Supplementary Text

Supplementary Materials and Methods

DMPK Constructs

mRFP-C1 was made by replacing the EGFP fragment in pEGFP-C1 (CLONTECH) with an mRFP fragment that was amplified using NheI-RFP-Fw and BglII-RFP-Rv. A genomic fragment of human *DMPK* covering last 2 exons was amplified by PCR using a primer set of BglII-DMPK-ex15-Fw and XhoI-DMPK-3UTR-Rv, digested by BglII and XhoI, and inserted into the BglII-SalI sites of mRFP-C1. The CTG repeat tract of DMPK was replaced with a SalI restriction site by PCR-mediated mutagenesis using DMPK3UTR-SalI-Fw and DMPK3UTR-SalI-Rv. The resulting vector was designated as mRFP-DMPK3'. Two oligonucleotides, TCGA-(CTG)20-C and TCGAG-(CAG)20, were purchased from SIGMA Genosys. Expansion of an interrupted CTG repeat was done as previously described (9). After expansion of multiple cycles, we obtained interrupted CTG (iCTG) fragments of 40, 120, 480, and 960 repeats. These fragments were inserted into the SalI site of mRFP-DMPK3'. The resulting vector containing iCTG480 was designated as mRFP-DMPK3'-iCTG480 or DM480 for short. The other clones containing different iCTG lengths were named in a similar manner. We confirmed that DM480 can induce foci formation of EGFP-fused MBNL1. To make a DMPK cDNA with a shorter CTG tract, TCGA-(CTG)20-C and TCGAG-(CAG)20 were annealed and directly cloned into the SalI site of mRFP-DMPK3'. We obtained a clone containing CTG18, which was designated as mRFP-DMPK3'-CTG18 (DM18). The structures of RFP-DMPK3'-CTG18 and RFP-DMPK3'-iCTG480 are illustrated in Supplementary Fig.S4. Primer sequences are listed in Supplementary Table S1.

Tpm2-based heterologous minigene

For making heterologous minigenes, *Tpm2* exon1-exon 2 was amplified by PCR using BamHI-Tpm2-ex1-Fw and XhoI-Tpm2-ex2-Rv. From this fragment, a fragment corresponding to 114 nt of exon 1 (starting from the initiation ATG) and subsequent 175 nt region of intron 1 was amplified by PCR using a primer set of BamHI-Tpm2-ex1-Fw and BglII-Tpm2-int1-Rv, digested by BamHI and BglII, and inserted into the BglII site of pEGFP-C1. Similarly, another fragment corresponding to exon 2 and its upstream region was amplified using a primer set of SalI-Tpm2-int1-Fw and XhoI-Tpm2-ex2-Rv, digested by SalI and XhoI, and inserted into the SalI site of pEGFP-C1 that had been fused with the *Tpm2* exon1-intron 1 fragment above. The resulting vector was named EGFP-Tpm2-ex1-2. Alternative exons with flanking regions such as *Clcn1* 451-720 (Fig. 5A) were inserted in the BglII-SalI site of pEGFP-Tpm2-ex1-2.

Rat *Actn1* minigene

An *Actn1* fragment covering exons EF1 to EF2 were amplified from rat genomic DNA by PCR using Actn1-Fw and Actn1-Rv (Supp. Table S1). This fragment was cloned into pEGFP-C1 as described in the case of the *Clcn1* minigene (see Materials and Methods of the main text). Splicing assay of *Actn1* was performed using FITC-GFP-Fw and Actn1-Rv. Because the size of NM-containing PCR product was close to that of SM-containing PCR product, PCR products were treated with NcoI to specifically cleave SM-containing product, resulting in a faster migration of the cleaved fragment. Similar to a previous report (S1), our CUG-BP, ETR-3 and CELF4 recombinant proteins regulated alternative exons of *Actn1* (Supp. Fig. S6B).

Cell culture and transfection

COS-7, Neuro2a, and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum. For minigene assays, cells were typically cultured in 12-well plates and transfected with 0.5 μg of plasmids for protein expression (or cognate empty vector) and 0.01 μg of plasmids for minigene expression using Fugene 6 (Roche) or Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. When multiple plasmids were transfected for protein expression, the total amount of plasmids was adjusted to 0.5 μg in total. In the case of RNAi experiments, when necessary, the culture medium of transfected cells was replaced with DMEM containing blasticidin (1 μg/ml) on the next day of transfection to minimize untransfected cells. Efficacy of RNAi-mediated knockdown for endogenous mRNAs was determined using either qRT-PCR or Western blot analysis.

