Mechanistic Determinants of MBNL Activity

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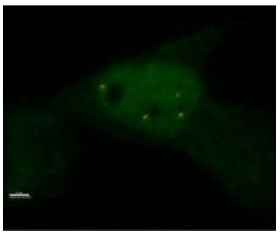
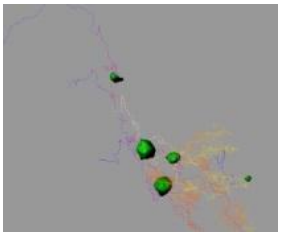
Supplementary Table S1 (.xlsx file). Detailed information about MBNL1, MBNL2 and MBNL3 genetic constructs.

Supplementary Table S2 (.xlsx file). Detailed information about tested AS events.

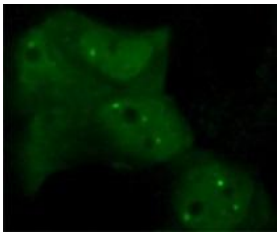
Supplementary Videos



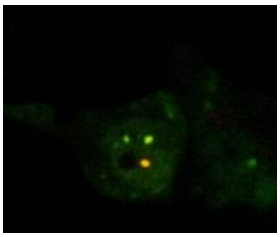
Supplementary Video S1 (.avi file). 16 hours time-lapse sequence of living HeLa cell expressing exogenous MBNL1 protein fused with GFP and CUG^{exp} (part 1).



Supplementary Video S2 (.avi file). 16 hours time-lapse sequence of living HeLa cell expressing exogenous MBNL1 protein fused with GFP and CUG^{exp} (part 2).



Supplementary Video S3 (.avi file). Fluorescence recovery after photobleaching (FRAP) of exogenous MBNL1 protein fused with GFP in CUG^{exp} foci.



Supplementary Video S4 (.avi file). Photoswitching of Dendra2 fluorescence protein fused with MBNL1 in CUG^{exp} foci.

Supplementary Material & Methods

(in this file)

Supplementary Figures

Supplementary Figure S1

Supplementary Figure S1

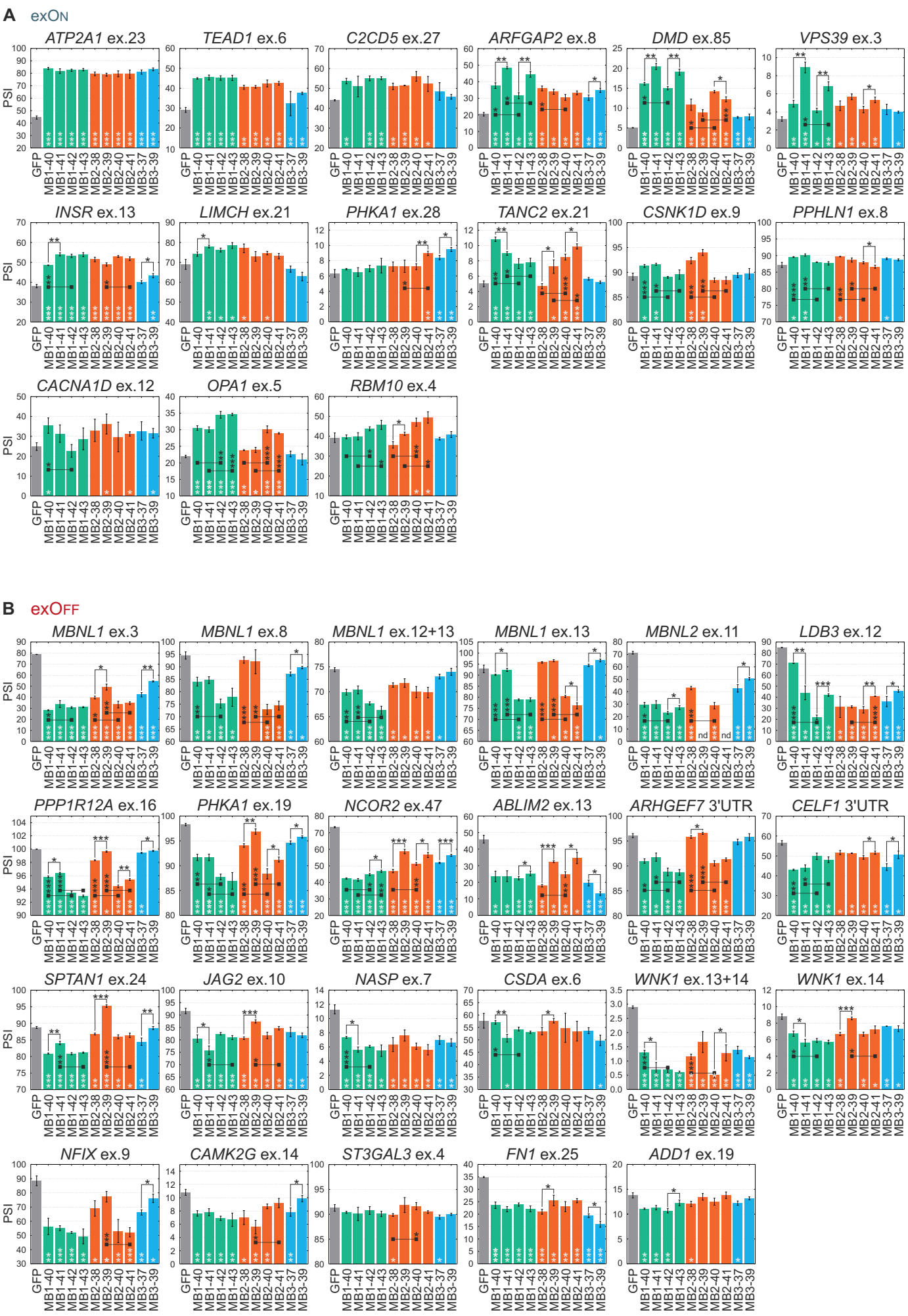
Exonic structure and expression pattern of MBNL paralogs.

(A) The exonic structure of MBNL paralogs. Exon enumeration derived from previous studies (1) and FasterDB (2). For more information see Supplementary Table S1.
(B) Gene expression level of *MBNL* paralogs and *CELF1* in 73 cell lines derived from FasterDB (2).
(C) Amino acid identity of MB1-43 (ex.54nt, ex.36nt and ex.95nt), MB2-40 (ex.54nt and ex.95nt) and MB3-39 (ex.36nt and ex.95nt) proteins. Zinc fingers, ex.54nt, ex.36 and ex.95 are highlighted on gray, blue, red and orange respectively. Various amino acids are highlighted.
(D) The level of exogenous MBNL proteins. Exogenous proteins were detected by anti-GFP antibody and normalized to GAPDH. Bars represent average expression level ± SD from two independent biological experiments.

References

(1) Pascual M, Vicente M, Monferrer L, Artero R. The Muscleblind family of proteins: an emerging class of regulators of developmentally programmed alternative splicing. Differentiation. 2006;74:65-80. doi:10.1111/j.1432-0436.2006.00060.x
(2) Mallinjoud P, Villemin JP, Mortada H, Polay Espinoza M, Desmet FO, Samaan S, et al. Endothelial, epithelial, and fibroblast cells exhibit specific splicing programs independently of their tissue of origin. Genome Res. 2014;24:511-521. doi: 10.1101/gr.162933.113.

Supplementary Figure S2



Supplementary Figure S2

The effect of 10 different MBNL isoforms on endogenous AS events.

