing.

Cell culture, transfection, and cell viability assay. Mouse neuronal cells, Neuro-2a (American Type Culture Collection; CCL-131), were plated into 96-well cell culture clusters at a density of 5×10^3 cells/well in 100 μL of culture medium. Proliferating Neuro-2a cells were allowed to attach for 24 h before transfection. Lipofectamine 2000 (Invitrogen) was used for introduction of exogenous plasmid DNA or siRNA into Neuro-2a cells according to the manufacturer's instructions. At 48 h after transfection, CellTiter-Blue (Promega) was added to each well, followed by incubation for 2 h and then measurement with a SpectraMax M5 or Gemini EM microplate reader (MolecularDevices) with 560-nm excitation and 590-nm emissions. All of the transfection experiments were repeated in triplicate.

RNA-binding assays and identification of rGGGGCC repeat RNA-binding proteins. All of the manipulations were performed at 4 °C or on ice. Mouse spinal cord was washed twice with ice-cold PBS and homogenized in lysis buffer [20 mM Hepes (pH 7.4), 1 mM $MgCl_2$, 150 mM NaCl, 1 mM EDTA, and 0.1% Triton X-100] supplemented with RNase and protease inhibitors (Roche). The sample was then sonicated and centrifuged for 10 min at 10,000 × *g* to pellet nuclei and debris, and the supernatant was collected and used for RNA-binding assays. Biotinylated $rGGGGCC_{10}$ repeat RNAs were synthesized and purified by HPLC (Integrated DNA Technologies). For the binding reaction, biotinylated RNAs (300 μM) were captured with prewashed Dynabeads M-280 Streptavidin (Invitrogen) and incubated with spinal cord lysates in 1× binding buffer [20 mM Tris·HCl (pH 7.6), 300 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, and 10% glycerol] for 4 h and washed five times with 1× binding buffer. The same amounts of biotin and lysates were used as in the control binding reactions. The captured RNA–protein complex was separated by SDS/PAGE gel and stained with Coomassie blue. Bands were excised from the gel, and MS was performed at the Emory Proteomics Core. For the competition assay, the amount of the molar excess unlabeled RNA probe was added to the binding reaction before addition of the labeled probe.

Expression and purification of recombinant proteins. pGEX-2TK, pGEX-Pur α (mouse and *Drosophila*), and pGEX-hnRNP A2/B1 were individually transformed into the *Escherichia coli* expression strain BL21 (DE3). Overexpression of recombinant proteins was induced by 100 μM isopropyl-β-othiogalactopyranoside (IPTG) at an optical density of 0.6 and allowed to grow for 7 h at room temperature. Bacteria expressing corresponding proteins were lysed in Pierce B-PER Protein Extraction Reagents (Thermo Scientific). Purification of GST-tagged Pur α, hnRNP A2/B1, and GST alone was carried out by affinity chromatography using glutathione immobilized to a matrix Sepharose. Samples were then treated with DNase and RNase before use in the RNA-binding experiments. The purity and identity of protein were determined with 12% SDS/PAGE.

RNA-binding protein immunoprecipitation, western blot analysis, and quantitative RT-PCR. All manipulations of the lysates were performed at 4 °C or on ice. Neuro-2a cells were collected and homogenized in 1 mL ice-cold lysis buffer [10 mM Tris (pH 7.4), 300 mM NaCl, 30 mM EDTA, and 0.5% Triton X-100] supplemented with RNase and protease inhibitors (Roche). Nuclei and debris were pelleted at 10,000 × *g* for 10 min, after which the supernatant was collected and protein concentrations were determined by the Bradford assay (Bio-Rad). Equal amounts of lysate (1 mg) were precleared for 2 h with 100 μL of recombinant protein A agarose (Invitrogen). Anti–FLAG-M2 monoclonal antibody (Sigma-Aldrich) or normal mouse IgG (Millipore) was incubated with recombinant protein A agarose at 4 °C for 2 h and washed three times with lysis buffer. The precleared lysates were immunoprecipitated with antibody-coated recombinant protein A agarose at 4 °C for 2 h and then washed five times with lysis buffer. The precipitated complexes were used for Western blot analysis and RNA isolation.

