Supporting Information

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SI Experimental Procedures

Antibodies and Chemicals.

Anti-SERT Ab (SAB4200039; Sigma) 1:1,000

Anti–B-actin Ab (sc 1616; Santa Cruz Biotechnology) 1:5,000

Anti-phosphotyrosine Ab (sc 7020; Santa Cruz Biotechnology) 1:3,000

Anti-hnRNPK Ab (RN019P; MBL International) 1:2,000

Secondary Ab (1:1,000)–Anti goat IgG-HRP (sc 2020; Santa Cruz Biotechnology).

Anti-rabbit IgG-HRP (7074; Cell Signaling)

Anti-mouse IgG-HRP (7076; Cell Signaling)

S100B (S1677; Sigma)

PP2 (041M4602V; Sigma)

Cell Culture. Rat C6 astroglioma cells and Human embryonic kidney cells were obtained from American Type Culture Collection and cultured in DMEM containing 10% (vol/vol) FBS, glutamax, and 1× Pen/Strep antibiotics (Life Technologies). RN46A cells were a generous gift from Scott Whittemore (1). All experiments used undifferentiated RN46A cells cultured at 33 °C in DMEM containing 10% FBS and 1× Pen/Strep. Primary cortical astrocyte cultures were prepared from postnatal (P)1–P3 mouse pups (2). After mechanical and enzymatic dissociation, cells were plated on polyethyleneimine-coated 12-well plates at a density of 1×10^6 cells per well in DMEM with 10% FBS and 1× P/S overnight, after which medium was changed to Neurobasal medium with 1 mM L-glutamax and G-5 supplement (Invitrogen). After 5–7 d in glial cells were subcultured on fresh polyethyleneimine-coated plastic dishes for 2 wk before experiments. All cells were cultured in humidified 5% CO_2 at 37 °C, and all cell culture reagents are from Life Technologies.

Transfections. Cells were plated 24 h before transfection by using Lipofectamine 2000 (Life Technologies) for 48 h before analyses.

Ribonucleotide Probe Cross-Linking Assays. ***Probes.*** Overlapping ribonucleotide (RNA) probes comprising the most highly conserved portion of the human SERT distal polyadenylation element were synthesized with a 5′ biotin modification and tetra-ethyleneglycol (TEG; 15 atom) spacer (IDT). Probe sequences are as follows: SERT1 5′-Biotin-TEG-GGCCCAUUACAAAUAUAUGAAUUCCCCAAAUUUUUCUUAC-3′; SERT2 5′-Biotin-TEG-CCCCAAAUUUUUCUUACAUUAACAAAUUCUACCAACUCAA-3′.

Preparation of cytosolic protein extracts. Protein extracts were prepared from adult mouse brain and cultured cells. Mouse brains were dissected to obtain midbrain tissue including the serotonergic raphe nuclei or nonraphe containing frontal cortex. Cultured cells were trypsinized, harvested, and briefly centrifuged (500 × *g* for 5 min) to collect cells. Tissue or cells were homogenized in 2 volumes of ice-cold lysis buffer (20 mM Hepes, 400 mM NaCl, 20% glycerol, 1.5 mM $MgCl_2$, and 1 mM DTT) with 1× protease inhibitor mixture (Calbiochem) and 1× phosphotase inhibitor (Calbiochem) by using a polytron and then centrifuged at 14,000 × *g* for 30 min at 4 °C to remove debris. Supernatants were then divided into aliquots at a final concentration of 10 mg/mL in lysis buffer after determining the protein concentration with BCA protein assay kit (Pierce) and stored −80 °C.

UV cross-linking. Cytosolic extracts (10 μg) were combined with biotinlyated RNA probe (1 nM final concentration) in the presence or absence of 100× (100 nM) unlabeled probe in 1.5-cc microfuge tubes. Samples were UV cross-linked (254 nm, 1,650 mJ of total energy; Stratalinker, Agilent Technologies) on ice. Samples were then denatured for SDS/PAGE by boiling for 5 min in NuPAGE LDS Sample Buffer (Invitrogen). Twenty microliters of this solution was then electrophoretically separated on 10% SDS/PAGE (Invitrogen). UV cross-linked complexes were transferred to polyvinylidene fluoride (PVDF) membrane (Millipore) at 75 V for 3 h at 10 °C. Biotinlyated probes on the membrane were detected by using Brightstar biodetect (Ambion) following the manufacturer's instructions. Apparent molecular masses of the cross-linked complexes were calculated based on their migration relative to prestained broad-range molecular mass standard markers (GE).