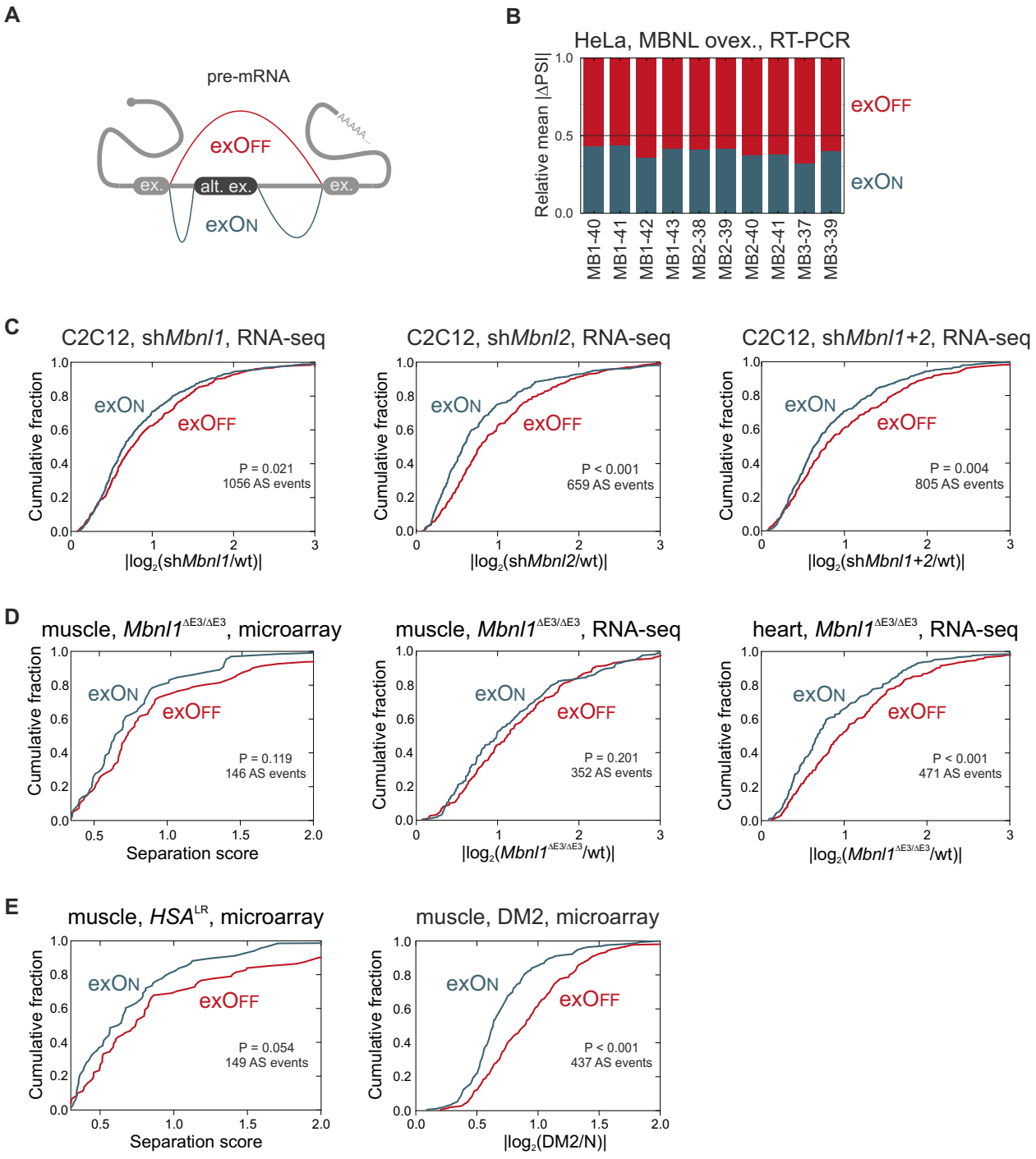
HeLa cells were transfected with 10 MBNL constructs. Splicing pattern of 38 AS events were analyzed by RT-PCR. To standardize alternative exon enumeration we applied numbering from FasterDB (1). For more information see Supplementary Table S2. As a measure of AS changes we used the PSI parameter.

(A) Splicing pattern of exons positively regulated by MBNL proteins (exON) and **(B)** exons negatively regulated by MBNLs (exOFF). Bars represent average PSI ± SD from three independent experiments and statistical significance was determined by the Student's t-test (∗ $P < 0.05$, ∗∗ $P < 0.01$, ∗∗∗ $P < 0.001$). White stars refer to statistical difference between particular MBNL isoforms and GFP control. Black stars refer to statistical differences between comparable MBNL isoforms having or not sequence encoded by alternative ex.54nt (matched by horizontal lines on bars), ex.36nt and ex.95nt (matched above bars).

References

(1) Mallinjoud P, Villemin JP, Mortada H, Polay Espinoza M, Desmet FO, Samaan S, et al. Endothelial, epithelial, and fibroblast cells exhibit specific splicing programs independently of their tissue of origin. Genome Res. 2014;24:511-521. doi: 10.1101/gr.162933.113.

Supplementary Figure S3



Supplementary Figure S3

Higher impact of MBNL proteins on alternative exon exclusion (exOFF) than on exon inclusion (exON).

(**A**) Schematic representation of exOFF and exON.

(**B**) Comparison of relative mean for exON/exOFF splicing changes (|ΔPSI|) of 38 endogenous AS events in HeLa cells transfected with 10 different MBNL isoforms. Global analysis of exON and exOFF changes in (**C**) C2C12 with silencing of *Mbnl* paralogs determined by RNA-seq (1), (**D**) heart and skeletal muscles of *Mbnl1*-knockout mouse (*Mbnl1*$^{\Delta E3/\Delta E3}$) determined by RNA-seq and microarray (1,2), (**E**) skeletal muscles of DM1 mouse model (*HSA*^{LR}) and DM2 patients analyzed by microarrays (2,3). For all analyses the fold change was used as a measure of splicing strength for the indicated number of AS events. The statistical significance was determined by Mann-Whitney U test. All comparisons revealed higher average changes for exOFF than exON.

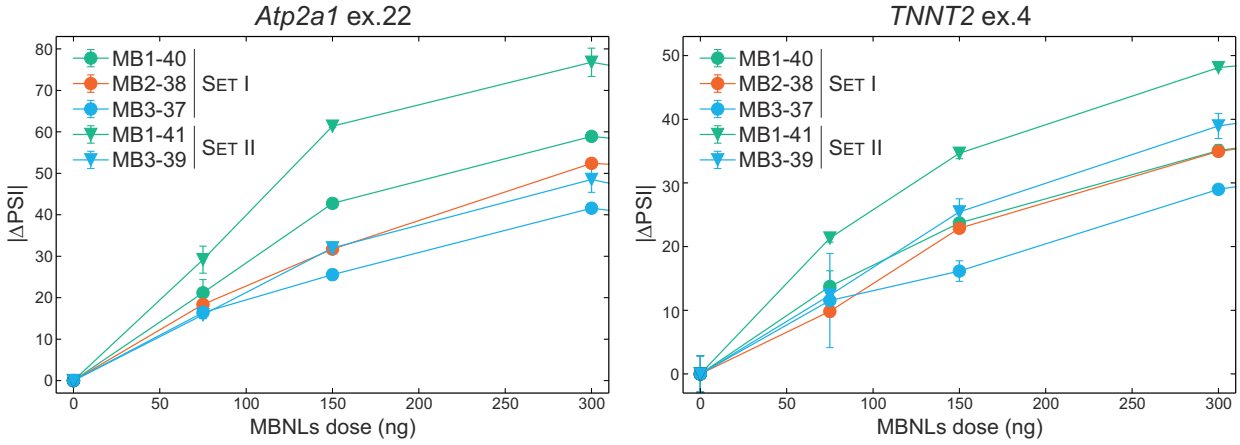
References

(1) Wang E, Cody N, Jog S, Biancolella M, Wang T, Treacy D, et al. Transcriptome-wide regulation of pre-mRNA splicing and mRNA localization by muscleblind proteins. Cell. 2012;150:710-724. doi: 10.1016/j.cell.2012.06.041.

(2) Du H, Cline M, Osborne R, Tuttle D, Clark T, Donohue J, et al. Aberrant alternative splicing and extracellular matrix gene expression in mouse models of myotonic dystrophy. Nat Struct Mol Biol. 2010;17:187-193. doi: 10.1038/nsmb.1720.

(3) Nakamori M, Sobczak K, Puwanant A, Welle S, Eichinger K, Pandya S, et al. Splicing biomarkers of disease severity in myotonic dystrophy. Ann Neurol. 2013;74:862-872. doi: 10.1002/ana.23992.