Western blot analyses were performed using anti-Pur α antibody (ab79936; Abcam) or anti–TDP-43 antibody (10782-1-AP; ProteinTech Group). For quantitative RT-PCR (qRT-PCR), the immunoprecipitated RNAs were isolated using TRIzol (Invitrogen) and cleaned with an RNeasy MinElute Cleanup Kit (Qiagen). RNAs were quantified using absorbance at 260 and 280 nm (NanoDrop) and reverse-transcribed with oligo(dT) 12-18 and SuperScript III (Invitrogen). qRT-PCR was performed with gene-specific primers for EGFP and Power SYBR Green PCR Master Mix (Applied Biosystems).

In vitro RNA-binding assay. T4 polynucleotide kinase (New England BioLabs) was used to radiolabel the $rGGGGCC_{10}$ repeat at the 5′ end with ^{32}P-γ-ATP, which was purified by ethanol precipitation. The radiolabeled $rGGGGCC_{10}$ repeat was allowed to refold by heating at 95 °C for 2 min, followed by incubation at 37 °C for 1 h. For each concentration of $rGGGGCC_{10}$ repeat, 100 nM purified recombinant protein was incubated with radiolabeled RNA in 1× binding buffer [20 mM Tris·HCl (pH 8.0), 15 mM NaCl, and 2.5 mM $MgCl_2$] in a total volume of 50 μL at 30 °C for 60 min. Binding solutions were passed through MF-Millipore membrane filters (0.45 μm, type HA; Millipore) and washed three times in 4 mL of ice-cold wash buffer [50 mM Tris-HCl (pH 7.4) and 20 mM KCl]. The membranes were dried for 2 min at 25 °C, then immersed into ScintiVerse BD Mixture (Fluka). Liquid scintillation was counted for 1 min using a LS6500 Multipurpose Scintillation Counter (Beckman Coulter). All assays were performed in triplicate. The dissociation constant, K_d, for each enzyme was de-

termined by nonlinear regression, taking into account ligand depletion using Prism version 5.00 for OS X GraphPad Software.

Drosophila *genetics and activity assay.* Male flies were individually placed in *Drosophila* Activity Monitoring System (TriKinetics) testing chambers that had been capped with regular food at one end. The flies were grown on a 12-h:12-h light:dark cycle at 25 °C for 4 wk. Locomotor data were collected in the light cycle, also at 25 °C. Locomotor activity averages of each day during the first and fourth wks were calculated. Data were assayed in triplicate with 32 flies at a time (n = 96 per genotype).

Drosophila *scanning electron microscopy.* To obtain scanning electron microscopy (SEM) images, whole flies were dehydrated in gradient concentration ethanol (25%, 50%, 75%, and 100%), dried with hexamethyldisilazane (Sigma-Aldrich), coated by electric field and argon gas, and analyzed with an ISI DS-130 LaB6 SEM/STEM microscope.

Statistical analyses. All cell culture data are presented as mean cell viability relative to the control condition. Statistical significance was determined using two-sample, two-sided t tests. Fly locomotion is presented as mean activity relative to the control fly. All statistical analyses were performed with using Prism version 5.00 for OS X (GraphPad Software).

