Supplementary Information

Experimental Methods

7 Supplementary Figures

Dataset 1: 16 Supplementary Tables.

Experimental methods

Animals: Pregnant CD1 mice (Charles River Laboratories) were housed singly on a 12-hour light/dark cycle with ad libitum access to food and water. All experiments were performed during the light part of the diurnal cycle. Housing, animal care and experimental procedures were consistent with the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute.

Primary neuronal culture and transfection of lncRNA gapmeRs: Primary hippocampal cultures were prepared from the brains of embryonic day 18 mice. Cells were plated at a density of 5×10^5 on poly-D-lysine-coated (700 µg/ml) dishes and glass coverslips. Cultures were plated in Neurobasal medium (Invitrogen) supplemented with 10% fetal bovine serum and penicillin/streptomycin mix and grown in Neurobasal medium supplemented with 2% B27 (Invitrogen), 0.5 mM glutamine, and penicillin/streptomycin mix at 37°C in 5% CO_2. Antisense LNA gapmeRs against GM12371 and a non-targeting gapmeR control were designed using a design program by EXIQUON (now QIAGEN) and were synthesized. The following gapmeRs were used in

electrophysiology, imaging and gene expression analysis. gapmeR B2 5' GCTTGATTCAGTTGGT 3'; gapmeR B3 5' CCTTGGAAATTGCTA 3' and control non targeting gapmeR 5' AACACGTCTATACGC 3'. Briefly, gapmeRs were introduced to primary hippocampal neurons (7-14 days *in vitro* (DIV)) using Lipofectamine RNAiMAX or Lipofectamine 2000 (Invitrogen) according to manufacturer's guidelines. One day before transfection, fresh culture medium was prepared and mixed evenly with the old medium. One half of the mixed media were left with the cells for transfection, and the other half was saved for media replacement after transfection when using Lipofectamine 2000 for 2 hrs. of transfection and cells were then returned to the saved culture medium. Briefly, for the transfection of cells growing in the single well of a 24-well format dish, 6 pmol of gapmeR was mixed with 1 µl of Lipofectamine RNAiMAX in 100 µl of Neurobasal medium and incubated for 15 minutes. The complexes of RNAi and Lipofectamine RNAiMAX were added to the cells in incubation at 37°C in 5% CO_2.

Patch Clamp Electrophysiology: Whole-cell patch-clamp recordings were performed using an Axon Multiclamp 700 b amplifier, 1440A Digidata digitizer and pClamp software (Axon Instruments, Foster City, CA). Recordings were made at 50 kHz, and subsequently filtered at 5 kHz. Current and voltage recordings were obtained from primary culture hippocampal neurons. The recordings were conducted blindly, and three groups were used during these experiments; nontargeting gapmeR control, gapmeR B2 and B3 to knockdown GM12371. Hippocampal neurons were cultured for at least 8 to 10 days to allow an extensive synaptic network to develop before recordings were made. Recordings were done on 9-12 DIV. Spontaneous excitatory post-synaptic

potential currents (sEPSCs) were recorded in hippocampal neurons in culture under voltage clamp. The current clamp was recorded only to identify the health of the neurons. Only neurons with resting membrane potential of more than -40 mV were used for our analysis. Action potential was recorded in current clamp mode, I=0. Membrane potential was maintained at -40 mV in current clamp mode. Extracellular bath solution (EBS) for the recordings contained 135 mM NaCl, 10 mM glucose, 3 mM $CaCl_2$, 2 mM KCl, 2 mM $MgCl_2$, and 5 mM Hepes, pH adjusted to 7.3-7.4 with NaOH, and 300-315 mOsm with sucrose.

Patch-pipettes were pulled from borosilicate glass with micropipette puller (Sutter Instrument Co.) and filled with an intracellular solution containing 100 mM K-gluconate, 1.7 mM KCl, 0.6 mM EGTA, 5 mM $MgCl_2$, 10 mM Hepes, 4 mM ATP, and 0.1 mM GTP, adjusted to pH 7.2 with NaOH and 300-315 mOsm with sucrose (1). Once a neuron was patched, the seal was monitored, and if the transient potential was less than 100 pA, the recording was not used for analysis. All experiments were performed at room temperature (22-24^0 C). The frequency and amplitude of EPSCs were analyzed using the template match search (pClamp) and basic statistical analysis performed to extract average amplitude and frequency. The amplitude and frequency of EPSCs expressed as a percentage of baseline level, calculated from an average of 5 minutes of the baseline-recording period. The degree of the amplitude as well as the frequency of EPSCs for each experiment was measured as the average of 5 minutes during the recording period. The shown ‘n’ values refer to the number of neurons recorded. Results are presented as mean ± SEM throughout the text unless otherwise noted. The

term significant denotes a relationship with $p<0.05$ determined using an unpaired Student's t-test, and One-Way ANOVA followed by Tukey's multiple comparison test.

