Supplementary Information for

Regulatory Discrimination of mRNAs by FMRP Controls Mouse Adult Neural Stem Cell Differentiation

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SI Materials and Methods

Adult neural stem cell isolation and culture

All animal procedures were performed according to protocols approved by the University of Wisconsin-Madison Animal Care and Use Committee. Adult-derived NSCs were isolated from the dentate gyrus (DG) of 8- to 10-week-old male *Fmr1* KO mice (FVB/N Fmr1^{tm1Cgr} mice, Jackson) and wild-type (WT) littermate controls based on our published method (1). Briefly, DG were dissected using forceps and 27gauge needle (BD, #305109) and placed in Hank's balanced salt solution (HBSS, Invitrogen, #14025-126) on ice. Tissue was spun down and digested using MACS Neural Tissue Dissociation kit (Miltenyi Biotech, #130-090-753). After dissociation with a fire-polished glass pipette, cells wFere filtered through a 70-μm cell strainer (BD Falcon, #252350) and washed with HBSS, the single-cell suspension from each sample was collected and cultured in proliferation medium [Neurobasal medium containing B27 serum-free supplement (Invitrogen, #17504-044), 10 ng/ml basic fibroblast growth factor (FGF-2, PeproTech, #K1606), 10 ng/ml epidermal growth factor (EGF, PeproTech, #A2306), 1% Antibiotic-Antimycotic, and 2 mM L-glutamine], in a 5% CO_2 incubator at 37°C. Half of the medium was replaced every two days. Independently isolated cells served as biological replicates.

Ribosome profiling

Cycloheximide (CHX, Sigma-Aldrich, #C7698) was added into cultured aNSCs to a final concentration of 100μg/ml, and cells were incubated at 37 °C for 10 min to stabilize ribosomes. Then, cells were transported to cold room and spun down gently at 1000g for 3 min and the supernatant was carefully discarded. Cells were washed twice with ice-cold PBS containing 100μg/ml CHX and were spun down at 1000g for 3 minutes. The supernatant was carefully removed to collect the cell pellet. The cell pellet was immediately stored and kept in -80 °C freezer for later analysis.

Frozen cell pellets were thawed in ice-cold polysome lysis buffer [20mM Tris-HCl pH7.4, 5mM $MgCl_2$, 100mM KCl, 1mM DTT, 100μg/ml CHX, 25U/ml Turbo DNaseI (Ambion, #AM2238), 1% TritonX-100 in nuclease-free water] and lysed by trituration through a 25-G need for 10 times. After 10-min incubation on ice, lysates were clarified by centrifugation at 14,000g 4 °C for 10min. The supernatants were collected and the amounts of nucleic acids (A_{260} units) were measured with Nanodrop (Thermo Fisher Scientific). For each sample, cytoplasmic

RNA for RNA-seq was purified from one fourth of the lysate with TRIzol LS reagent (Invitrogen, #10296028). The other three fourths of the lysate was digested with 100ng/A_{260} RNase A (Ambion, #AM2270) and 60U/A_{260} RNAse T1 (Thermo Fisher Scientific, #EN0542) at 25°C for 30min. The digestion was stopped by adding 50U SUPERase In RNase inhibitor (Ambion, #AM2694) and chilling on ice. Digested lysates were loaded on 10%-50% sucrose gradients prepared in 1X polysome buffer (20mM Tris-HCl pH7.4, 5mM $MgCl_2$, 100mM KCl, 1mM DTT, 100µg/ml CHX in nuclease-free water). After the ultracentrifugation in a SW41Ti rotor (Beckman Coulter) at 35,000 rpm 4°C for 2.5 hours, gradients were fractionated at 1.5 ml/min and 12 sec collection interval through a fractionation system (Brandel) that continually monitored A_{260} values. Monosome fractions were identified, pooled, and extracted with TRIzol LS.

For the cytoplasmic RNA-seq, 500ng total RNA per sample was used for the library preparation with the Ovation RNA-Seq System V2 (NuGEN, #0304) following manufacturer's instructions. HL-dsDNase (ArcticZymes, #70800-201) method was used to eliminate DNA contamination and cDNA was fragmented with M220 Covaris Focused-ultrasonicator for a target median fragment size of 300bp (peak power: 50.0, duty factor 20.0, cycles/burst: 200, treatment time: 75sec). qPCR test was performed with EvaGreen Dye (Biotium, #31000-T) to determine the optimal PCR cycles.

For the ribosome profiling samples, libraries were prepared following the published protocols (2). Briefly, rRNA was depleted from the purified monosomal RNA samples with RiboZero (Illumina, #MRZG12324). Remaining RNA samples were separated on a 15% TBU gel (National Diagnostics, #EC-833) and the ribosome footprints were size-selected based on the 26 and 34nt markers. RNA was eluted from the crushed gel pieces in RNA elution buffer (300mM NaOAc pH5.5, 1mM EDTA, 0.25% SDS) at room temperature overnight, filtered with Spin-X Centrifuge Tube Filters (Corning, #8162) and precipitated with equal volume of isopropanol. Recovered RNA was dephosphorylated with T4 Polynucleotide Kinase (NEB, #M0201S) and ligated with preadenylated adaptor in miRCat®-33 Conversion Oligos Pack (IDT) using T4RNL2Tr.K227Q ligase (NEB, #M0351L). Reverse transcription (RT) was performed with primers containing 5nt-barcode and 8nt-unique molecular identifier (UMI) and SuperScript III (Invitrogen, #18080-044) in 1X first-strand buffer without $MgCl_2$ (50 mM Tris-HCl, pH 8.3, 75 mM KCl). RT products were separated on a 10% TBU gel and the 130-140nt