Avidin affinity purification of cross-linked proteins. Cytosolic extracts from C6 cells (15 mg of protein per reaction) were cross-linked to probes as described above and diluted into 2 mL of binding buffer (PBS containing 0.1% Tween-20), mixed with 50 μL of monomeric avidin magnetic beads (Bioclone) and incubated overnight at 4 °C with mixing. Probe–protein complexes were washed five times with 1× PBS. Proteins were eluted by incubating with 2 mM D-biotin in PBS for 10 min at room temperature. Eluates were pooled and resuspended in 4× NuPAGE LDS Sample Buffer (Life Technologies) and separated by 8% SDS/PAGE. Proteins were visualized by silver staining (Invitrogen) following the manufacturer's instructions. Designated bands were excised and subjected to in-gel tryptic digestion.

Liquid chromatography/mass spectrometry. Avidin affinity-purified proteins were separated on a 1D SDS/PAGE, and the gel was visualized by silver staining. The corresponding bands were excised, cut into ~1 mm × 1 mm small pieces, and destained. In-gel trypsin digestion was carried out in 37 °C for 16 h with sequencing grade-modified trypsin (Promega) under constant shaking according to a previous protocol (3). Peptides were extracted with 5% formic acid/50% acetonitrile in aqueous solution three times by using 30 min of incubation with constant shaking and 30 min of sonication in a water bath sonicator followed by extraction with 100% acetonitrile. The extracts were combined, dried by vacuum centrifugation, and resuspended in 20 μL of 5% acetonitrile/0.1% trifluoroacetic acid aqueous solvent before mass spectrometry (MS) analysis.

For each in-gel trypsin digested sample, 8 μL was injected onto an LC/MS system consisting of an 1100 series HPLC, HPLC-Chip Cube MS interface, and 6520 Series Q-TOF mass spectrometer (Agilent Technologies). The HPLC-Chip contains a 40-nL enrichment column and a 43 mm × 75 μm analytical column packed with Zorbax 300SB-C18 (5-μm particles). Peptides were loaded onto the enrichment column with 97% solvent A and 3% solvent B with a flow rate of 4 μL/min. Solvent A consists of 0.1% formic acid and solvent B of 90% acetonitrile and 0.1% formic acid. Peptides were eluted with a gradient from 3 to 45% solvent B in 20 min, followed by a steep gradient to 90% solvent B for 5 min at a flow rate of 0.3 μL/min and a 10-min equilibrium with 3% solvent B. Mass spectra were acquired in the positive-ion mode with automated data-dependent MS/MS fragmentation on the five most intense ions from precursor MS scans and every selected precursor peak was analyzed twice within 30 s.

PKL files were created with the Spectrum Mill (Rev A.03.03.084b SR4b) Data Extractor for MS/MS spectra that could be assigned to at least four *y*- or *b*-series ions. Scans with the same precursor ±0.5 *m*/*z* were merged within a time frame of ±15 s,

charges up to a maximum of five were assigned to precursor ions, and the ^{12}C peak was determined by the Data Extractor. Swiss-Prot database (November 2011 release) was searched for tryptic peptides with a mass tolerance of ±20 ppm for the precursor ions and a mass tolerance of ±50 ppm for the fragment ions with one fixed modification of cysteine carboxymethylation and a variable modification of methionine oxidation. Two trypsin missed cleavages were allowed during the database search. The threshold for peptide identification was set as a score of ≥10, an SPI% (the percentage of scored peak intensity) of ≥60%, and the value of forward score minus reverse score is larger than 2. These stringent search criteria resulted in <1% of false positive rate. In the peptide list, only the highest scoring member of each peptide group was listed and only peptides with a charge state of 2, 3, and 4 were reported. All of the MS/MS spectra were manually validated, and the spectra with low quality fragmentations were discarded. The proteins with correct molecular masses as the ones in the gel were regarded as positive identification.

RNA immunoprecipitation. The Magna RIP system (Millipore) was used for all analyses according to the manufacturer's standard protocol on ~1×10^7 cells or 100 mg of brain tissue. An aliquot of each lysate was used to assess the quantity of target sequences before immunoprecipitation. These samples were processed in parallel with immunoprecipitates through all steps after immunoprecipitation. Normal rabbit serum included with Magna RIP reagents was used as a negative control. Immunoprecipitation was carried out by using a RNA immunoprecipitation (RIP)-certified anti-hnRNPK antibody at 5 μg/IP (MBL International; RN019P).