Sholl and Spine analysis: After 72 hours of transfection of hippocampal neurons using pEGFPN1, GFP expressing live neurons were observed and images were collected at room temperature in the light microscopy facility at the Max Planck Florida Institute, using a confocal microscope (LSM 780; Carl Zeiss; Plan Neofluor 63X/1.3 N.A. Korr differential interference contrast M27 objective in water). Z-stack images were acquired using ZEN 2015 (64 bit) software (Carl Zeiss) and dendritic arbors were manually traced and later quantified by Sholl analysis FIJI (Image J, NIH). A series of concentric circles were drawn from the center of the soma at intervals of 20μm, with the radius of the outermost circle set at 100μm. The maximum value of sampled intersections reflecting the highest number of processes/branches in the arbor was calculated and the number of intersections plotted against distance from the soma center in μm.

In order to analyze spines, images were captured using a 63X objective. Secondary branches from basal and apical dendrites were selected as regions of interest from the original image stack, using MATLAB software. Values obtained for spine density and spine morphology i.e., the number of different spine types viz. mushroom, thin or stubby were used for the analysis. One-way ANOVA followed by Tukey test was carried out to evaluate the statistical difference amongst the groups.

Next Generation Sequencing and Data Analysis: RNAseq and analysis were done

following Scripps Florida Genomics core protocol as described previously (2). Analysis of the RNAseq data was performed using Tophat2 (v.2.0.9) (3) and the Cufflinks suite (v.2.1.1) (4). Trimming of the data was performed using the FastX-Toolkit (v.0.0.13) (Toolkit1 by Hannon Lab.). The reads were trimmed using the quality scores (cut-off Phred score = 28). The trimmed reads were aligned to the *Mus musculus* genome build NCBIM37 using Tophat. Tophat was run using its default parameters, which include a maximum of 2 mismatches, a maximum gap length of 2 gaps and an edit distance of 2 for the final alignment.

After the alignment, Cufflinks was utilized to calculate the FPKM values. The default parameters were also used to run cufflinks, except for one parameter "–max-bundle-frags" which was changed to 100,000,000. Cufflinks measures transcript abundances in Fragment Per Kilobases of transcripts per million fragments mapped. The alignment data were then processed and quantified using HTSeq (5). The raw read counts generated in HTSeq were used to identify differentially expressed genes in the R Bioconductor package DESeq (6). HTSeq count was run with "–stranded = no" option, and the ID attribute option, "-i" set to "gene_name." DESeq uses the total size of each library to normalize the raw read counts and perform calculations on fold change as well as significance based on *p*-value and adjusted *p*-value. The comparisons in this case are between samples of two groups, GM12371 knockdown and gapmeR control. lncRNA transcripts were extracted from the Ensembl annotation file (NCBIM37). RNAseq data can be accessed from NCBI BioProject PRJNA356039 (https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA356039).

Ingenuity Pathway Analysis (IPA): IPA software (Qiagen-IPA) was used to identify enriched canonical pathways in the list of up regulated (log2 fold change >1, adjusted *p*-value < 0.05) and downregulated genes (log2 fold change <−1, adjusted *p*-value < 0.05) in the GM12371 knockdown compared to control. The upregulated and downregulated pathways were identified and sorted according to their statistical significance as the negative logarithm of *p*-values calculated by IPA.

Heatmap analysis plots: Normalized read counts were extracted from the DESeq results for the 50 genes flagged as the most differentially expressed and belonging to a specific pathway as identified by IPA. The normalized counts were given a pseudo count of 1 and log2 transformed and plotted using R (version 3.2.3).

Circos plot: Normalized read counts for all protein coding genes that belonged to three specific molecular mechanisms (cellular development, growth and differentiation) were separated from all other protein coding genes. Normalized read counts for lncRNAs made up a third category. These three separate tracks, in addition to the mouse cytoband information, were plotted using the RCircos library in R (version 3.2.3).

Quantitative Real-Time PCR (qPCR), Fluorescence in situ hybridization (FISH) and immunocytochemistry (ICC): qPCR was done as described previously (2,7). Quantification of each transcript was normalized to the mouse 18S reference gene following the 2−ΔΔCt method (8). Student *t*-test was used to select genes with statistically significant expression levels where **p*-value < 0.05, ***p*-value < 0.01, ****p*-

value < 0.001. The sequences of primers for the mRNAs and lncRNAs are given in the Supplementary Table S16. A 300 bp fragment of GM12371 was sub cloned into PCRII TOPO vector to produce DIG labeled antisense probes for fluorescence in situ hybridization (FISH). Tyramide SIGNAL Amplification (Thermo Fisher Scientific) was used following manufacturer's protocol to detect and image GM12371 in neuronal cultures. Anti-SOX 10 antibody [EPR4007] was obtained from Abcam and anti-alpha tubulin antibody (T8203) was obtained from SIGMA. ICC and western blot hybridization experiments were carried out as described previously (7).