region was selected. cDNA was eluted in DNA elution buffer (10mM Tris pH 8.0, 300mM NaCl, 1mM EDTA) at room temperature overnight, filtered, and precipitated with isopropanol. Purified cDNA was circularized with CircLigase (Epicentre, #CL4115K). cDNA derived from remaining rRNA was hybridized to biotin-labelled antisense probes (IDT) and further depleted with Dynabeads MyOne Streptavidin C1 (Invitrogen, #65001). Optimal PCR cycle was determined empirically by test PCR reactions with titrated cycle numbers. Final PCR amplification was performed with KAPA Library Amplification Kit (Kapa Biosystems, #KK2611) and 180-190bp products were size-selected on an 8% TBE gel. DNA was eluted in DNA elution buffer, filtered, and precipitated with isopropanol. The final library DNA was purified with AMPure XP beads (Beckman Coulter, #A63880). Oligos used for the library preparation are listed in SI Appendix (Table S3).

The size distributions of final libraries were measured by Fragment Analyzer (Advanced Analytical, performed by Molecular Biology Core Labs at UMMS). The concentrations were quantified with KAPA Library Quantification Kit (Kapa Biosystems, #KK4835). Libraries were pooled with equal molar ratios, denatured, diluted, and sequenced with NextSeq 500/550 High Output Kit v2 (Illumina, 75bp single-end runs for ribosome profiling, #FC-404-2005; 75bp pair-end runs for RNA-seq, #FC-404-2002) on a Nextseq500 sequencer (Illumina). The raw sequencing data reported in this paper have been deposited in GEO under accession number GSE112502.

Read mapping and quality control

For ribosome profiling data, individual samples were separated from the raw fastq files based on the barcode sequences. Adaptor sequences (TGGAATTCTCGGGTGCCAAGGAGATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCG) were removed with cutadapt (1.7.1). Trimmed reads were quality filtered and mapped to the mouse rRNA and then tRNA references with Bowtie2 (2.1.0). Unmapped reads were next mapped to the mm10 mouse genome with Tophat2 (2.0.9). PCR duplicates were marked based on the UMI sequences and only uniquely mapped reads without duplicates were retained with samtools (0.0.19) for the downstream analysis. For RNA-seq data, reads unmapped to rRNA and tRNA were mapped to mm10 genome with Tophat2, but only the Read 1 of the paired-end reads was used for the quality control and visualization purposes.

Bam files from four biological replicates were merged to reveal the global distribution of reads. Reads mapped to 5'UTR, CDS, and 3'UTR regions were counted after intersecting the bam files with bed annotation files (USCS genome browser) using bedtools (2.22.0). RPF length distribution, P-site offsets, and frame preference were calculated with plastid (0.4.8). Counts at each nucleotide position were extracted using P-sites of RPFs and 5'end of mRNA reads with +11 offset. For all the transcript-level analysis, one representative transcript isoform with the highest abundance for each gene was selected based on the WT RNA-seq data. Only transcripts with UTRs longer than 90nt, CDS longer than 300nt, and minimum 50 reads on CDS were included for the metagene analysis. Counts at each nucleotide position was first normalized to the mean read density on CDS and then averaged across the transcriptome to obtain the global pattern of read distributions.

Differential translation analysis

Cleaned bam files of RPFs were converted to fastq files with bedtools. Cleaned fastq files without rRNA or tRNA were used for RNA-seq quantification. For both ribosome profiling and RNA-seq, gene expression was quantified with RSEM (1.2.11) using the cleaned fastq files and Refseq (V69) mouse CDS without the first and last 30nt to avoid the translation initiation and termination peaks. Genes were filtered with minimum 10 reads across all replicates and then the read counts were batch-corrected with the Combat function in sva (3.24.4) using a full model matrix. Batch-corrected counts were normalized with trimmed mean of M values (TMM) method and used to identify differential expressed genes (DEGs) with anota2seq (1.0.0). Instead of the default setting, the priorities of translation and buffering groups were determined by the nominal p-values and were set to be higher than the priority of mRNA only groups. The minimum nominal p-value cut-off (0.05) that allows the identification of most possible FMRP CLIP genes as "Translation up" and "Buffering up" was used as the optimal cut-off (SI Appendix, Figure S1F). A permutation test was performed to estimate the false discovery rate (FDR) under the selected cut-offs (absolute fold changes > 1.2 and nominal p-value < 0.05).

GO analysis was performed with DAVID 6.8 using all genes past filtering in the dataset as the background. For the mRNA feature analysis, lengths of 5'UTR, CDS, and 3'UTR with minimum 20nt were extracted from the Refseq annotation file (UCSC genome browser). GC content was calculated based on the sequences. Minimum Free Energies (MEFs) were calculated by the RNAfold function in ViennaRNA package (2.1.6). Wilcoxon rank sum test was used to

compare the median of a feature measurement (e.g. mRNA expression) in a regulatory gene group (e.g. “Buffering up”) to the median of that feature in all genes used for DEG analysis. Bonferroni method was used for the multiple test correction. Expression changes of “mRNA up” and “mRNA down” groups were used to infer upstream regulators by Ingenuity Pathway Analysis (QIAGEN) using Ingenuity Knowledge Base (Genes Only) as the reference set. Read counts at each nucleotide position of a transcript were averaged across four biological replicates after the batch-correction. Processed counts were smoothed within a 30nt window and plotted to visualize gene expression changes. For the overlap analysis between genes in each regulatory group and FMRP CLIP genes, all the genes with mRNA expression comparable to that of DEGs in WT aNSCs were used as the background to correct the expression level bias. Statistical significance is calculated with a hypergeometric test with Bonferroni correction (SI Appendix, Table S2). For the transcripts with RPFs on 5’UTR, 5’UTR/CDS read ratios were calculated for both WT and *Fmr1* KO aNSCs and the fold changes were plotted.