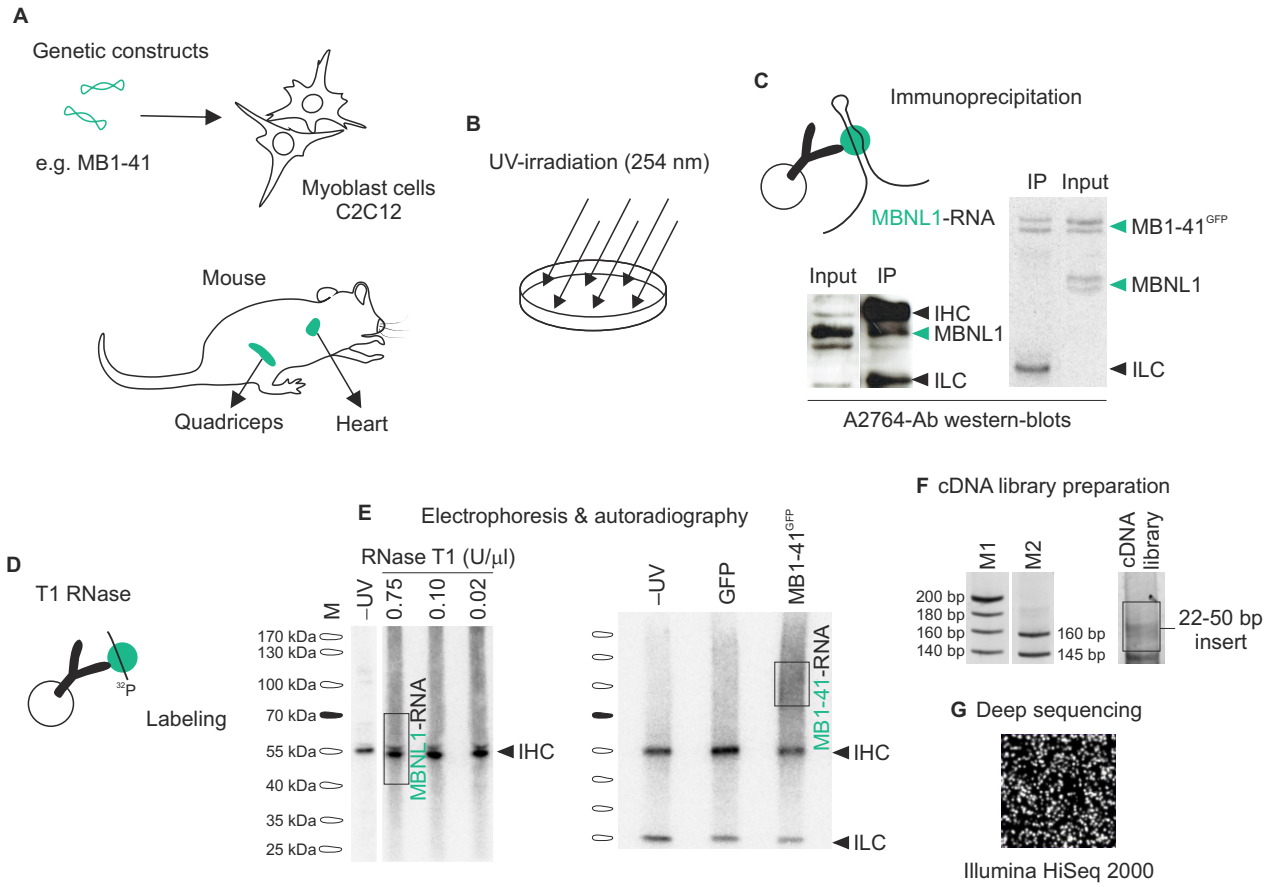
Supplementary Figure S4



Activity of different MBNL proteins from SET I and SET II on *Atp2a1* ex.22 and *TNNT2* ex.4 alternative splicing.

Bars represent average PSI ± SD from two independent experiments.

Supplementary Figure S5



CLIP-seq procedure for endogenous and exogenous MBNL1 study.

(A) C2C12 cells were transfected with exogenous MB1-40, MB1-41 and MB1-43 (Figure 1A). Heart and quadriceps were harvested from C57BL/6J mouse. Muscles were cut into thin slices.

(B) C2C12 and muscle slices were UV-irradiated.

(C) For endogenous MBNL1 and exogenous MBNL1 immunoprecipitation highly specific A2764 and anti-FLAG antibodies were used, respectively (IHC, ILC – Ig heavy/light chains).

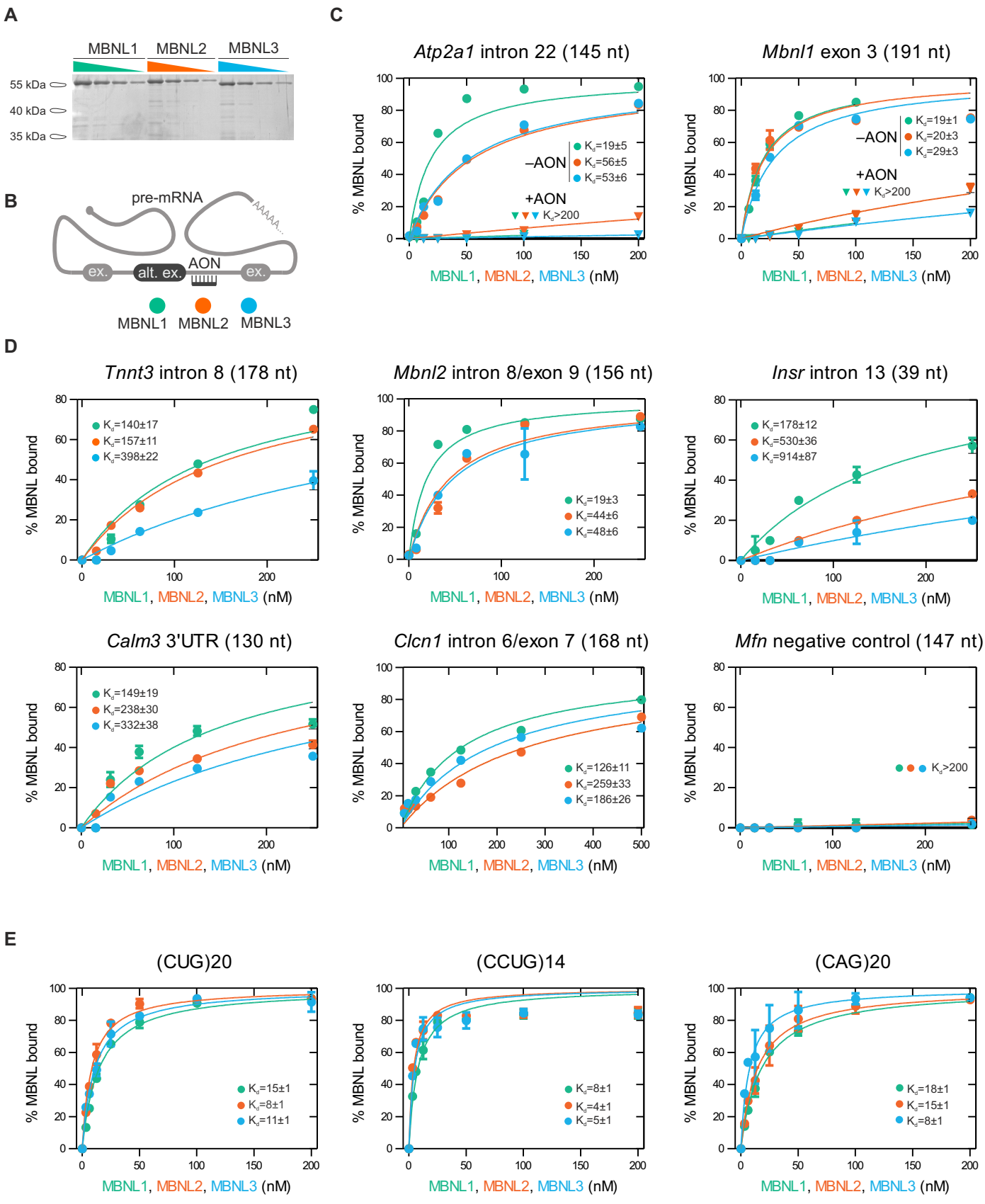
(D) Fixed complexes were treated with T1 RNase and RNA were radioactively labeled on 5' end.