Native Chromatin immunoprecipitation (ChIP): Native ChIP was performed as previously described (Kasinathan, et al., 2014) with some modifications. For ChIP analysis approximately 500,000 cells per well of a 6-well plate were plated. Four hours after plating, plating media was replaced with feeding media consisting of Neurobasal media supplemented with Glutamax, Penstrep, and 2% B27, and half of the media was replaced every 4 days until the time of experiments (DIV17). Prior to experiments, hippocampal neurons were treated with Forskolin (50μM) or DMSO (equal volume) for 30 minutes. Primary hippocampal neurons were washed with cold dPBS, then brought to 4^{o}C, gently lysed in cytoplasmic buffer (20 mM Tris-HCl pH 7.4, 150mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 0.5%Nonidet P-40, protease inhibitor cocktail, phosphatase inhibitor cocktail mixture) for 20 minutes, scraped and collected into tubes for centrifugation at 4000g for 5 minutes. The nuclear pellet was then washed in Nuclear wash buffer (10 mM HEPES pH7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M Sucrose, and complete protease inhibitor mixture) and lysed in RIPA buffer with protease and phosphatase inhibitor

mixture for 10 minutes, followed by centrifugation at 13,000g for 10 minutes. The pellet was resuspended in 500µl TM2 buffer (10 mM Tris, pH 7.4, 2 mM $MgCl_2$, 1× EDTA-free protease inhibitor cocktail) and prewarmed to 37 ^oC for 3 minutes, followed by addition of 12µl 0.2M Calcium Chloride and 200 U MNase, and incubation at 37 ^oC for 6 minutes. The sample was then treated with 24µl of 0.2M EGTA, brought to 4^oC and centrifuged at 2000 rpm for 10 minutes. The pellet was resuspended in 80T buffer (70 mM NaCl, 10 mM Tris, pH 7.4, 2 mM $MgCl_2$, 2 mM EGTA, 0.1% Triton X-100, 1× EDTA-free Complete Protease Inhibitor Cocktail) and triturated with a 27-gauge needle 10 times, and then pelleted. 20% was saved as input control. The remaining sample was split evenly into IgG antibody (Pierce) control group or H3K27ac antibody (Active Motif) group for overnight incubation with rotation at 4^oC. The next day the samples were incubated with precleared protein A/G beads for one hour and pelleted. After multiple washes in 80T buffer, the protein/bead complexes were dissociated in Trizol, followed by phenol/chloroform extraction of DNA in the interphase layer and RNA in the aqueous layer. RNA was further precipitated by adding one-tenth volume of 0.5M sodium acetate, 5µl glycerol, and 2.5x volume absolute ethanol. Abundance and purity of DNA and RNA were assessed using Nanodrop.

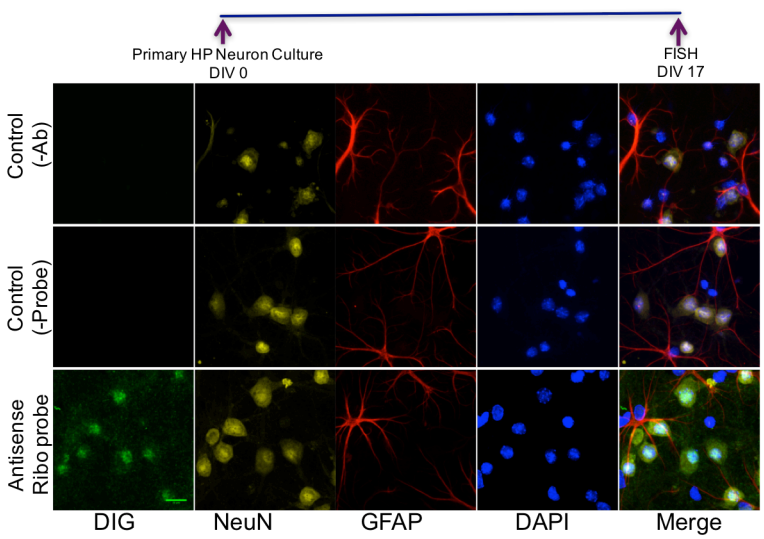
Supplementary References

1. Girotto, F. *et al.* High dose folic acid supplementation of rats alters synaptic transmission and seizure susceptibility in offspring. *Scientific reports* **3**, 1465, doi:10.1038/srep01465 (2013).
2. Kadakkuzha, B. M. *et al.* Transcriptome analyses of adult mouse brain reveal enrichment of lncRNAs in specific brain regions and neuronal populations. *Frontiers in cellular neuroscience* **9**, 63, doi:10.3389/fncel.2015.00063 (2015).

3. Kim, D. *et al.* TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome biology* **14**, R36, doi:10.1186/gb-2013-14-4-r36 (2013).
4. Trapnell, C. *et al.* Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nature biotechnology* **28**, 511-515, doi:10.1038/nbt.1621 (2010).
5. Anders, S., Pyl, P. T. & Huber, W. HTSeq--a Python framework to work with high-throughput sequencing data. *Bioinformatics* **31**, 166-169, doi:10.1093/bioinformatics/btu638 (2015).
6. Anders, S. & Huber, W. Differential expression analysis for sequence count data. *Genome biology* **11**, R106, doi:10.1186/gb-2010-11-10-r106 (2010).
7. Liu, X. A. *et al.* New approach to capture and characterize synaptic proteome. *Proceedings of the National Academy of Sciences of the United States of America* **111**, 16154-16159, doi:10.1073/pnas.1401483111 (2014).
8. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402-408, doi:10.1006/meth.2001.1262 (2001).