FMRP CLIP data re-analysis

FMRP CLIP data from age P11 and P13 mouse brain polysomes were downloaded from GEO (GSE45148) and processed as described in the original study (3). The centers of CLIP tags were used as the putative FMRP binding sites. Sequences of +/- 50nt windows surrounding CLIP peaks with more than 5 tags on transcripts with more than 50 tags were extracted. The enriched motifs were identified with the DREME function in MEME suite (4.9.1).

shRNA plasmid construction

Lenti-shNdn was cloned using lentivirus-sh-Control vector as the backbone (4). Briefly, shNdn (5′- GCTAACCGTGAAATCACCAAG -3′) was designed for targeting mouse *Ndn*. The shNC sequence (5′-GGAATCTCATTCGATGCATAC-3′) was published previously (4). The shRNA sequences together with the loop sequence (TCAAGAG), U6 promoter and restriction sites (HpaI and ClaI) were cloned using two rounds of PCRs with Lenti-U6 promoter-sh-Control vector as the template. The U6-shRNA cassettes were then cloned into lenti-U6-sh-Control vector via HpaI and ClaI restriction sites using the In-fusion kit (Clonetech, #638909) according to its instructions. All plasmid constructs were verified by DNA sequencing. Primers used for cloning are listed in SI Appendix (Table S3).

Lentivirus production

Lentivirus production was performed as described previous (5). Briefly, lenti-viral DNA was co-transfected with packaging plasmids pMDL, REV and pCMV-Vsvg into 5×15 cm dishes of HEK293T cells using the calcium phosphate method. The medium containing lentivirus was collected at 36 and 60 hours post-transfection, pooled, filtered through a 0.2-μm filter, and concentrated by ultracentrifugation at 19,000 rpm for 2 hours at 4°C using a SW27 rotor (Beckman). The virus was washed once and then resuspended in 100 μl PBS. We routinely obtained 1×10^9 infectious lentiviral particles /ml. To study the effects of *Ndn* on the proliferation and differentiation of aNSCs, 1×10^7 viral particles were added to the aNSCs cultured in proliferating condition on a 10-cm dish. After a 2-day incubation, infected aNSCs were trypsinized and plated on 24-well plates (Fisher, #87721), at a density of $1X10^5$ cells/well, for the proliferation or differentiation analysis.

Proliferation and differentiation assays

Proliferation and differentiation of aNSCs were analyzed as described (4). We used only early passage cells (between passage 4 and 10) and only the same passage numbers of WT and *Fmr1* KO cells. For each experiment, duplicate wells of cells were analyzed, and results were averaged as one data point (N=1). At least 3 independent biological replicates were used (N=3) for statistical analyses. To study cell proliferation, we dissociated neural stem cells with trypsin and plated them on 24-well plates with poly-L-ornithine (Sigma-Aldrich, #P3655) and Laminin (BD Biosciences, #354232)-coated coverslips at a density of $1X10^5$ cells/well in proliferation medium (see above). At 18 hours post-plating, 5μM 5- bromo-2'-deoxyuridine (BrdU, Sigma-Aldrich, #B5002) was added into the culture medium for 6 hours. aNSCs were then washed with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature, followed by immunohistochemical analysis. To detect BrdU incorporation, fixed cells were pretreated with 1M HCl for 30 min at 37°C, washed with borate buffer, pH 8.5, for 30 min, and followed by the standard immunocytochemical protocol (6). For the differentiation assay, at 24 hours post-plating, cells were changed into differentiation medium, which is Neurobasal medium containing 5 μM forskolin (Sigma-Aldrich, #F-6886) and 1 μM retinoic acid (Sigma-Aldrich, #R-2625) for 4 days. Upon fixation with 4% paraformaldehyde, the coverslips were subjected to our standard immunohistochemistry protocol (6).

Immunocytochemistry

Immunocytochemistry staining was carried out as described (4, 6). Briefly, cells were fixed with 4% paraformaldehyde for 20 min, and then washed with Tris-buffered saline (TBS, 50 mM Tris-Cl, pH 7.5, 150 mM NaCl) for 30 min. Cells were preblocked using TBS containing 2% normal goat serum (VECTOR, #S-1000) and 0.1% Triton X-100 for 30 min, followed by overnight incubation with primary antibodies. After washing with TBS, cells were incubated with fluorescent secondary antibodies, followed by counterstaining with the fluorescent nuclear dye 4', 6 -dimidino-2'-phenylindole dihydrochloride (DAPI, Roche Applied Science). The coverslips were mounted with polyvinyl alcohol (PVA) mounting medium with DABCO (Sigma-Aldrich, #10981) and stored in cold and dark before and during analysis. The numbers of marker-positive cells ($BrdU^+$, $Tuj1^+$ or $GFAP^+$) and total lenti-infected cells (shRNA-GFP^+) were quantified using an Olympus BX51 microscope equipped with a MicroFire digital camera (Optronics) and a motorized stage using 20X objective lens. The quantification was carried out using an unbiased stereology method with assistance from Stereo Investigator software (MBF Biosciences). The percentage of differentiated cells was calculated as the number of marker-positive cells divided by the total number of GFP^+ cells. At least 3 independently viral infected cell cultures were analyzed for statistical analysis