Target sequences were quantified by real-time quantitative PCR (qPCR) using an ABI 7900HT apparatus. All assays were designed against species-specific sequence of target genes and optimized to ensure >95% efficiency. Relative quantification was performed by comparing the threshold cycle (Ct) for target amplification to a standard curve prepared using a serial dilution of pooled input samples.

RNA samples from immunoprecipitates were treated with DNase I to remove any contaminating genomic DNA in a 10-μL volume according to the manufacturer's protocol (Sigma) and reverse transcribed by using random hexamers (20nmol) and 25 units of M-MuLV reverse transcriptase (NEB). All reverse transcriptions were performed in duplicate, and all qPCR assays were performed in quadruplicates for each cDNA sample.

qPCR Assays. All qPCR was performed by using either SYBR green detection (Power SYBR Green PCR Master Mix (ABI) or prevalidated Taqman Assays using TaqMan Universal Master Mix II, with UNG (ABI) as specified. For SYBR green assays, all reactions were in 5 μL with 1 μL of cDNA template and 10 pmol of each primer. Specificity of target amplification was assessed by evaluation of a dissociation curve after amplification. Taqman assays were used as provided by ABI at a 1× final concentration.

RNA isolated by immunoprecipitation was treated with DNase I and reverse transcribed by using random primers. Resulting cDNA was used as template for real-time qPCR. Relative abundance of target sequences was determined by comparison of samples with a dilution series of cDNA from pooled samples of total cellular RNA before RIP run in parallel with all experiments. The relative abundance of target sequences in immunoprecipitates was compared with their abundance in diluted input samples (IPs were volume adjusted to allow direct comparison of input and immunoprecipitated samples) for quantitative evaluation of target sequence immunoprecipitation.

Human assays:

SERT coding (total SERT mRNA)

hSERTCoding F: 5′-GGTGATGAAAACCACCCTATCAT-3′

hSERTCoding R: 5′-CCCTGTTCTCTCCTACGCAGTT-3′

hSERTpA-B (SERT mRNA containing the distal polyadenylation element)

pA-BF: 5′-TCAGGAAAGGAAGATGTAAGAAGCTAA-3′

pA-BR: 5′-AGAATACACTGAAACAACAATATACACAGACT-3′

Thermal cycles:

50 °C × 2 min

95 °C × 10 min

95 °C × 15 s

61 °C × 90 s × 40 cycles

humanGAPDH (glyceraldehyde 3-phosphate dehydrogenase)

Human GAPD (GAPDH) Endogenous Control (ABI)

Thermal cycles:

50 °C × 2 min

95 °C × 10 min

95 °C × 15 s

60 °C × 1 min × 40 cycles

Mouse assays:

SERT proximal polyadenylation element (total SERT mRNA)

pA-AF: 5′-CCAAGCTGATGATGTAAGGTCTTT-3′

pA-AR: 5′-GTCACCAGCTAATGTGGCAGTAA-3′

SERT distal polyadenylation element (SERT mRNA containing the distal polyadenylation element)

pA-BF: 5′-GACGTAAAGACGCTACACTGAAAACT-3′

pA-BR: 5′-GGCCCGGAGTGTTGGAAT-3′

mouse GAPDH (glyceraldehyde 3-phosphate dehydrogenase)

mGAPDHF: 5′-CATGGCCTTCCGTGTTCCTA-3′

mGAPDHR: 5′-ATGCCTGCTTCACCACCTTCT-3′

mouse hnRNPK (heterogeneous ribonucleoprotein K)

hnRNPKF: 5′-GCACAGTATTTGCTGCAGAACA-3′

hnRNPKR: 5′-CCTTCAGTTCTTCACTAGTCTTA-3′

Thermal cycles for all mouse assays:

50 °C × 2 min

95 °C × 10 min

95 °C × 15 s

60 °C × 1 min × 40 cycles

Rat assays:

SERT proximal polyadenylation element (total SERT mRNA)

pA-AF: 5′-GCCTTCAATGTTCATGAATACATAAA-3′

pA-AR: 5′-GGGTTCTAGGAGATTCCAGCTATA-3′

SERT distal polyadenylation element (SERT mRNA containing the distal polyadenylation element)

pA-BF: 5′-GGTGTATATGTGTGAGCTATTGTGTCTGT-3′

pA-BR: 5′-ACAGTTTGAAATGGACCTGGAGTAT-3′

rat GAPDH (glyceraldehyde 3-phosphate dehydrogenase)

rGAPDHF: 5′-AAGTTCAACGGCACAGTCAAG-3′

rGAPDHR: 5′-ACATACTCAGCACCAGCATCAC-3′

rat hnRNPK (heterogeneous ribonucleoprotein K)

hnRNPKF: 5′-GCACAGTATTTGCTGCAGAACA-3′

hnRNPKR: 5′-CCTTCAGTTCTTCACTAGTCTTA-3′

rat COX2 (cyclooxygenase 2)

rCOX2F: 5′-CTGCCTCAATTCAGTCTCTCAT-3′

rCOX2R: 5′-GTCTGGAGTGGGAGGCACTT-3′

rat TPH1 (tryptophan hydroxylase 1)

rTPH1F: 5′-CCAGAGGACCCTGCTTTGAT-3′

rTPH1R: 5′-GTGCACCACTTGCATGCTATC-3′

rat TPH2 (tryptophan hydroxylase 2)

rTPH2F: 5′-GGTACGCTTCCTGAAGTGCTT-3′

rTPH2R: 5′-GACACATTCACAGTTCCAAGATT-3′

rat VMAT2 (vesicular monoamine transporter 2)

rVMAT2F: 5′-CCACCCACCTGAATGGTTGTA-3′

rVMAT2R: 5′-GCTTTAGTGACTGCTGCCGAT-3′

rat 5HT1AR (serotonin receptor 1A)

r5HT1ARF: 5′-CGCTGCGCTGATCTCGCTCA-3′

r5HTR1ARR: 5′-GGGATGGAGATGAGAAAGCCA-AT-3′

rat 5HT2BR (serotonin receptor 2)

r5HT2BRF: 5′-CCTCGGGAGTGAATCCTTTGAT-3′

r5HT2BRR: 5′-ATTGCAGGTGATGTACCTGCC-3′

Thermal cycles (for all rat assays):

50 °C × 2 min

95 °C × 10 min

95 °C × 15 s

60 °C × 1 min × 40 cycles

hnRNPK Transfections. For all transfection experiments, C6 cells were seeded 1 d before transfection or treatment at a density of 1×10^4 cells per well in 12-well plates (Costar). Transections used Lipofectamine 2000 (Life Technologies) with plasmid DNA. Cells were incubated for 48 h before analysis.

Wild-type rat hnRNPK in pCMV-SPORT6 cassette (MRN1768-9144781; Open Biosystems) was used for overexpression. For hnRNPK knockdown, a pRFP C-RS shRNA plasmid containing a rat anti-hnRNPKshRNA element (OriGene) was used.

Western Blotting and Immunoprecipitation. For Western blotting and immunoprecipitation, protein lysates were prepared in RIPA lysis buffer (50 mM Tris·HCl at pH 7.4, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 0.5% Na-deoxycholate, 0.1% SDS, and 1% Triton X-100). Total cellular protein or immunoprecipitates (1 μg/1 μL) were suspended in 4× NuPAGE LDS Sample Buffer (Life Technologies), boiled, run on precast 10% SDS/PAGE gels (Life Technologies), and transferred to PVDF membrane (Millipore). Specific proteins were immunodetected by using commercially available antibodies and immunoreactive bands were detected by using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). For immunoprecipitation, protein lysates (100 μg) were incubated with antibody and 30 μL of Agarose beads at 4 °C overnight. Resulting immunocomplexes were washed five times with RIPA buffer and resuspended in 4× NuPAGE LDS Sample Buffer.

MicroRNA-16 Binding Assay. The hCOX2 3′ UTR from 11 bases 5′ of the stop codon to 463 bases 3′ of the stop codon were amplified by using Phusion high fidelity polymerase (Finnzyme) and the following primers: hCOX2F 5′-CGACTGAACTGTAGAAGTCT-AAT-3′; hCOX2R 5′-GCACTGATACCTGTTTTTGTTTGAT-3′.