Supplementary Figures

A



B

GM12371 Sequence and locations of gapmeR 2 (in blue) and gapmeR 3 (in red)

GAAGCTGGCTGGCTGAGAATAAGAAAATACAGTATGTGAGCACTTGGAGGAGAAACTGTAGGT
TGCAATAGTGGAAGGGAGGAGGATTTTGAGCAAAAGAAGAAACTTGTGTTGTTAGGAGCTGCG
ACCACGCTATTCATTGCCTGAGTAGCCAAGCATGTTAAGGTTTCTGCTGGAAAGGAAGACATCA
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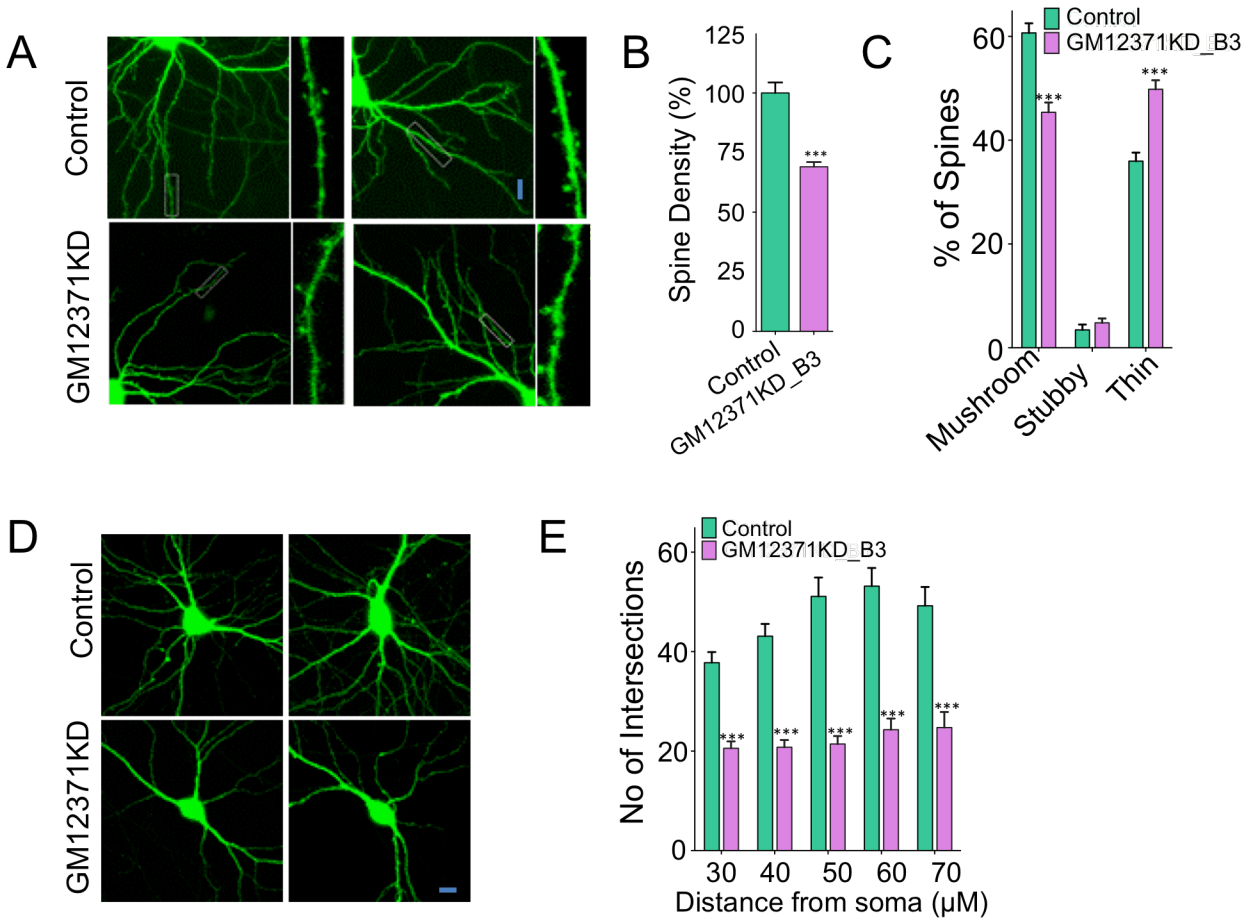
CAGTGTTGGAAAACAGTGTATTCTCTAAATAAAAAAGAGAAAAATTTAAATTAAAAGA

Supplementary figure S1. Analysis of GM12371 lncRNA. A. Fluorescence *in situ* hybridization (FISH) analysis to visualize the expression of GM12371 in hippocampal neurons. Confocal projection images are shown. DIG labeled RNA prepared from cloned 300 bp fragment of GM12371 was used in the FISH analysis. No probe and no anti DIG antibody were used as specificity controls. DIG: Digoxigenin, Ab: Antibody. B. Recognition site of gapmeRs B2 and B3. GM12371 sequence and recognition site of B2 and B3 are shown.