Immunohistochemistry. In brief, immunohistochemistry analyses were performed using 9-μm paraffin-embedded sections from human postmortem hippocampus and cerebellum. Antigen retrieval was performed by microwaving in citrate buffer (10 mM; pH 6) for 5 min. Endogenous peroxide activity was blocked with 3% hydrogen peroxide in 100% methanol for 5 min at 40 °C. Sections were then incubated with 2% normal horse serum in 0.1 M Tris-buffered saline (TBS) (pH 7.5) with 0.25% Brij in TBS (pH 7.5) for 15 min at 40 °C. Adjacent sections were incubated with anti-p62 (GP62-N, 1:250 dilution; Progen), anti-PURA antibody (LS-B6784; LSBio), or anti pTDP-43 antibody (TIP-PTD-P02, 1:8,000 dilution; Cosmo Bio). All primary antibodies were diluted in 1% BSA in 0.25% Brij in TBS (pH 7.5), with overnight incubation at 4 °C. The next day, sections were incubated with biotinylated secondary antibody (1:200 in 0.25% Brij in TBS) for 30 min at 37 °C, followed by avidin–biotin peroxidase complex (prepared as directed; Vector Laboratories) for 60 min at 37 °C. Sections were developed using a 3,3′-diaminobenzoic acid (DAB) peroxidase substrate kit (Vector Laboratories) and counterstained with hematoxylin. Staining and rating of all human immunohistochemistry was performed by individuals blinded to the disease or carrier status of cases.

All *Drosophila* tissue was fixed in 4% paraformaldehyde (Electron Microscopy Sciences). *Drosophila* eye cryosections was prepared as described previously (1). All tissue was washed three times in 1× PBS and blocked in normal goat serum (Electron Microscopy Sciences). Primary antibodies included rabbit anti-ubiquitin (1:100 dilution; Dako), mouse anti-ubiquitin (Ubi-1, 1:100 dilution; Novus Biologicals), rabbit anti-Pur α (1:500 dilution) (2). Anti-rabbit or anti-mouse antibodies conjugated with Alexa Fluor 568 or FITC (1:500 dilution; Molecular Probes) were used for immunostaining. Images were captured with a Zeiss LSM 510 Meta confocal microscope and Zeiss software.

1. Park JJ, Cunningham MG (2007) Thin sectioning of slice preparations for immunohistochemistry. *J Vis Exp* 3:194.

2. Jin P, et al. (2007) Pur alpha binds to rCGG repeats and modulates repeat-mediated neurodegeneration in a Drosophila model of fragile X tremor/ataxia syndrome. *Neuron* 55(4):556–564.