The hSERT 3′ UTR from immediately 3′ of the stop codon to 689 bases 3′ of the stop codon was cloned by using Phusion high fidelity polymerase (Finnzyme) and the following primers: hpolyAF 5′-CACACTCACCGAGAGGAAA-3′; hpolyAR 5′-TGA-GTTGGTAGAATTTGTTA-3′.

Amplified fragments were cloned into pCR2.1 TOPO (Life Technologies) by using the standard protocol. Clones were sequenced to verify insert direction and correct sequence.

Plasmids were linearized with HindIII, purified by using minielute reaction clean up kit (Qiagen), and used as a template for in vitro transcription with T7 RNA polymerase (ABI) with biotinylated UTP. Labeled 3′ UTR's were purified by using RNA mini Quick Spin Columns (Roche Diagnostics).

Oligonucleotides for microRNA (miR)-16: 5′-UAGCAGCA-CGUAAAUAUUGGCG-3′ or anti–miR-16: 5′-CGCCAAUA-UUUACGUGCUGCUA-3′ were synthesized by IDT and 5′ end-labeled by using T4 polynucleotide kinase (Thermo Scientific) and γ-labeled [^{32}P]-ATP (Perkin-Elmer).

miR-16 Expression. miR-16 was quantified by qPCR using Cells-to-Ct reagents and a miR-16 Taqman Cells-to-Ct microRNA assay (Life Technologies). An aliquot of each lysate was analyzed for rat GAPDH according to the reverse transcription and qPCR protocol detailed above.

1. White LA, et al. (1994) Distinct regulatory pathways control neurofilament expression and neurotransmitter synthesis in immortalized serotonergic neurons. *J Neurosci* 14(11 Pt 1):6744–6753.
2. Yamasaki K, et al. (2003) Neurons but not glial cells show reciprocal imprinting of sense and antisense transcripts of Ube3a. *Hum Mol Genet* 12(8):837–847.
3. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat Protoc* 1(6): 2856–2860.

Fig. S1. Sequence conservation in the 3′ UTR of the serotonin transporter mRNA. Human and mouse sequences were aligned with ClustalW (www.ebi.ac.uk/Tools/msa/clustalw2/). The sequence is presented 5′ to 3′ with reference to the SERT mRNA sequence. Positions conserved between mouse and human are marked with an asterisk. The proximal polyadenylation signal is highlighted in orange, and the distal polyadenylation signal is in blue. The region of the distal polyadenylation sequence element covered by the oligonucleotide probes is marked by red asterisks.

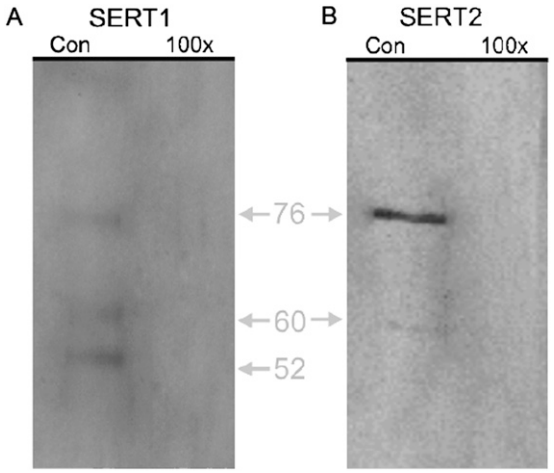

Fig. S2. Avidin affinity purification of SERT distal polyadenylation element binding proteins. Silver-stained gel after avidin affinity purification of oligoribonucleotide probe-binding proteins. Cytosolic extracts from C6 cells were UV cross-linked to oligoribonucleotide probes (1 nM) in the absence (Con) or presence (100×) of excess unlabeled probe. Cross-linked proteins were affinity purified and run on SDS/PAGE. (*A*) SERT1 probe. Approximate molecular masses of purified proteins are labeled by gray arrows and are similar to specific labeled proteins from crude cytosolic extracts (60 kDa and 76 kDa). An additional band of ∼52 kDa was present after purification. (*B*) SERT2 probe.