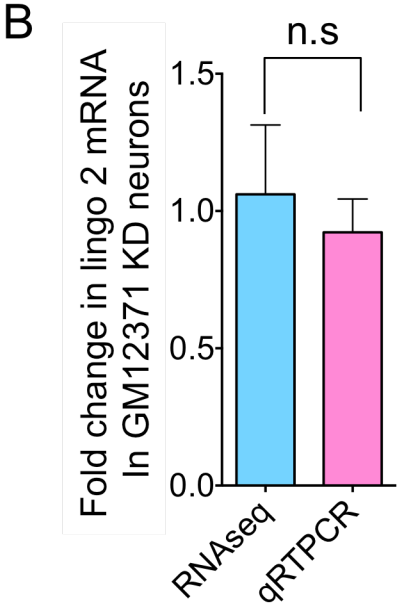
Supplementary figure S2. GM12371 regulates spine density, spine morphology and dendritic tree complexity in DIV 10-12 hippocampal neurons. A: Two representative confocal projection images of spines collected are shown. Space bar 20 μm. gapmeR B3 was used to knockdown GM12371. A nontargeting gapmeR was used as control for comparisons. **B:** Quantification of total spine density. Bar graphs show number of spines per 100 μm of distal dendrites quantified in GM12371 knockdown and control neurons; **C:** Quantitation of specific changes in spine morphology. Number of neurons analyzed for gapmeR control = 32, gapmeR B3= 54. **D:** Sholl analysis to assess the effect of GM12371 knockdown on dendritic tree complexity. Shown are

confocal projection images of EGFP expressing hippocampal neurons following transfection by nontargeting gapmeR, gapmeR B3. **E:** Bar graphs show number of intersections at varying distances from soma. Number of neurons analyzed for gapmeR control=23, gapmeR B3=25. Error bars are SEM, "*" is $p<0.05$, Unpaired two-tailed t test, KD: knockdown; DIV: days in vitro; HP: hippocampus.

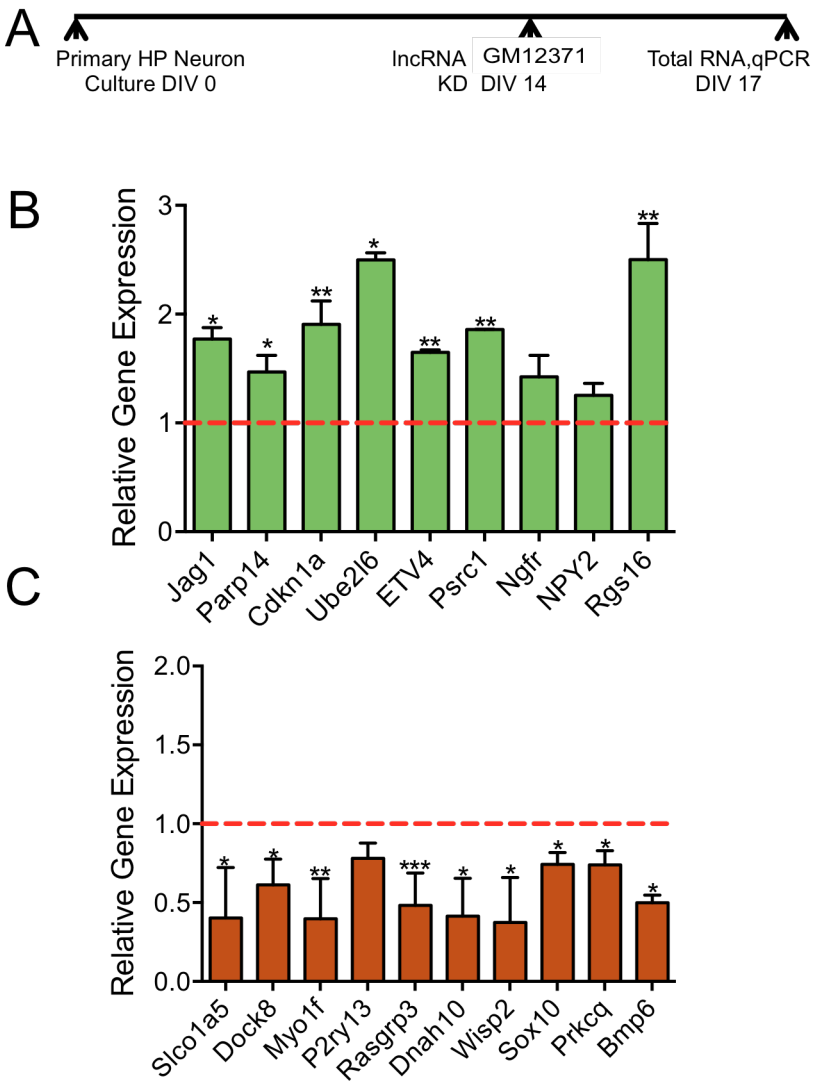
A

Genomic location of lncRNA GM12371 and mRNA, lingo2.