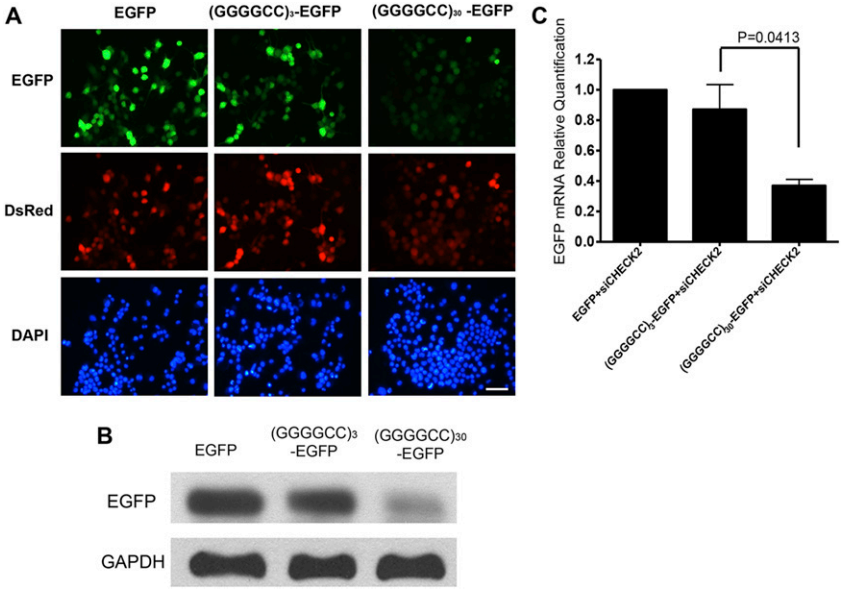

Fig. S1. Expression of EGFP mRNA and protein in Neuro-2a cells transfected with $(GGGGCC)_n$-EGFP–containing constructs. (*A*) Fluorescent light microscopy images of Neuro-2a cells transiently transfected with EGFP, $(GGGGCC)_3$-EGFP, and $(GGGGCC)_{30}$-EGFP constructs. (Scale bar: 100 μm.). (*B*) Western blots using anti-GFP and anti-GAPDH antibodies in Neuro-2a cells cotransfected with the siCHECK2 vector and $(GGGGCC)_n$-EGFP–containing constructs. (*C*) Quantification of EGFP mRNA by qRT-PCR using sequence-specific primers relative to the GAPDH housekeeping gene.

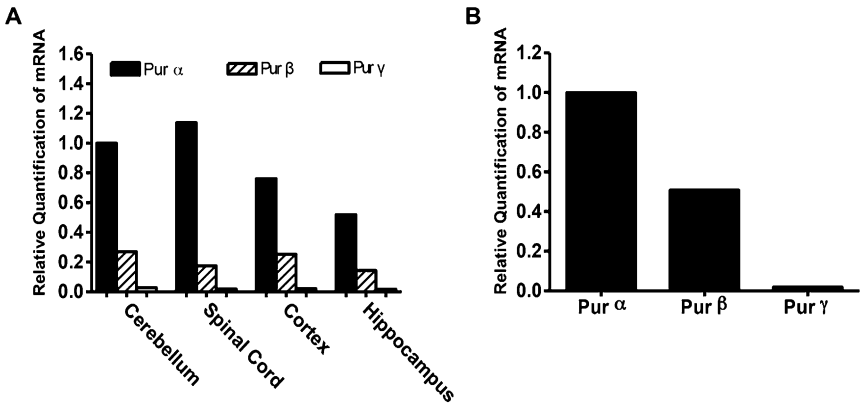

Fig. S2. Quantification of Pur α, Pur β, and Pur γ transcript levels in mouse brain and Neuro-2a cells. (*A*) qRT-PCR of Pur α, Pur β, and Pur γ using sequence-specific primers from RNA extracted from mouse cerebellum, spinal cord, cortex, and hippocampus. All quantification is provided relative to the GAPDH housekeeping gene. (*B*) qRT-PCR of Pur α, Pur β, and Pur γ using sequence-specific primers from RNA extracted from Neuro-2a cells. All quantification is provided relative to the GAPDH housekeeping gene.