(E) Complexes were separated on denaturing polyacrylamide gel and electrotransferred on membrane. After autoradiography RNA fragments were isolated from selected membrane part. M – ladder.

(F) cDNA libraries were prepared and **(G)** sequenced on Illumina HiSeq 2000.

For more information see Supplementary Material & Methods.

Supplementary Figure S6



Supplementary Figure S6

Comparison of affinity of three MBNL paralogs to selected RNA fragments.

(A) MBNL1, MBNL2 and MBNL3 recombinant proteins quantification on polyacrylamide gel.

(B) Experimental strategy of antisense oligonucleotide (AON) usage for masking the MBNL-binding sites.

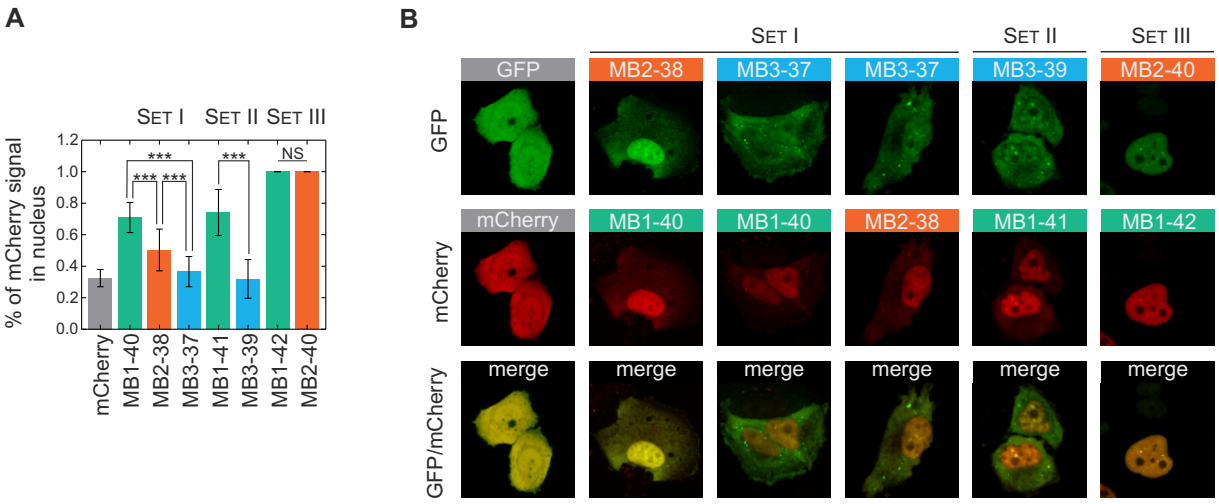
(C–E) Results of *in vitro* filter binding assays. For more details see Supplementary Material & Methods.

(C) Dissociation constants (K_d) of two selected RNA fragments, *Atp2a1* intron 22 and *Mbnl1* exon 3, significantly increase after blocking the MBNL1-specific binding site by short antisense oligonucleotide (+AON) compare to control experiments (−AON). For RNA and AON sequences see Supplementary Material & Methods.

(D) Different K_d for interaction between MBNL paralogs and selected RNA fragments known to be a target for MBNLs. Fragment of *Mfn* transcript was used as a negative control.

(E) MBNL paralogs have very high affinity to transcripts consisting of CUG, CCUG and CAG repeats.

Supplementary Figure S7

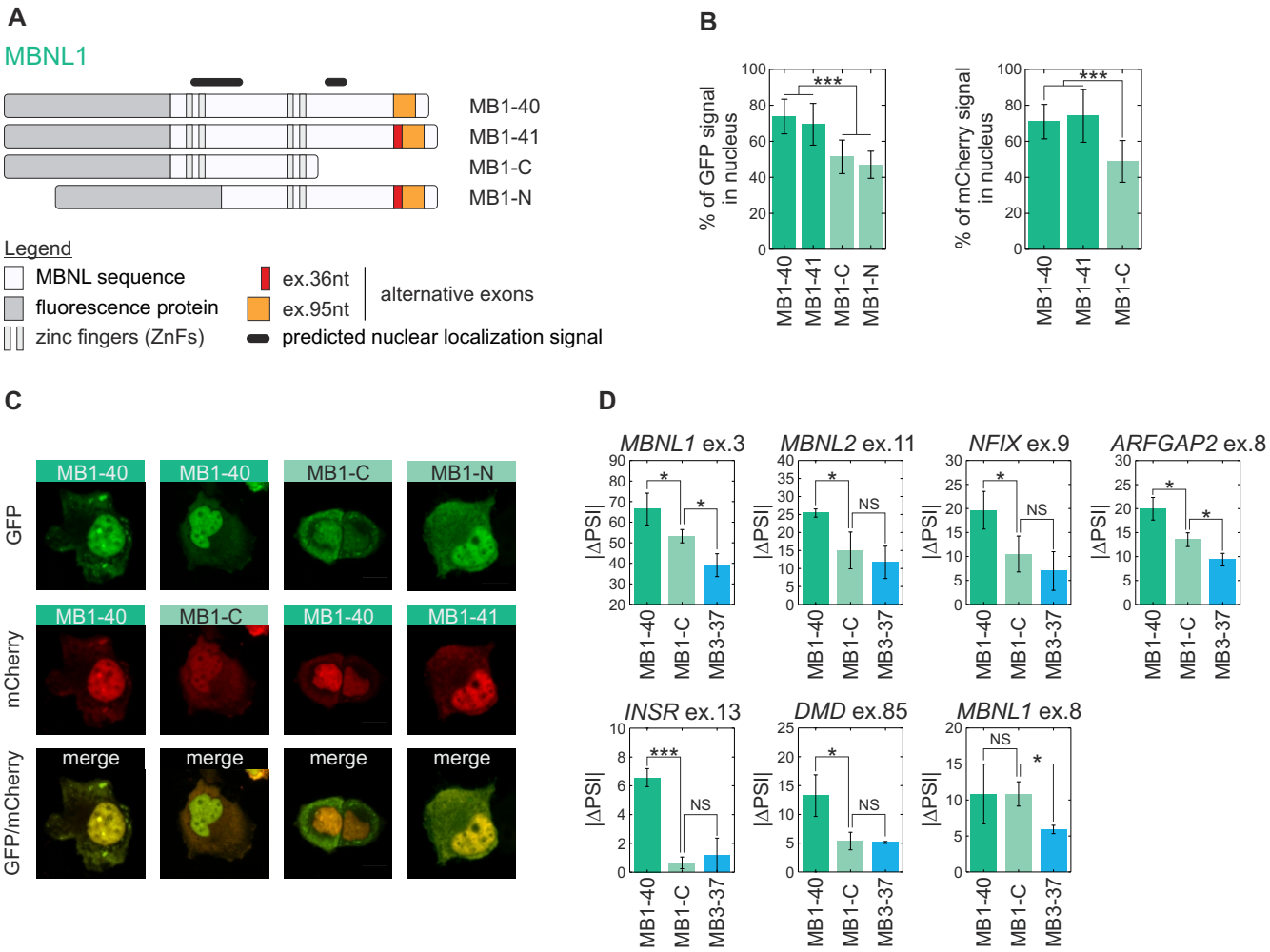


Distinct localization of MBNL paralogs in nucleoplasm and cytoplasm.

(A) The percent of nucleoplasmic signal for mCherry fused MBNL paralogs belonging to SET I, SET II and SET III were determined by quantitative confocal microscopy analysis of mCherry fluorescence signal. Bars represent mean from entire volume of about 50 cells. Statistical significance was assessed by the Student's t-test (NS for $P \geq 0.05$ and *** for $P < 0.001$).