B

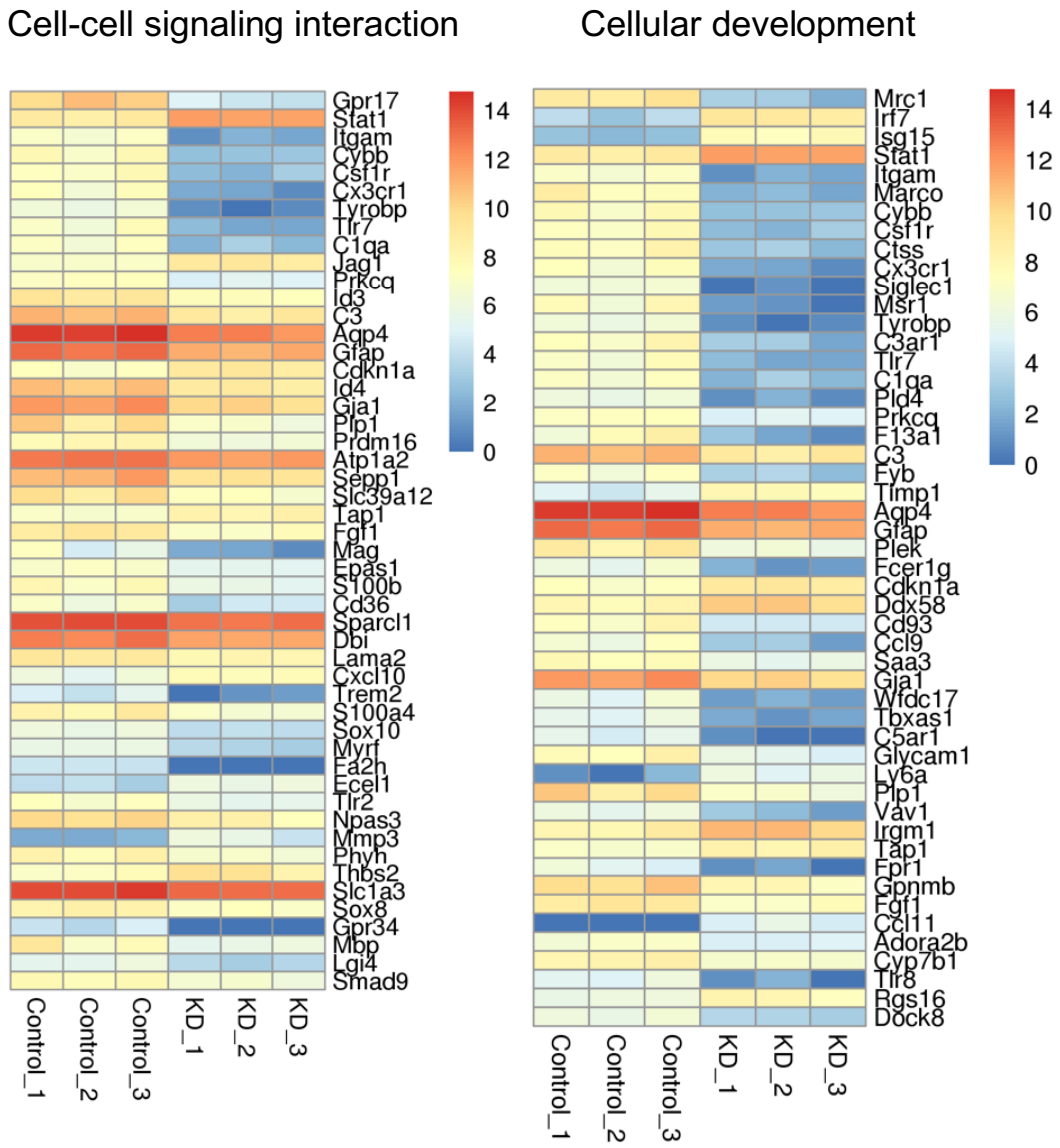


Supplementary figure S3. Analysis of GM12371 and its putative cognate mRNA target lingo2. A: Chromosomal position of GM12371 and lingo2. **B:** Expression of lingo 2 is not regulated by GM12371. Bar graph shows fold changes in lingo2 levels following GM12371 knockdown (using gapmeR B3, see Figure 1) compared to control (non-targeting gapmeR). Error bars are SEM, n=3 for RNAseq and n=5 for qPCR, Students' t test. n.s: non-significant.