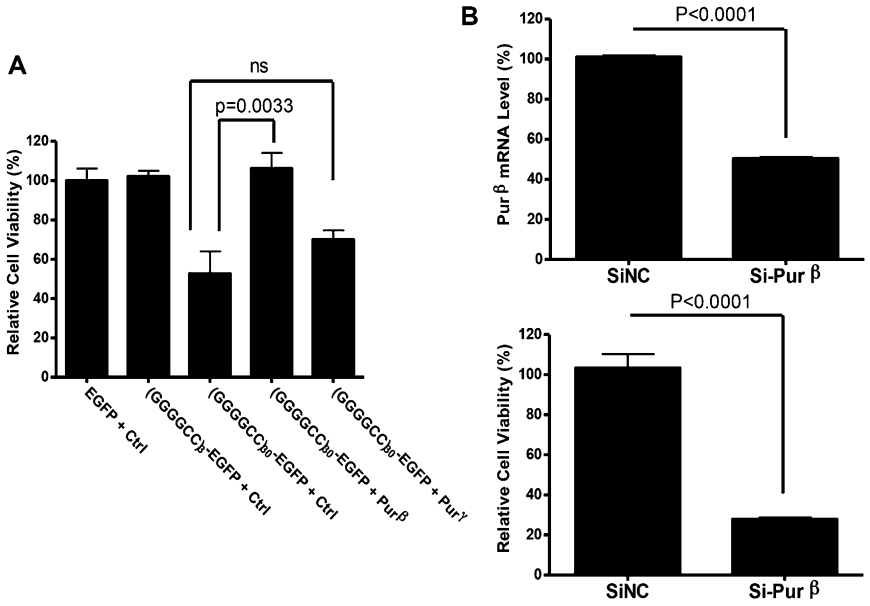

Fig. S3. Overexpression of Pur β suppresses rGGGGCC-mediated cell death in mammalian cells. (*A*) Expression of Pur β, but not of Pur γ, attenuates rGGGGCC repeat-mediated neuronal cell death in Neuro-2a cells. Cell viability of Neuro-2a cells cotransfected with GGGGCC repeat-expressing constructs and Pur β or Pur γ expression vectors was assessed. A significant reduction in cell viability for Neuro-2a cells transfected with the $(GGGGCC)_{30}$ construct and control vector versus $(GGGGCC)_3$ construct or control EGFP expression vector was detected ($P < 0.0001$, *t* test). Expression of Pur β in the presence of $(GGGGCC)_{30}$ construct resulted in significantly greater cell viability ($P = 0.0033$, *t* test). Expression of Pur γ in the presence of the $(GGGGCC)_{30}$ construct had no significant impact on cell viability ($P = 0.1963$, *t* test). (*B*) Knockdown of Pur β by siRNA induces cell death in Neuro-2a cells. Transient transfection of siRNA against Pur β significantly reduced both Pur β mRNA levels (*Upper*) ($P < 0.0001$, *t* test) and cell viability (*Lower*) ($P < 0.0001$, *t* test) as detected by qRT-PCR.