(B) Distinct nucleoplasmic and cytoplasmic distribution pattern of isoform pairs visualized by coexpression of MBNL proteins fused with GFP and mCherry (SET I and SET II). Isoforms having ex.54nt localize exclusively in the nucleus (SET III). Free GFP and mCherry proteins have an equal distribution pattern.

Supplementary Figure S8



Truncated MBNL1s.

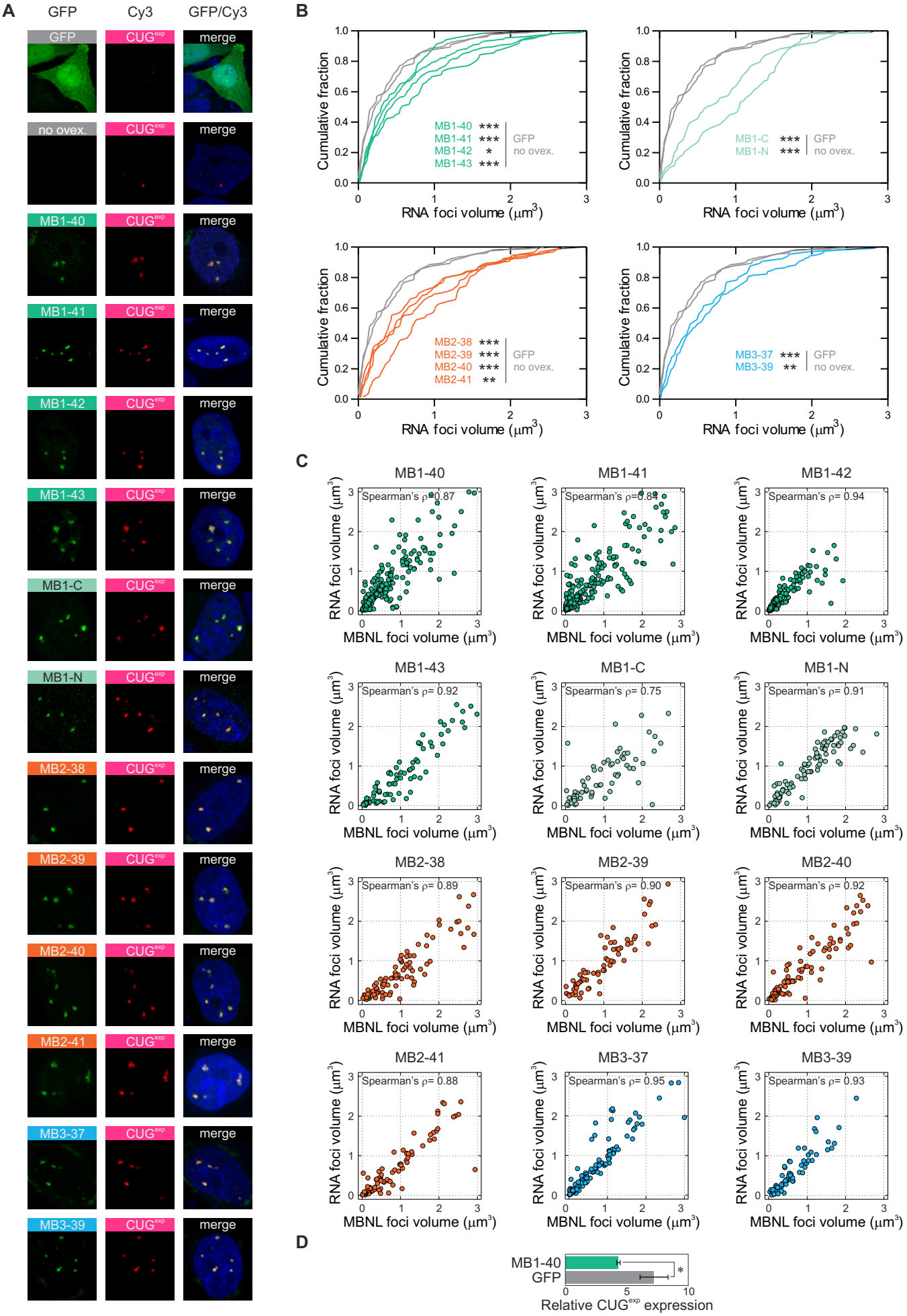
(A) Scheme of expression constructs containing sequences of fluorescence protein (GFP, mCherry) and two full length (MB1-40, MB1-41) and two truncated MBNL1 proteins without C-terminal domain (MB1-C) and without first ZnF tandem domain located on N-terminus (MB1-N). Both, MB1-C and MB1-N lacked predicted nuclear localization signals.

(B) Reduced nucleoplasmic distribution of both MB1-C and MB1-N compared to full length MBNL1 isoforms. Cellular distribution was determined by a quantitative confocal microscopy analysis of GFP and mCherry fluorescence signal. Bars represent means and standard deviations derived from analysis of about 100 cells. Statistical significance was assessed by the Student's t-test (NS for $P \geq 0.05$ and *** for $P < 0.001$).

(C) Distinct nucleoplasmic and cytoplasmic distribution pattern visualized by coexpression of MBNL isoforms fused with GFP and mCherry.

(D) For seven AS events the MB1-C reveals similar splicing activity to MB3-37 than MB1-40. Selected AS events in previous analysis had significantly different |ΔPSI| values for MBNL1 and MBNL3. Represented means and standard deviations were derived from three biological replicas. Statistical significance was assessed by the Student's t-test (NS for $P \geq 0.05$ and *** for $P<0.001$).

Supplementary Figure S9



Supplementary Figure S9

MBNL proteins influence the CUG^{exp} foci volume.

Combined analysis of signals from RNA foci visualized by fluorescent *in situ* hybridization (FISH, Cy3 labeled probe) and GFP signal.

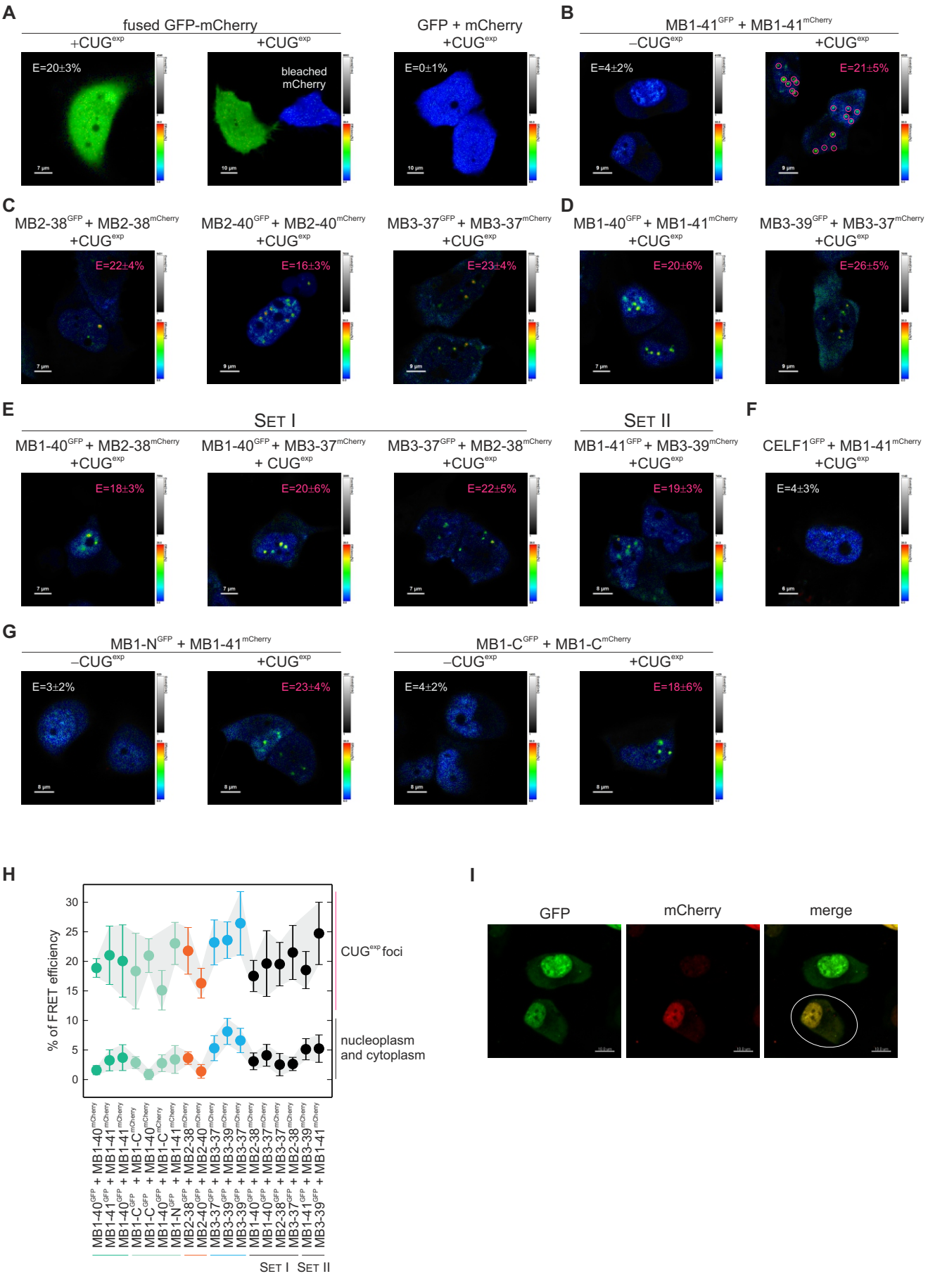
(A) All tested MBNL isoforms colocalize with CUG^{exp} in discrete foci.