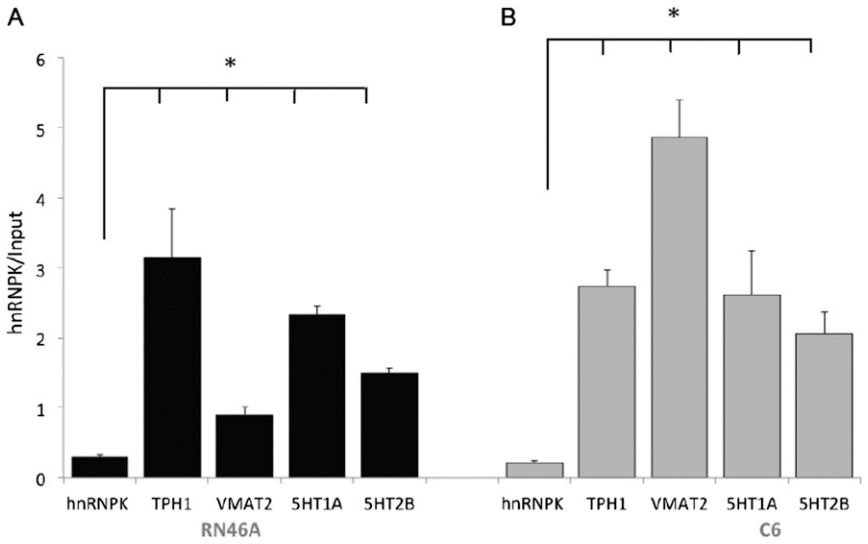

Fig. S3. hnRNPK RNA immunoprecipitation of related serotonergic mRNAs. Cell lysates were immunoprecipitated (IP) with an anti-hnRNPK antibody. Relative abundance of target sequences in hnRNPK immunoprecipitates (hnRNPK) was assessed by qPCR and compared with their abundance in total lysate (Input) to determine the degree of hnRNPK interaction with the various target mRNA species. The hnRNPK mRNA was quantified as a negative control. *$P < 0.05$. Error bars, SEM. (*A*) RN46A cells were used for immunoprecipitation. All targets present in RN46A lysates were enriched in hnRNPK immunoprecipitates significantly more than the hnRNPK mRNA. We attempted to assess TPH2 but were unable to amplify its mRNA in either input or immunoprecipitated samples. (*B*) C6 cells were used for immunoprecipitation. All targets present in C6 lysates were enriched in hnRNPK immunoprecipitates significantly more than the hnRNPK mRNA. We attempted to assess TPH2 but were unable to amplify its mRNA in either input or immunoprecipitated samples.

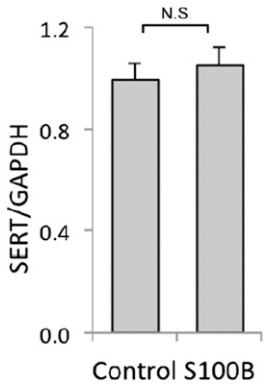

Fig. S4. S100B treatment of C6 cells does not increase expression of SERT mRNA. C6 cells were treated with S100B (1 nM) for 48 h, conditions that increased expression of SERT protein. Total SERT mRNA was quantified by real-time PCR and normalized to GAPDH. There were no significant changes in expression of SERT mRNA in response to S100B treatment, suggesting that increases in SERT protein were due to enhanced translation of the SERT mRNA. N.S., nonsignificant.

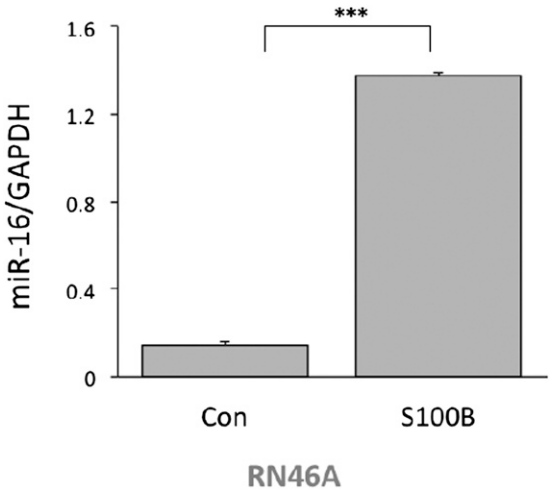

Fig. S5. S100B treatment of RN46A cells induces expression of miR-16. RN46A cells were treated with S100B (1 nM) for 48 h. miR-16 expression was quantified by using a Cells-to-Ct assay (Life Technologies). An aliquot of each lysate was also used for standard reverse transcription and qPCR assay for GAPDH to allow normalization of miR-16 expression to total RNA. ***$P < 0.005$.