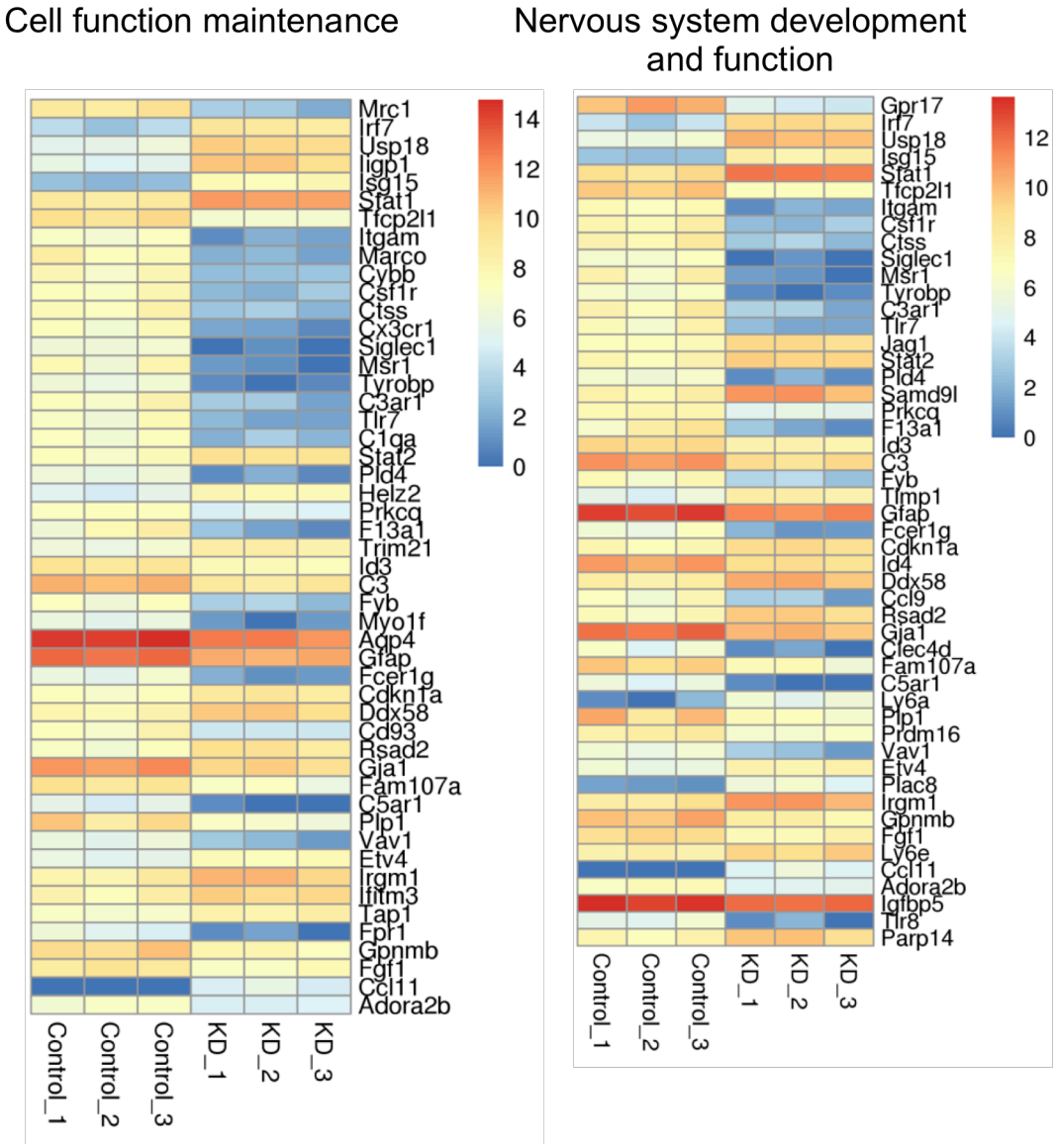


Supplementary figure S4. Validation of RNAseq data. A. Experimental outline. **B&C** qPCR analysis of relative expression changes in up and downregulated genes, selected from RNAseq data, in GM12371 knockdown (using gapmeR B3) neurons compared to control (nontargeting gapmeR). n=6 for both upregulated **(B)** and downregulated **(C)** genes. Data was normalized to 18srRNA levels. Error bars are SEM, "*" is $p<0.05$,