(B) Overexpression of MBNL paralogs significantly increase CUG^{exp} foci volume measured by FISH for ~130 foci. There is no difference in CUG^{exp} foci volume distribution between cells transfected with GFP and without protein overexpression (no ovex.). Statistical significance was assessed by Mann-Whitney U test ($*$ for $P < 0.05$, $**$ for $P < 0.01$ and $***$ for $P < 0.001$).

(C) Correlation between RNA foci volume with the volume of MBNL isoforms (measured as GFP signal). The Spearman's ρ is between 0.75 to 0.95. FISH and GFP fluorescence measurements were performed using quantitative confocal microscopy.

(D) Exogenous CUG^{exp} transcript expression/stability is not elevated after MBNL1 overexpression. HeLa cells were cotransfected with CUG^{exp} encoding transcript with MB1-40 or GFP. Bars represent average expression level ± SD measured by semi-quantitative RT-PCR from two independent experiments. Statistical significance was determined by the Student's t-test ($*$ for $P < 0.05$).

Supplementary Figure S10



Supplementary Figure S10

Comparative analysis of FRET efficiency (E) between GFP and mCherry fused with different MBNL proteins.

(**A**) GFP-mCherry fusion protein with nine amino acid linker show ~20% E and after mCherry photobleaching there is no FRET. No FRET is also observed in cells cotransfected with GFP and mCherry constructs.

(**B**) E value for the pair of full length MB1-41 in CUG^{exp} foci is as high as in positive GFP-mCherry control. In absence of CUG^{exp} E value measured in nucleoplasm or cytoplasm is slightly above background. High E is also observed in foci for (**C**) homologous and (**D**) heterologous pairs of MBNL paralogs as well as (**E**) pairs of MBNL paralogs in SET I and SET II.

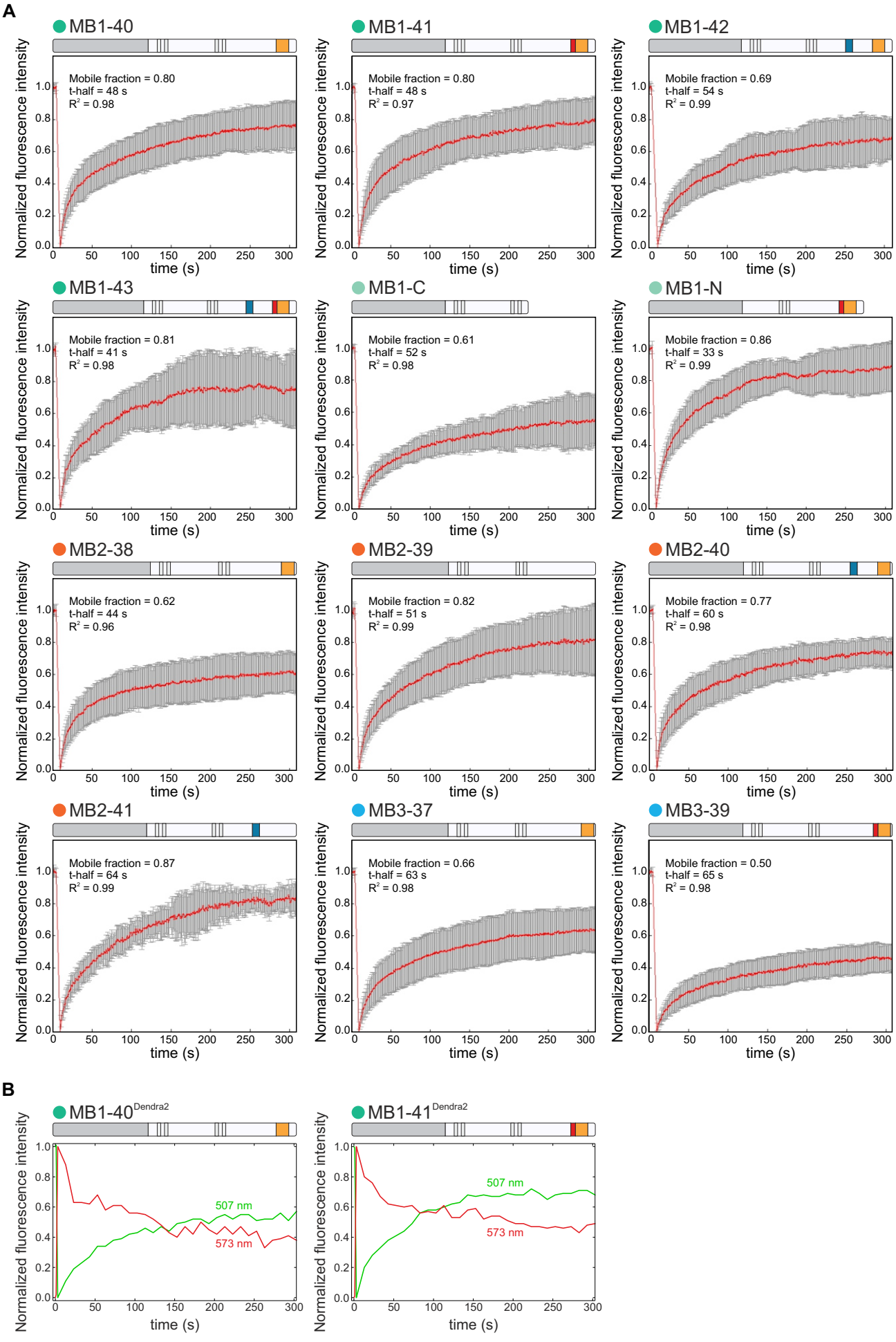
(**F**) There is no FRET in foci of cells cotransfected with MB1-41 and CELF1 which do not colocalize with CUG^{exp}. For this pair of proteins observed E value is as low as in cells without CUG^{exp}.

(**G**) E values for truncated MBNL1 (MB1-C and MB1-N) that are lacking in sequences important for multimerization or without two ZnFs are similar to those observed for full length MBNL proteins.

(**H**) Quantification of data from all experiments. Represented means and standard deviations were derived from 10-20 FLIM measurements.

(**I**) Coexpression of MBNL proteins fused with GFP and mCherry visualized by confocal microscopy. Only selected cells with equal fluorescence emission of both GFP and mCherry labeled MBNL isoforms were used for FRET experiments.