Ndn mRNA Stability Assay

aNSCs from WT and *Fmr1* KO mice were grown in proliferating condition (see above). 10 μg/ml of Actinomycin D (Sigma-Aldrich) was added based on published protocol (4) and total RNA was isolated at various time intervals. The *Ndn* mRNA level was normalized to *Gapdh* mRNA as measured by quantitative PCR.

RNA purification and quantitative PCR

Total RNA was purified from cells using the TRIzol reagent (Invitrogen, #15596018) and then reverse transcribed to cDNA with the QuantiTect Reverse Transcription Kit (QIAGEN, #205311) following manufacturer's instructions. qPCR was performed with the iTaq universal SYBR Green supermix (Bio-rad, #1725122) on an Applied Biosystems quantitative PCR system with StepOne software. mRNA levels were normalized to the geometric mean of *Gapdh* and *Actb* levels for each sample and 2 technical replicates were averaged for each biological replicate. Primers are listed in SI Appendix (Table S3).

JC-10 assay

The mitochondrial membrane potential in WT and *Fmr1* KO aNSCs was determined by JC-10 Mitochondrial Membrane Potential Assay Kit (Abcam), following the manufacturer's protocol. Briefly, aNSCs were stained with JC-10 solution for 30 min at 37°C. After adding buffer B, aNSCs were imaged using an A1RSi confocal microscope system (Nikon) with a 20X objective. At least 50 cells were randomly selected from each cell line and the fluorescent intensities of F520 and F590 were measured after subtracting background pixel intensity in the same image using Image J software (NIH). The average intensity from each cell line (at least 50 cells) was counted as N=1 for statistical analysis. The mitochondrial membrane potential was assessed by quantifying the ratio between red fluorescence (590 nm) and green fluorescence (520 nm) intensities.

Oxygen consumption rate (OCR) analysis

OCR analysis was performed using the Seahorse XF Cell Mito Stress Test Kit and an Agilent Seahorse XF-24 Analyzer based on the protocol provided by the vendor. Briefly, we plated $1x10^4$ aNSCs per well in a 96-well plate. At 24 hours post-plating, we changed the medium to assay medium provided in the kit and preincubated for 1 hour before the assay. The next steps were performed following the protocol provided in the kit without alteration. The data were generated and analyzed by the XF-24 analyzer.

Quantification and statistical analysis

Statistical details of each experiment are included in the figure legends. Statistical analysis was performed using ANOVA and Student's t test, unless specified, with the Graphpad software. Two-tailed and unpaired t-test was used to compare two conditions. Two-way ANOVA was used for comparison among multiple experimental conditions. Tukey post hoc tests were used when comparing among each condition. For *Ndn* mRNA stability analysis, two-way ANOVA was used for comparison of the different decay rates. All data were shown as means± SEM. Probabilities of $p<0.05$ were considered as significant. For cell culture experiments, independently isolated cells served as biological replicates and 3 independent biological replicates were used (N = 3) for statistical analyses. A permutation test was performed in R to estimate the false discovery rate (FDR) under the selected cut-offs (absolute fold changes > 1.2 and nominal p-value < 0.05) for identifying DEGs. Hypergeometric tests with Bonferroni correction were performed in R for analyses of overlaps of gene groups. Variance of genomic data is shown in box and whisker

plots. Wilcoxon rank sum tests with Bonferroni correction were performed in R for changes among regulatory groups.