SDS-PAGE and Western blot analysis

Harvested cells were lysed with RIPA buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 % Triton X-100, 0.5 % sodium deoxycholate, 1mM EDTA (pH 8.0)] supplemented with SDS at the final concentration of 2 % (RIPA2.0) and protease inhibitors (Complete Protease Inhibitor Cocktail, Roche). The lysates were sonicated using Branson Sonifier for 15 seconds at the output setting of seven. Protein concentration of each sample was determined using Micro BCA Protein Assay Reagent (PIERCE Biotechnology). After adjusting the concentration of samples by adding appropriate amount of RIPA2.0, samples were boiled for five minutes. SDS-PAGE was performed using 5-20 % gradient gels (e-PAGEL, ATTO). After electrophoresis proteins were transferred to a PVDF membrane at 150 mA for 1 hour. The membrane was immersed in TBST buffer [150 mM NaCl, 20 mM Tris-HCl (pH7.5), 0.05 % Tween-20] containing 5% skim milk for at

least 30 minutes to reduce non-specific detection and then treated with a primary antibody overnight at 4 ^{o}C. Following TBST wash for five minutes at least three times, the membrane was treated with secondary antibody for one hour at room temperature. After washing with TBST three times, the membrane was exposed to capture protein images by LAS-1000 using ECL (GE Healthcare UK Ltd.) or SuperSignal West Dura Extended Duration Substrate (Pierce). Antibodies were used at the dilution rate indicated as follows; 1:1000 for mouse monoclonal anti-myc (Invitrogen), 1:2000 for rabbit polyclonal anti-GFP (598, MBL), 1:2000 for rabbit monoclonal anti-GFP (Roche), 1:200 for mouse monoclonal anti-CUGBP1 (3B1, Santa Cruz Biotechnology), 1:5000 for mouse monoclonal anti-β-actin (AC-15, SIGMA-ALDRICH), and 1:200 for goat polyclonal anti-LaminB (M-20, Santa Cruz Biotechnology).

Neuro2a-based cell lines stably expressing EGFP-MBNL1$_{40}$

Neuro2a cells were transfected with EGFP-MBNL1$_{40}$ and were cultured in DMEM containing 0.4 μg/ml G418 for one week. Selected cells were sorted by their fluorescent intensity and each cell was separated into a well of 96-well plates. After further selection by 0.4 μg/ml G418, proliferated clones were collected as cell lines. Multiple independent cell lines were established. The expression of EGFP-MBNL1$_{40}$ was confirmed by Western blot using anti-GFP and anti-MBNL1 antibodies. One of cell lines, A6, exhibited relatively high expression of EGFP-MBNL1$_{40}$.

Ribonucleoprotein immunoprecipitation (RIP)

Neuro2A or A6 cells were cultured in 6-well plates and fixed by 1% paraformaldehyde in PBS for 10 minutes, followed by a treatment of 0.25 M glycine for 5 minutes. After washing with PBS three times, cells were collected and frozen. Cells were lysed in

RIPA buffer containing 1x protease inhibitor (Complete Protease Inhibitor Cocktail, Roche), 0.1 u/μl RNase inhibitor (SUPERaseIn, Ambion). Cell lysate was sonicated for 30 seconds six times with intervals of one minute and then fractioned by centrifuge at 16000 x g for 15 minutes. Supernatant fraction was pre-cleared with protein A-conjugated magnetic beads (Dynabeads Protein A, Invitrogen) at 4 ^{o}C for 1.5 hours. A portion of pre-cleared lysate (typically 60 μl of 600 μl lysate) was collected as input. The other portion of pre-cleared lysate was subjected to immunoprecipitation using protein A-magnetic beads pretreated with polyclonal anti-GFP antibody (MBL, 2μl per sample) or rabbit IgG overnight at 4 ^{o}C. Beads were washed 4 times with high-stringent RIPA buffer containing 1 M NaCl, 1 M urea, then reverse-crosslinked in an elution buffer containing 50 mM Tris-HCl (pH 7.4), 20 mM DTT, 1 % SDS, 1 mM EDTA, and 1 x protease inhibitor at 70 ^{o}C for 1 hour. The input fraction was mixed with an equal amount of 2 x elution buffer and reverse-crosslinked together with IP fraction. RNA was purified by phenol/chloroform extraction followed by 2-propanol precipitation. RNA was treated with RNase-free DNase (RQ1, Promega), and subjected to chloroform extraction followed by 2-propanol precipitation. Using purified RNA, cDNA was synthesized by reverse-transcription using Revertra Ace -α-. The amount of co-precipitated RNA fragments was quantified by SYBR green-based qPCR. Primer sequences are listed in Supplementary Table S3.