Supplementary Figure S11



Supplementary Figure S11

Differences in mobility of MBNL isoforms between CUG^{exp} foci and nucleoplasm based on fluorescence recovery after photobleaching (FRAP) experiments.

(**A**) All tested MBNL isoforms are mobile, but their mobile fractions are different (0.50-0.87). T-half values are also various (33-65 s). FRAP experiments were performed for ~23 cells for each model. We analyzed a few foci in each nucleus. Raw data were analyzed with easyFRAP software (1). We applied full scale normalization and single exponential fitting equation ($R \geq 0.96$). See Supplementary Video 3.

(**B**) Photoswitching of Dendra2 fused with MBNL1 isoforms. Photoconverted (red, emission 573 nm) protein is passing out whereas unconverted (green, emission 507 nm) protein flow into the foci. Represented results were derived from five independent experiments. See Supplementary Video 4.

References

(1) Rapsomaniki MA, Kotsantis P, Symeonidou IE, Giakoumakis NN, Taraviras S, Lygerou Z. easyFRAP: an interactive, easy-to-use tool for qualitative and quantitative analysis of FRAP data. Bioinformatics. 2012;28:1800-1801. doi: 10.1093/bioinformatics/bts241.

Supplementary

Material & Methods

(A) List of primers for genetic constructs generation.

Primers for MBNL2 constructs generation.

1. GCGAT**GAATTC**GGCTTTGAACGTTGCCCCAGTCA (**EcoRI**, forward for MBNL2)
2. CGTTTCTGC**GGATCC**TTTCAGAATTAT (**BamHI**, reverse for MB2-38, MB2-40)
3. ACTACATCTT**GGATCC**GCATGCAGTT (**BamHI**, reverse for MB2-39, MB2-41)

Primers for MBNL3 constructs generation.

1. GCGAT**GAATTC**GACGGCTGTCAATGTTGCCCTGAT (**EcoRI**, forward for MBNL3)
2. TAACTCTGC**GGATCC**GAATTTCAGCTGAT (**BamHI**, reverse for MBNL3)

Primers for mCherry constructs generation.

1. GCGAT**GCTAGC**ATGGTGAGCAAGGGCGAGGA (**NheI**, forward for MBNL1, MBNL2 and MBNL3)
2. ATCGC**CTCGAG**CCTTGTACAGCTCGTCCATGC (**XhoI**, reverse for MBNL1)
3. ATCGC**AAGCTT**TCTTGTACAGCTCGTCCATGC (**HindIII**, reverse for MBNL2 and MBNL3)
4. GCGAT**AAGCTT**TGGTGAGCAAGGGCGAGGAG (**HindIII**, forward for EGFP-mCherry)
5. ATCGC**GAATTC**CTTGTACAGCTCGTCCATGC (**EcoRI**, reverse for EGFP-mCherry)

Primers for Dendra2 constructs generation.

1. GCGAT**GCTAGC**ATGAACACCCCGGGAATTAA (**NheI**, forward for MBNL1)
2. ATCGC**CTCGAG**CCTGGCTGGGCAGGGGGCTG (**XhoI**, reverse for MBNL1)

Primers for recombinant MBNL2 generation.

1. TCGAT**GGATCC**TCGGCTTTGAACGTTGCCC (**BamHI**, forward)
2. TTTCTG**GAATTC**CCATGACTGTGGCCGCGG (**EcoRI**, reverse)

Primers for recombinant MBNL3 generation.

1. TCGAT**GGATCC**TCGACGGCTGTCAATGTTGC (**BamHI**, forward)
2. TTTCTG**GAATTC**CCATGGCAGAGGCAGCTGAA (**EcoRI**, reverse)

Conditions for all Pfx50 (Invitrogen) PCRs

94°C/2 min; 35 cycles {94°C/15 s; 60°C/15 s; 68°C/45 s} 68°C/5 min.

(B) RNA fragments and oligonucleotides used in filter binding assays.

***Atpta1* intron 22** (mouse)

Primer set for long product amplification (Ta = 58°C)

Forward: GGTGGGATTTTCTCCCAACCT

Reverse: GCTGTATCTCCTTGCGCATC

Primer set for promoter containing template generation:

Forward: TAATACGACTCACTATAGGGCACCCCTGCGGTCACT

Reverse: AGCAGACTGGACGGCCA

Figure 3C and Supplementary Figure S6 *in vitro* transcript (145 nt)

gggcaccccugcggucacuugcgcgcgcucgcagcucucccuggagccacu**gcugcuguugccacugcccgc**uuccacaugagggccaugccguuacugugggcacuggcagcugugaggcuguguccuggccguccagucugcu

Antisense oligonucleotide (AON)

gcgggcagtggcaacagcagc

***Mbnl1* exon 3** (mouse)

Primer set for long product amplification (Ta = 60°C)

Forward: GATGGCTGGCTGCAATATGCC

Reverse: CCTAGGGCAATGGCAGATACTC

Primer set for promoter containing template generation:

Forward: TAATACGACTCACTATAGGTTGGTACTAAGAAGTGCCT

Reverse: CCTAGGGCAATGGCAGATACTC

Figure 3C and Supplementary Figure S6 *in vitro* transcript (191 nt)

GGGUUGGUACUAAGAAGUGCCUUUCCUGACGUCUCUGCUGCUUGGAACCGCUUCUAGAGCAGCCUC**UGCUUUUGCCUUGCUUGCUGC**CAGCUAGACUGACGACAGCACAUCCGCCCUCCACCUCUAGCCCAGACACCCCAUUUCUACUUCUAAUCAGGAGAAAAGCUCUGAGUAUCUGCCAUUGCCCUAGG

Antisense oligonucleotide (AON)

gcagcaagcaaggcaaaagca

***Mbnl2* intron 8/exon 9** (ex.54nt) (mouse)

Primer set for long product amplification (Ta = 60°C)

Forward: TGTGTATGCAAGCTGCTGTT

Reverse: ACAAATCTCGGCTGGTTCTCT

Primer set for promoter containing template generation:

Forward: TAATACGACTCACTATAGGGTTCCCATGATGCACCTCTGC

Reverse: CAGGTCTACAGTTGCTTCGA

Figure 3C and Supplementary Figure S6 *in vitro* transcript (156 nt)

ggguucccaugaugcaccucugcuugcuguuuaccuguauguuaauucgcuugaaucccauuggcccacugcc
aucaugugcucgcugccuguuauuaaaagACUCAGUCGACUGCCAAAGCACUGAAGCG
ACCUCUCGAAGCAACUGUAGACCUG

Figure 3E (**wt**) *in vitro* transcript (92 nt)

gggucguuugaaucccauuggcccacugccaucaugugcucgcugccuguuauuaaaagACUCAGUCG
ACUGUCAAAGCACUGAAGCGACCa

Figure 3E **mut#1** *in vitro* transcript (92 nt)

gggucguuugaaucccauuggcccacugucaucaugugcucgcugccuguuauuaaaagACUCAGUCG
ACUGUCAAAGCACUGAAGCGACCa

Figure 3E **mut#2** *in vitro* transcript (92 nt)

gggucguuugaaucccauuggcccacugccaucaugugaucgaugacuguuauuaaaagACUCAGUCG
ACUGUCAAAGCACUGAAGCGACC

Tnnt3 **intron 8** (mouse)

Primer set for long product amplification (Ta = 60°C)

Forward: GAAGGTAGAGACCGGCTTCG

Reverse: GTCACTGAGGAACCATGCCA

Primer set for promoter containing template generation:

Forward: TAATACGACTCACTATAGGCGTGCCTGCCTCTGCTGTT

Reverse: TTCCTCGTCCTCCTCCCGCTCC

Figure 3C and Supplementary Figure S6 *in vitro* transcript (178 nt)

ggacagcaaguauccaugaaggggaggagaccuaugggaagguguaggagcugcccugccacccucaggcagg
gggcucugugcgugccugccucugcuguuccauguggccacugcugcuguguggccucugcacuucugcagc
ugcguggcuuuuuucuugcaugugcgcuugugc

Calm3 **3´UTR** (mouse)

Primer set for long product amplification (Ta = 60°C)