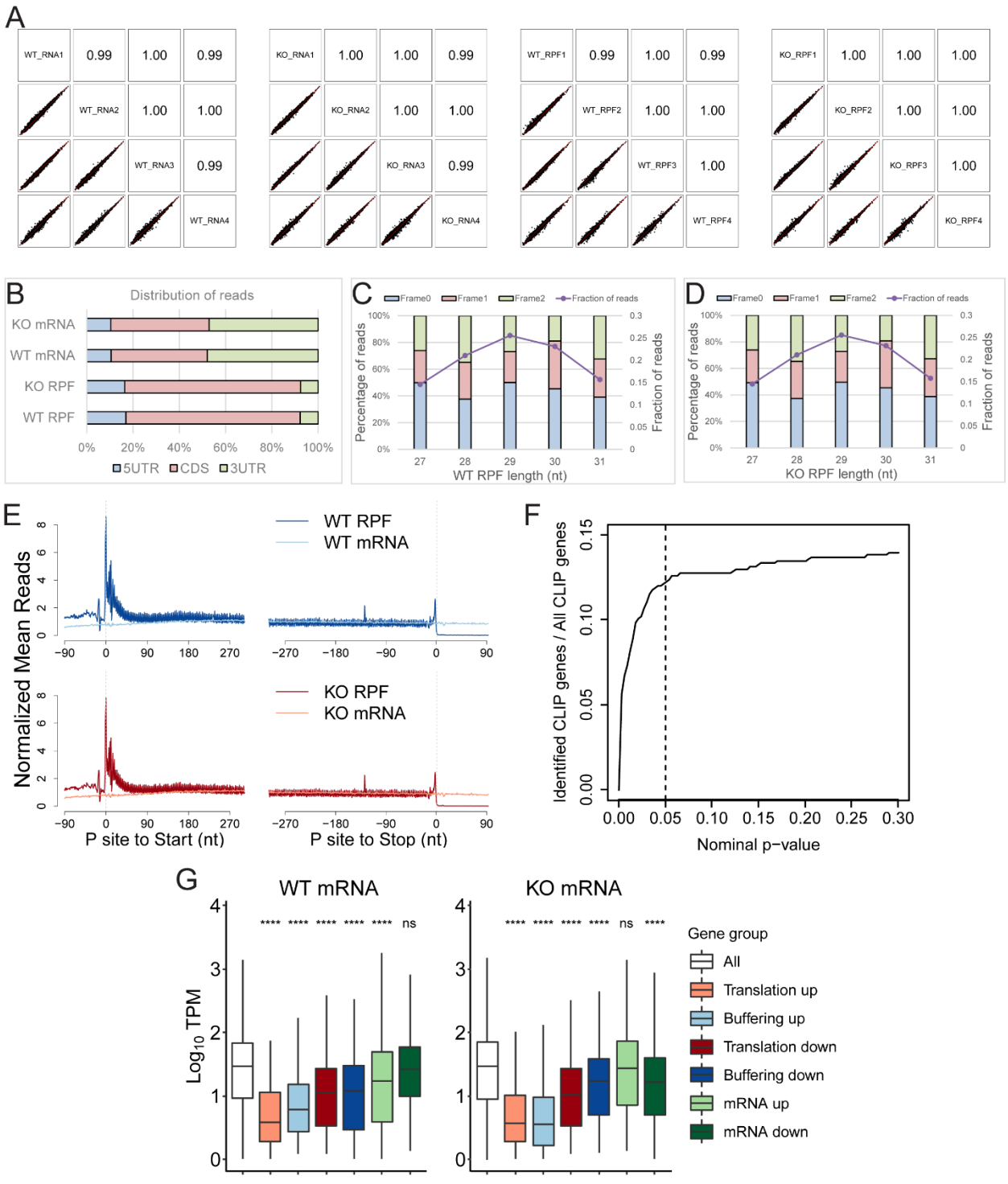
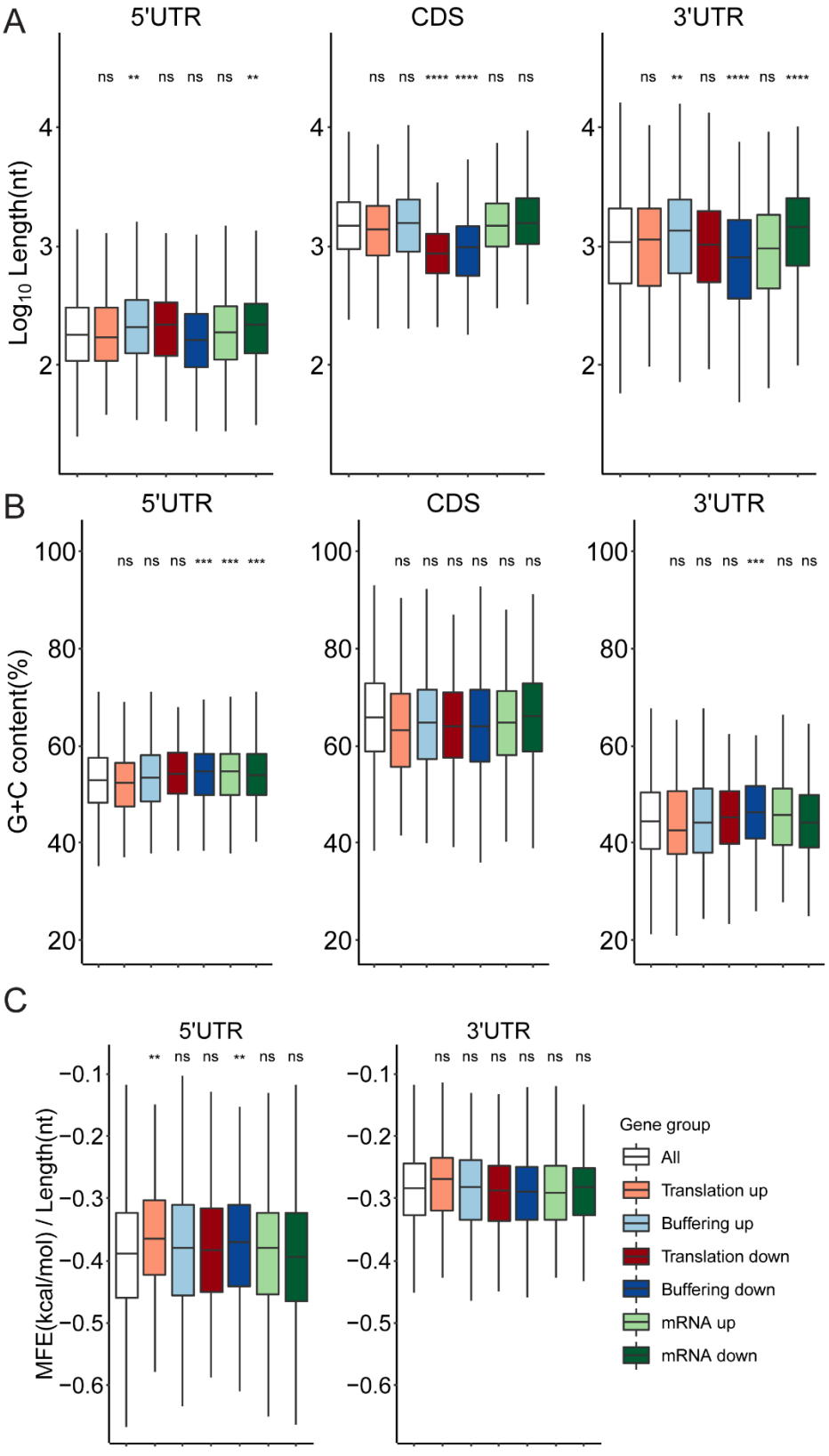