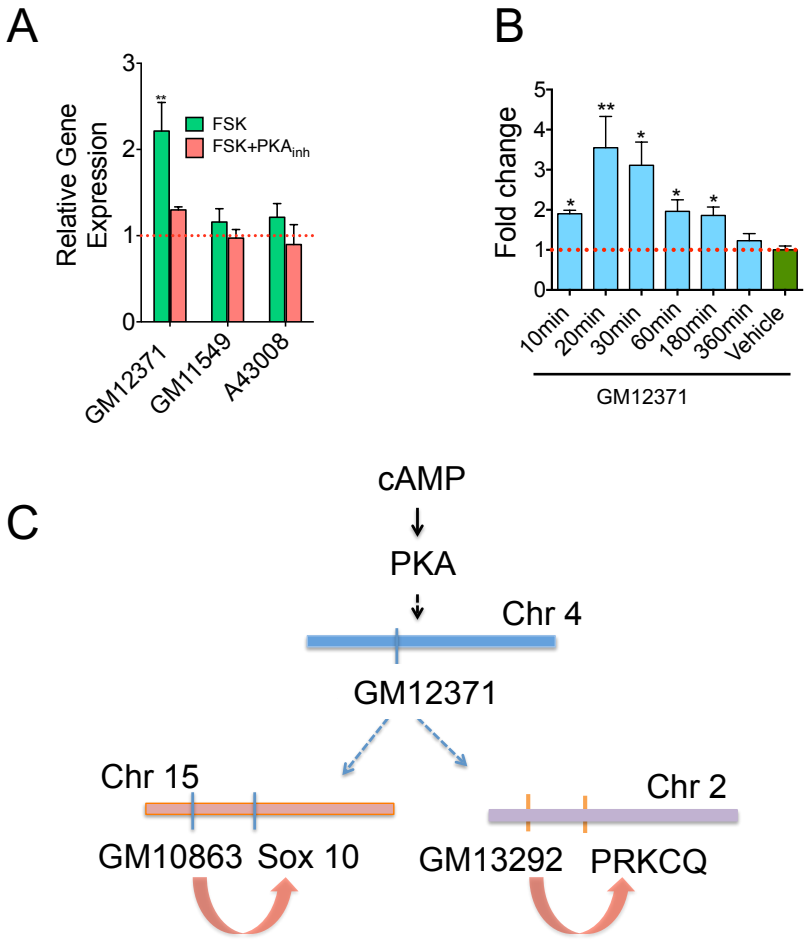
Unpaired two-tailed t test. DIV: days in vitro, KD: knockdown. Data analysis shown in Supplementary table S8.



Supplementary figure S5. GO analysis of differentially expressed mRNAs (adjusted *p* value <0.05) identified from RNAseq analysis of GM12371 knockdown. Heat maps show top 50 differentially expressed mRNAs in category “Cell-Cell signaling interaction and cellular development”.



Supplementary figure S6. GO analysis of differentially expressed mRNAs (adjusted *p* value <0.05) identified from RNAseq analysis of GM12371 knockdown. Heat maps show top 50 differentially expressed mRNAs in category "Cell function maintenance and Nervous system development and function".



Supplementary figure S7. cAMP-PKA regulation of expression of GM12371.

FSK: Forskolin, PKA inh (14-22 amide, a cell permeable PKA inhibitor). **A**. qPCR analysis of relative changes in expression of lncRNAs GM12371, GM11549, and A43008 within 30 minutes of forskolin (50 μM) exposure and in the presence of PKA inhibitor. **B.** Time course analysis of GM12371 expression in response to forskolin or vehicle (DMSO) exposure. Data was normalized to 18srRNA levels. Error bars are SEM."*" is $p<0.05$, One-Way ANOVA followed by Tukey test. **C***:* Cartoon showing *trans* mechanism of action of GM12371. The two lncRNA-mRNA pairs transcribed from

different chromosomes (2 and 15) are targets of GM12371 transcribed from chromosome 4.

Supplementary tables

Supplementary table S1. Characterization of expression and knockdown of lncRNA GM12371 in hippocampal primary neurons (refer Figure 1).

Supplementary table S2. Role of GM12371 in synapse density, morphology and dendritic tree complexity and in hippocampal neurons (refer Figure 2)

Supplementary table S3. Analysis of effect of GM12371 knockdown on lingo2 mRNA by qPCR (refer Supplementary Figure S3).

Supplementary table S4. Mapping statistics of RNAseq analysis of control and GM12371 knockdown neurons.

Supplementary table S5. RPKM values of mRNAs identified by RNAseq analysis of control and GM12371 knockdown neurons.

Supplementary table S6. Differentially expressed mRNAs following GM12371 KD identified from RNAseq data.

Supplementary table S7. Differentially expressed lncRNAs following GM12371 KD identified from RNAseq data.

Supplementary table S8. Characterization of RNAseq data by qPCRs. List of genes analyzed, their fold change, SEM and p values are shown (refer Supplementary Figure S4).

Supplementary table S9. List of canonical pathways that are downregulated following GM12371 knockdown by Ingenuity Pathway Analysis of differentially expressed mRNAs. 68 pathways that are statistically significant ($p<0.05$) are shaded.

Supplementary table S10. List of canonical pathways that are upregulated following GM12371 knockdown by Ingenuity Pathway Analysis of differentially expressed mRNAs.

Supplementary table S11. List of pathways that are involved in diseases and functions that are downregulated following GM12371 knockdown by Ingenuity Pathway Analysis of differentially expressed mRNAs.

Supplementary table S12. List of pathways that are involved in diseases and functions that are upregulated following GM12371 knockdown by Ingenuity Pathway Analysis of differentially expressed mRNAs.

Supplementary table S13. Characterization of lncRNA: mRNA pairs by qPCR analysis. List of genes analyzed, their fold change, SEM and p values are shown (refer Figure 3).

Supplementary table S14. Regulation of GM12371 by cAMP signaling. qPCR analysis of data shown in Supplementary Figure S7 is shown.

Supplementary table S15. Characterization of regulation of Sox10 expression by lncRNAs (refer Figure 6).

Supplementary table S16. Sequences of oligonucleotide primers used in this study.