Supplementary Reference

S1. Gromak N., Matlin A.J., Cooper T.A., Smith C.W. (2003) Antagonistic regulation of alpha-actinin alternative splicing by CELF proteins and polypyrimidine tract binding protein. *RNA*, **9**, 443-456.

Supplementary Figure Legends

Supplementary Fig. S1. MicroRNA-based RNAi in Neuro2a cells.

(**A**) Effects of RNAi vectors against an exogenous N-terminal fragment of murine Mbnl1 or Mbnl2 fused with EGFP. RNAi vectors were co-transfected with either EGFP-Mbnl1-N or EGFP-Mbnl2-N. The expression of EGFP-fused proteins was analyzed by Western blot using an anti-GFP antibody. Anti-actin staining was used as a loading control. (**B**) Endogenous mRNA level of Mbnl1 and Mbnl2 in the Neuro2a cells treated with RNAi vectors. Relative amount of mRNA was quantified by qRT-PCR using primers that can amplify either Mbnl1 or Mbnl2. The amount of Gapdh mRNA was also quantified and used for normalization. Bars represent normalized relative expression levels of Mbnl1 or Mbnl2 as indicated. (**C**) Effects of RNAi vectors against exogenous CELF proteins fused with EGFP. The effects of RNAi vectors are shown as in **A**. (**D**) Endogenous protein level of Cugbp1 in the cells treated with miCugbp1 vectors. Cugbp1 protein level was detected by Western blot analysis using 3B1 monoclonal antibody. Anti-LaminB staining was used as a loading control.

Supplementary Fig. S2. MicroRNA-based RNAi in HeLa cells.

(**A**) Results of splicing assay of the *Clcn1* minigene in HeLa cells. Cotransfected constructs are indicated. Overexpression of MBNL1 reduced inclusion of exon 7A, whereas the knockdown of endogenous MBNL1 increased the inclusion. (**B**) Effects of RNAi vectors against exogenous EGFP-fused MBNL1 or MBNL2 as in Supplementary Fig. S1A. (**C**) Endogenous mRNA level of MBNL1 in the HeLa cells treated with miRNA-based RNAi vectors. Data are shown as in Supplementary Fig. S1B.

Supplementary Fig. S3. Splicing analysis of a minigene containing an artificial ESE.

A variant minigene derived from 6/7 did not respond to MBNL1. The 6/7(ESE) mutant was made by substituting the first 12 nucleotide of exon 7 in the 6/7 minigene with a GAA repeat (Left). Splicing assay results of 6/7(ESE) (Right). MBNL1 did not alter the pattern of splicing of 6/7(ESE). V: empty vector. M: MBNL1-myc.

Supplementary Fig. S4. Structure of DMPK constructs.

A DMPK fragment containing last two exons and an intron between them was inserted in the BglII-SalI restriction sites of mRFP-C1 to make RFP-DMPK3'. Pure CTG18 and interrupted CTG480 were inserted into the SalI site of RFP-DMPK3', producing RFP-DMPK3'-CTG18 (DM18) and RFP-DMPK3'-iCTG480 (DM480), respectively.

Supplementary Fig. S5. A Neuro2a-based cell line stably expressing EGFP-MBNL1$_{40}$.

(**A**) Expression of EGFP-MBNL1$_{40}$ in the cell line A6. Protein expression was verified using polyclonal anti-GFP antibody. (**B**) Splicing pattern of endogenous *Clcn1* in N2a and A6 cells. *Clcn1* was amplified using Clcn1-40Fw and Clcn1-Rv (Supp. Table S1) and was visualized by ethidium bromide staining. (**C**) Immunoprecipitation of EGFP-MBNL1$_{40}$ in RIP analysis. EGFP-MBNL1 was immunoprecipitated by polyclonal anti-GFP antibody, but not by control IgG. EGFP-MBNL1 was detected using monoclonal anti-GFP antibody.

Supplementary Fig. S6. Expression levels of transfected proteins and an analysis of *Actn1*splicing.

(**A**) Expression of transfected MBNL and CELF proteins in COS-7 cells. Overexpressed proteins were detected using anti-myc antibody. (**B**) Splicing assay results of the *Actn1* minigene in Neuro2A cells. Rat *Actn1* minigene was co-transfected with CELF proteins or an empty vector. The splicing patterns and the quantified data are shown. Note that the PCR products were treated with NcoI to facilitate the separation of PCR products.