Figure S1. Quality Control of Ribosome Profiling Data. Related to Figure 1.

(A) Scatter plot and correlation coefficient of gene expression (regularized-log transformed counts) between biological replicates to show the high reproducibility.

(B) Distribution of ribosome protected footprints (RPFs) and RNA-seq reads on different mRNA regions. Reads are aggregated from all four biological replicates.
(C) Distribution of RPF length and frame preference in WT aNSCs.
(D) Distribution of RPF length and frame preference in *Fmr1* KO aNSCs.
(E) Metagene plot of RPFs and mRNA reads in WT (upper panel) and *Fmr1* KO (lower panel) aNSCs. Reads are mapped across the entire transcriptome, and aligned at the annotated start and stop codons. The read densities at each nucleotide position are averaged using the P sites of RPFs and 5'ends of mRNA reads.
(F) Plot shows the fraction of CLIP genes identified as "Translation up" or "Buffering up" with an increasing nominal p-value cut-off to determine the optimal p-value cut-off for the differential gene expression analysis.
(G) mRNA expression levels (TPM, transcript per million) in different regulatory groups were compared to those of all genes used for DEG analysis (ns: not significant, **** $p<0.0001$, Wilcoxon rank sum test after multiple test correction with Bonferroni method).


A
5'UTR
CDS
3'UTR
Log10 Length(nt)
B
G+C content(%)
C
MFE(kcal/mol) / Length(nt)
Gene group
All
Translation up
Buffering up
Translation down
Buffering down
mRNA up
mRNA down

1

Figure S2. Features of mRNAs in Different Regulatory Groups. Related to Figure 1.

(A) Boxplots of lengths of 5'UTR, CDS, and 3'UTR in different regulatory groups.

(B) Boxplots of GC contents of 5'UTR, CDS, and 3'UTR in different regulatory groups.

(C) Boxplots of length normalized minimum folding energy (MFE) of 5'UTR and 3'UTR in different regulatory groups. All results were from the comparisons to all genes used for DEG analysis (ns: not significant, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, Wilcoxon rank sum test after multiple test correction with Bonferroni method).

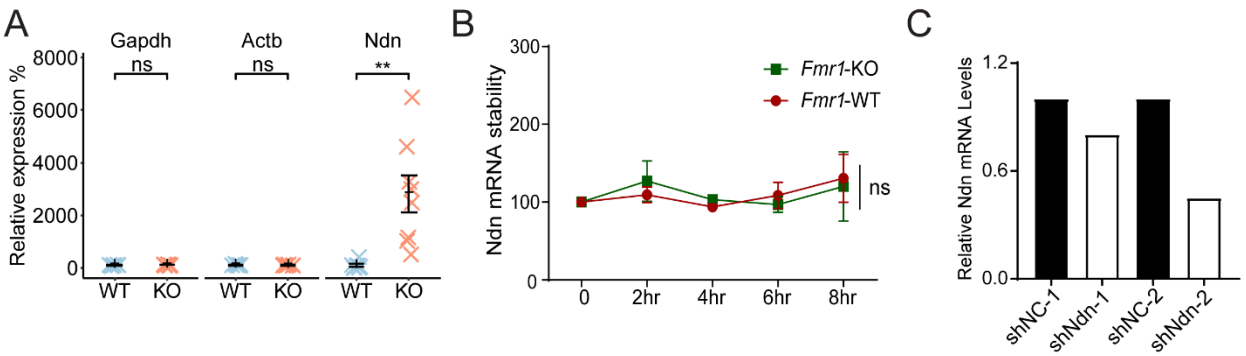


Figure S3. *Ndn* mRNA level and stability in aNSCs. Related to Figure 2.

(A) RT-qPCR validation of *Ndn* mRNA changes. Data are represented as mean± SEM (N=8, ** p<0.01, two-tailed Student's t-test).

(B) *Ndn* mRNA stability in WT and *Fmr1* KO aNSCs treated with actinomycin D to inhibit transcription. The percentage of *Ndn* mRNA remaining in the aNSCs was quantified using qPCR. Comparisons of the different decay rates were performed by two-way analysis of variance (ANOVA) (N=3). ns: not significant. Data are represented as mean ± SEM.

(C) Quantitative analysis of *Ndn* mRNA in WT aNSCs after shRNA knockdown. N=2.