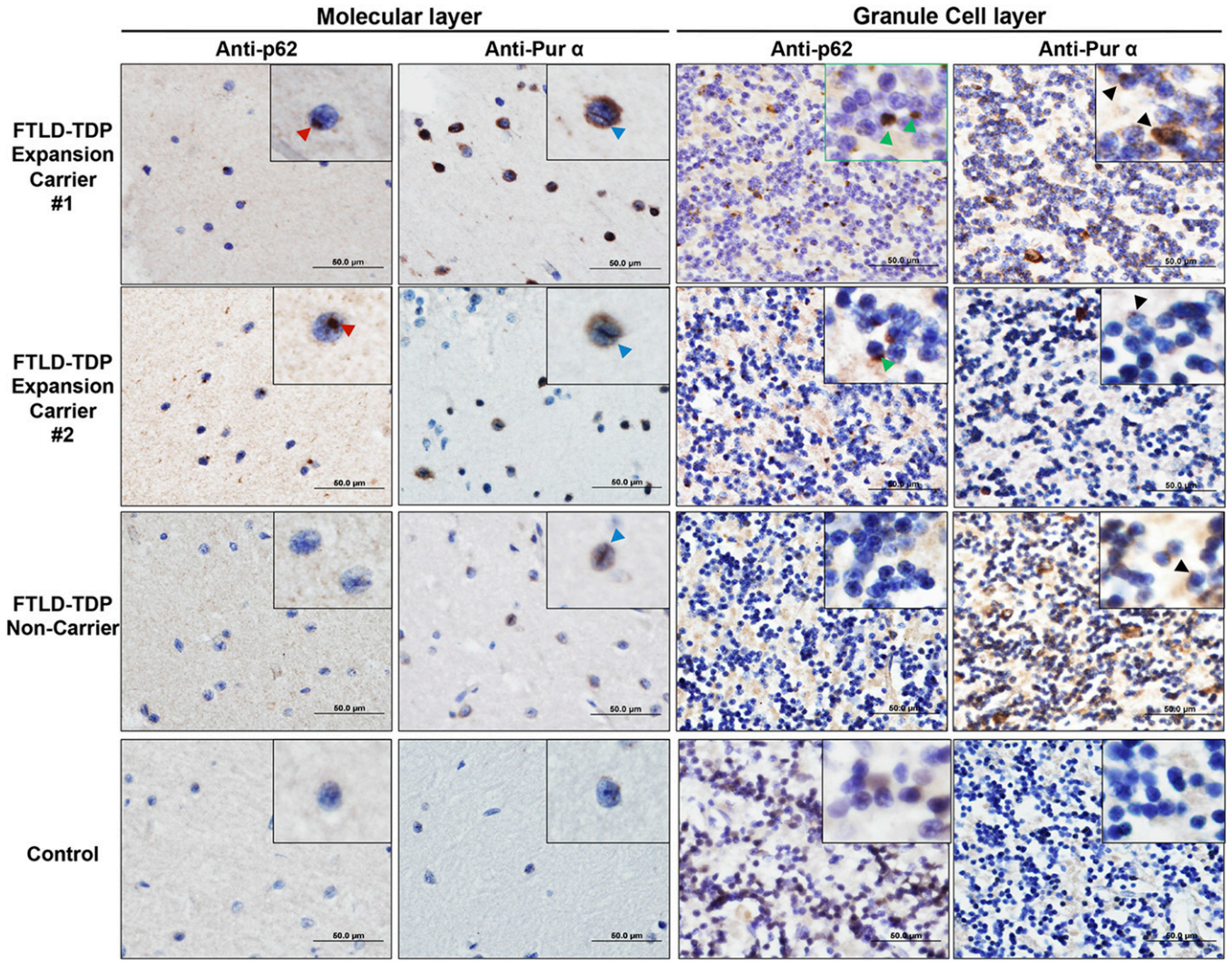

Fig. S4. Pur α forms inclusions in human cerebellum of FTLD-TDP cases with and without expanded rGGGGCC repeats. Molecular and granule cell layers of the cerebellum in individuals with FTLD-TDP who were carriers and non-carriers of expanded GGGGCC repeats and controls were stained for p62 and Pur α. In the molecular layer, p62-positive, TDP-negative intranuclear inclusions (red arrowhead) were observed in two of four expansion carriers, but not in noncarriers or controls. In the molecular layer, Pur α staining of intranuclear inclusions (blue arrowheads) was seen in three of four expansion carriers, in two of four noncarriers, and in one of six age-matched controls. In the granule cell layer, p62-positive TDP-negative inclusions (green arrowhead) were detected in four of four expansion carriers, in two of four noncarriers, and in two of six age-matched controls. In the granule cell layer, Pur α inclusions (black arrowheads) were observed in two of four expansion carriers, in two of four noncarriers, and in four of six controls; inclusions were subjectively more rare and less consistent in controls compared with expansion carriers or noncarriers.

Table S1. Identification of rGGGGCC repeat-binding proteins by MS

Description	Reference	SC	TP	UP	Predicted mass, kDa
Pur α	NP_033015.1	300	58	50	35
Pur β	NP_035351.1	114	36	25	34
Pur γ isoform a	NP_690034.1	30	15	2	40
Pur γ isoform b	NP_001091703.1	28	13	0	37
hnRNP C1/C2 isoform 1	NP_058580.1	27	14	0	34
hnRNP C1/C2 isoform 2	NP_001164452.1	27	14	0	33
Guanine nucleotide-binding protein G(o) subunit alpha isoform A	NP_034438.1	24	14	0	40
Nucleolysin TIAR	NP_033409.1	18	12	6	43
RNA-binding protein 4B	NP_079993.2	13	13	4	40
RNA-binding protein 4	NP_033058.2	11	11	2	40
Nucleolysin TIA-1 isoform 2	NP_001157550.1	11	8	0	42
Dual-specificity mitogen-activated protein kinase kinase 1	NP_032953.1	11	9	7	43
Poly(rC)-binding protein 1	NP_035995.1	10	9	7	37

SC, spectral count; TP, total peptide count; UP, unique peptide count.
See Dataset S1 for a complete list of rGGGGCC repeat binding identified by MS.

Other Supporting Information Files

Dataset S1 (XLSX)