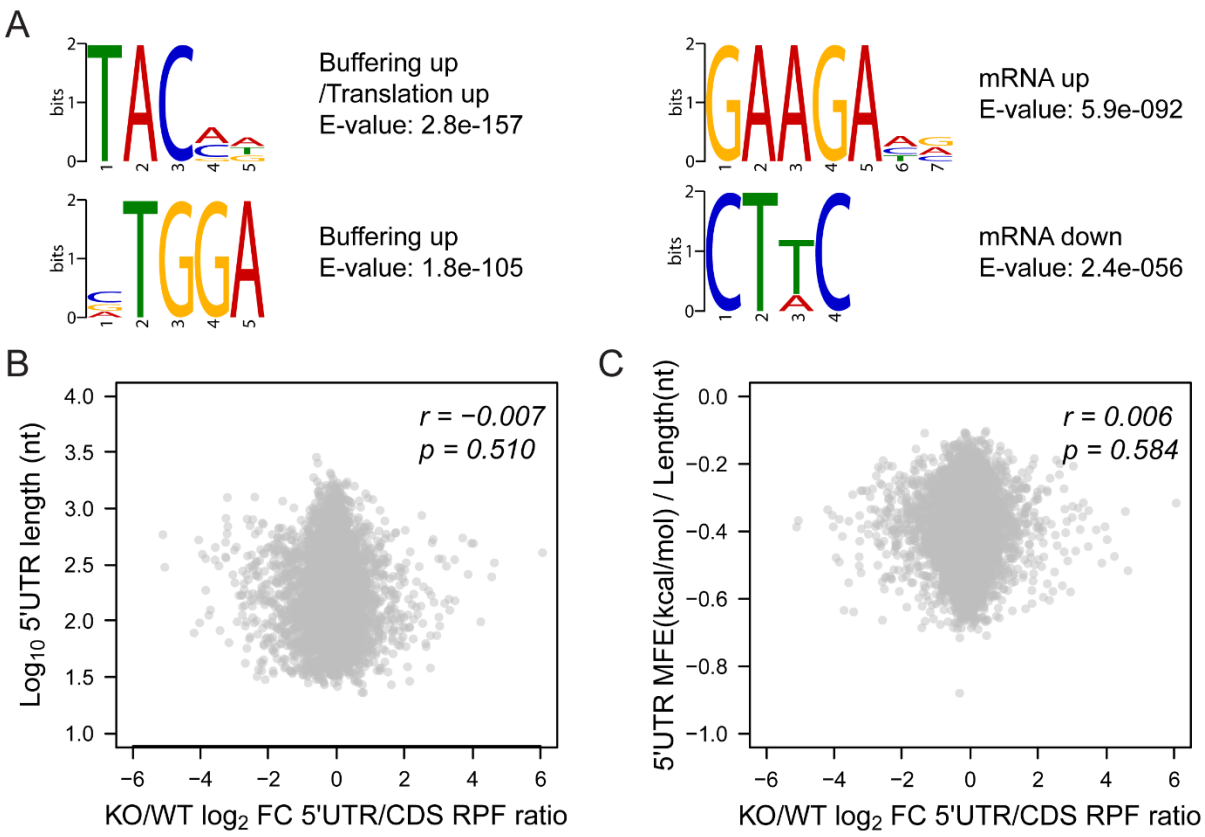


Figure S4. FMRP Binding Motifs and uORF Translation. Related to Figure 3.

(A) Combinatory analysis of FMRP CLIP and ribosome profiling data reveals distinct candidate motifs in different regulatory groups.

(B) Scatter plot with Pearson correlation coefficient and p-value shows a lack of correlation between KO/WT changes of 5'UTR/CDS RPF ratio and the length of the 5'UTR.

(C) Scatter plot with Pearson correlation coefficient and p-value shows a lack of correlation between KO/WT changes of 5'UTR/CDS RPF ratio and the complexity of the 5'UTR.

Table S1. Summary of mapping yields. Related to Figure 1.

Sample	Total Reads	rRNA Reads	rRNA %	tRNA Reads	tRNA %	Uniquely mapped	Uniquely mapped %
WT_RPF_Rep1	37,841,903	8,694,381	22.98	2,912,298	7.70	18,337,209	48.46
WT_RPF_Rep2	41,311,283	10,006,002	24.22	3,140,614	7.60	18,093,937	43.80
WT_RPF_Rep3	27,651,227	5,213,243	18.85	3,271,504	11.83	14,529,114	52.54
WT_RPF_Rep4	31,416,924	5,075,301	16.15	4,438,494	14.13	14,376,247	45.76
KO_RPF_Rep1	42,707,349	9,699,709	22.71	5,490,768	12.86	18,440,087	43.18
KO_RPF_Rep2	50,285,938	11,882,349	23.63	5,465,369	10.87	22,821,986	45.38
KO_RPF_Rep3	31,273,724	5,781,299	18.49	3,945,658	12.62	15,664,530	50.09
KO_RPF_Rep4	31,428,176	5,430,823	17.28	4,378,329	13.93	15,554,931	49.49
WT_RNA_Rep1	21,710,592	6,178,898	28.46	8	0.00	13,438,120	61.90
WT_RNA_Rep2	23,910,876	5,477,893	22.91	13	0.00	16,139,504	67.50
WT_RNA_Rep3	24,733,196	6,045,557	24.44	9	0.00	13,790,886	55.76
WT_RNA_Rep4	24,215,619	5,839,474	24.11	21	0.00	15,240,569	62.94
KO_RNA_Rep1	22,786,198	6,255,282	27.45	6	0.00	14,422,508	63.29
KO_RNA_Rep2	22,336,008	4,676,356	20.94	3	0.00	15,456,899	69.20
KO_RNA_Rep3	22,277,445	5,499,488	24.69	15	0.00	14,241,759	63.93
KO_RNA_Rep4	23,636,135	6,054,969	25.62	16	0.00	15,056,693	63.70

Table S2. Overlaps between genes in each regulatory group and FMRP CLIP genes for the hypergeometric tests. Related to Figure 3.

	Translation up	Others
CLIP	30	800
Non-CLIP	212	11453
Hypergeometric test with Bonferroni correction: p-value = 0.001		
	Translation down	Others
CLIP	4	826
Non-CLIP	250	11415
Hypergeometric test with Bonferroni correction: p-value = 1.0		
	Buffering up	Others
CLIP	72	758
Non-CLIP	506	11159
Hypergeometric test with Bonferroni correction: p-value = 6.186e-08		
	Buffering down	Others
CLIP	6	824
Non-CLIP	596	11069
Hypergeometric test with Bonferroni correction: p-value = 1.0		
	mRNA up	Others
CLIP	30	800
Non-CLIP	484	11181
Hypergeometric test with Bonferroni correction: p-value = 1.0		
	mRNA down	Others
CLIP	63	767
Non-CLIP	615	11050
Hypergeometric test with Bonferroni correction: p-value = 0.005		

Table S3. Oligonucleotides used in this paper. Related to SI Materials and Methods.

Description	**Sequence (5'-3')**
Primer: *Gapdh* Forward	AAGGTCATCCCAGAGCTGAA
Primer: *Gapdh* Reverse	CTGCTTCACCACCTTCTTGA
Primer: *Actb* Forward	TCTTTGCAGCTCCTTCGTTG
Primer: *Actb* Reverse	ACGATGGAGGGGAATACAGC
Primer: *Fmr1* Forward	CGCGGTCCTGGATATACTTC
Primer: *Fmr1* Reverse	TGGAGCTAATGACCAATCACTG
Primer: *Nkx2-2* Forward	GCGACAACCCCTACACTC
Primer: *Nkx2-2* mature Reverse	TCCTTGTCATTGTCCGGTGA
Primer: *Nkx2-2* primary Reverse	GCTTCTTCCCCAAAACTCCC
Primer: *Ndn* Forward-1	CTTGTTCTTTGTATGGGACTGATG
Primer: *Ndn* Reverse-1	TTGACCTTTCTCTTGCTCAGG
Primer: *Ndn* Forward-2	AGGACCTGAGCGACCCTAAC
Primer: *Ndn* Reverse-2	TGCTGCAGGATTTTAGGGTCAAC
shRNA: NC	GGAATCTCATTCGATGCATAC
shRNA: *Ndn*	GCTAACCGTGAAATCACCAAG
Primer: shNdn cloning first round Forward	GAATTCGGATCCGTTAACCAGGAAGAGGGCCTATTTCCCAT
Primer: shNdn cloning first round Reverse	GAAATCACCAAGCTCTTGACTTGGTGATTTCACGGTTAGCCGGATCCTCGTCCTTTCCAC
Primer: shNdn cloning second round Forward	GAATTCGGATCCGTTAACCAGGAAGAGGGCCTATTTCCCAT
Primer: shNdn cloning second round Reverse	CTCCCAAGCTTATCGATACAAAAAAGCTAACCGTGAAATCACCAAGCTCTTGACTTGGTG
Marker: 26nt	AUGUACACGGAGUCGACCCAACGCGA/3Phos/
Marker: 34nt	AUGUACACGGAGUCGAGCUCAACCCGCAACGCGA/3Phos/
Adaptor:	rAppTGGAATTCTCGGGTGCCAAGG/ddC/

RT primer: B is a barcode nucleotide N is a random UMI nucleotide	/5Phos/GGBBBBBNNNNNNNNAGATCGGAAGAGCGTCGTG TAGGGAAAGAGTGT/iSp18/CTCGGCATTCCTGCTGAACCG CTCTTCCGATCTCCTTGGCACCCGAGAATTCCA
Library PCR primer: Forward * indicates a phosphorothioate bond	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTAC ACGACGCTCTTCCGATC*T
Library PCR primer: Reverse	CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCC TGCTGAACCGCTCTTCCGATC*T
rRNA depletion probe: Mm-28S-2685-2722	/5BiotinTEG/TTGGGCGCGCGCCGCGGCTGGACGAGGCGCC GCCGCCCT
rRNA depletion probe: Mm-28S-2927-2933	/5BiotinTEG/AGCGGGCCCCCGGTGGGGCGGGGGGCCCGG ACAC
rRNA depletion probe: Mm-28S-989-1011	/5BiotinTEG/CGGGGCCCGGTGGGGGGCGGGGCGGACTGT
rRNA depletion probe: Mm-28S-1014-1049	/5BiotinTEG/CCAGTGCGCCCCGGGCGTCGTCGCGCCGTCG GGTCC
rRNA depletion probe: Mm-28S-1077-1109	/5BiotinTEG/CGACGAAGCCGAGCGCACGGGGTCGGCGGC GAT
rRNA depletion probe: Mm-28S-449-484	/5BiotinTEG/GCAGTCCGCCCGGAGGATTCAACCCGGCGGC GCGCG

Datasets S1. Summary of differential gene expression analysis. Related to Figure 1.

Datasets S2. Full lists of enriched GO terms in Biological Process. Related to Figure 1.

Datasets S3. Full lists of enriched GO terms in Cellular Component. Related to Figure 1.

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