Forward: GACTGACGGACTCAACCCTG

Reverse: CTGAGCAGGGACAGGAATGG

Primer set for promoter containing template generation:

Forward: TAATACGACTCACTATAGGGACTGAGAATCTGATAAAGC

Reverse: GGAGACTGGGAATGGAAGGG

Figure 3C and Supplementary Figure S6 *in vitro* transcript (130 nt)

GGGACUGAGAAUCUGAUAAAGCACCAAAAGAUUUGUCCCAAGCUGCAU
GACUGCUCUCUCUCCUUCCUCCUGACUGUCCCUCCACGCCCUCACCGCU
UCCUUUGGCCUUCCCCUUCCAUUCCCAGUCUCC

Figure 3E (**wt**) *in vitro* transcript (91 nt)

ggGCACCAAAAGAUUUGUCCCAAGCUGCAUGACUGCUCUCUCUCCUUCC
UCCUGACUGUCCCUCCACGCCCUCACCGCUUCCUUUGGCCCC

Figure 3E (**mut#1**) *in vitro* transcript (91 nt)

ggGCACCAAAAGAUUUGUCCCAAGCUGCAUGACUGGUCUCUCUCCUUCC
UCCUGACUGUCCCUCCACGCCCUCACCGCUUCCUUUGGCCCC

Figure 3E (**mut#2**) *in vitro* transcript (91 nt)

ggGCACCAAAAGAUUUGUCCCAAGCUGCAUGACUGCUCUCUCUCCUUCC
UCCUGACUGUCCCUCCACGCCCUCACCACUUCCUUUGGCCCC

Figure 3E (**mut#3**) *in vitro* transcript (91 nt)

ggGCACCAAAAGAUUUGUCCCAAGGUGGAUGACUGCUCUCUCUCCUUCC
UCCUGACUGUCCCUCCACGCCCUCACCGCUUCCUUUGGCCCC

***Clcn1* intron 6/exon 7** (mouse)

Primer set for long product amplification (Ta = 58°C)

Forward: CCTTTCCATGTTTCCTCCTGTG

Reverse: ACCAAGGTAGGGAGGAAGTG

Primer set for promoter containing template generation:

Forward: TAATACGACTCACTATAGGCCCCCGTTCTTCTGTG

Reverse: AAAGTAGCATCCCACGCCCA

Figure 3C and Supplementary Figure S6 *in vitro* transcript (168 nt)

ggcccccgttcttctgtgcttcctgacacccatccacctggtttacataccacctgtctgtccccctctgccacctgcctcgccc
gtcgtgcttctctgttgcagACCGTGCCTGGGCAGCTTGATCTCCTGGTGCCAGCCTGT
GCAGTGGGCGTGGGATGCTACTTT

***Insr* intron 13** (mouse)

DNA template

GCTCTCCAGCACAGCTGCCCGCCGCATGCAAAAAGCCCTATAGTGAGTCG
TATTA

Primer set for promoter containing template generation:

Forward: TAATACGACTCACTATAGGG

Reverse: GCTCTCCAGCACAGCTG

Figure 3C and Supplementary Figure S6 *in vitro* transcript (39 nt)

gggcuuuuugcaugcggcgggcagcugugcuggagagca

negative control *Mfn* (mouse)

Primer set for long product amplification (Ta = 58°C)

Forward: TGTAGGGCTAGGACTTGGGG

Reverse: GCAGTCAGGGCTCAAAGGTA

Primer set for promoter containing template generation:

Forward: TAATACGACTCACTATAGGGCACCTATCAGTGACCTTTTTC

Reverse: GCAATGTTGGTTAGTAACCC

Figure 3C and Supplementary Figure S6 *in vitro* transcript (147 nt)

gggcaccuaucagugaccuuuuuccuucuaccaagucacuuguggggcuggguacuacuuaucuccuccagcug accuguuuauucuguuuccaugguagcagguguaaggcaugcaggauguuaguaggguuacuaaccaacauu gc

Conditions for all GoTaq Flexi DNA Polymerase (Promega) PCRs

94°C/2 min; 30-32 cycles {94°C/20 s; 55°C/20 s; 72°C/20 s} 72°C/5 min.

(C) Antisense oligonucleotides (AONs).

AONs used in cellular experiments were synthesized by RiboTask.

(AON) 2´-OMePS-*Atp2a1*	GCGGGCAGUGGCAACAGCAGC
(AON) 2´-OMePS-*Nfix*	CCAGCAAGCACAGGCAGCGGG
(AON) 2´-OMePS-*Ldb3*	CAGAAGCAGGCAGCAGCGGGG
(Ctrl.) 2´-OMePS-control	GGAGAAGCAAAAAGCAAGAGA

(D) Step-by-step protocol for Mbnl1-CLIP-seq on mouse tissues.

Procedure was developed based on the original HITS-CLIP protocol (1, 2).

1. Mouse tissue preparation and UV-crosslinking.

- Dissect skeletal muscle (quadriceps) and/or heart from 3-mounth old C57BL/6J buck mouse. Immediately freeze the samples in liquid nitrogen.
- Cut tissue into 60 μm slices and put into cryostat at -23°C. Tissue slices plate on 10 cm culture dishes with 25 ml frozen buffer: 1X HBSS (Gibco, cat no. 14185) and 10 mM HEPES pH 7.3 (BioShop, cat no. HEP003).
- Place the culture dish with frozen buffer directly on UVP CL-1000 device. Crosslink RNA-protein complexes within tissue slices by UV irradiation with 400 mJ/cm^2 at 254 nm.
- Culture dishes with crosslinked biological material should be stored at -80°C.

2. Magnetic bead preparation.

- Resuspend Dynabeads Protein G (Invitrogen, cat no. 10003D) and transfer 50 μl of solution to a fresh 1.5 ml tube.
- Wash beads three times with 100 mM Na-citrate pH 5.0. To separate beads keep tube 1 minute on a magnet (Dynal MPC).
- Resuspend beads in 50 μl 100 mM Na-citrate with 2.5 μl A2764 anti-Mbnl1 antibody (gift from Charles Thornton).
- Mix gently tube at 4°C, O/N.

3. Tissue lysis.

- Thaw buffer with tissue slices at 4°C and transfer to 50 ml conical tube.
- Centrifuge at 400 × g, 10 min, 4°C (Beckman, Allegra X-30R, rotor CO650).
- Discard supernatant, resuspend tissue pellet in 700 μl ice-cold PBS ant transfer to a clean 1.5 ml tube.
- Centrifuge at 400 × g, 10 min, 4°C (rotor F2402H)
- Discard supernatant, resuspend pellet in 400 μl ice-cold lysis & immunoprecipitation buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM EDTA pH 8.0, 0.1% NP-40, 0.05% Tween-20, 1 mM PMSF*, 2 mM benzamidine*). * add fresh.
- Homogenize resuspended pellet in glass Dounce homogenizer using loose (20 moves) and tight (35 moves) pestles respectively. Transfer homogenous suspension to a clean 1.5 ml tube.
- Centrifuge at 18,000 × g, 10 min, 4°C.

- Transfer supernatant to a clean 1.5 tube, add 10 µl of DNase RQ1 (Promega, cat no. M6101) and 10 µl of RNasin (Promega cat no. N2511).

4. Immunoprecypitation.

- Coated beads with A2764 antibody (step 2) wash three times with buffer: 0.1 mM Na-citrate pH 5.0/0.05 % Tween-20. Transfer beads to a clean 1.5 ml tube.
- Add prepared homogenous suspension (step 3) to washed coated beads.
- Rotate 15 rpm on Stuart rotator SB3 (BioCote) for 2 h at 4°C.
- Short spin the tube (Eppendorf MiniSpin) to remove liquid from the cup. Separate beads from the magnet (1 min) and discard supernatant.
- Wash beads five times with 700 µl of PTM buffer (PBS/0.05% Tween-20/1 mM $MgCl_2$). Each time use Stuart rotator, short spin and then separate on a magnet.
- Transfer beads with RNA-Mbnl1 complexes to a clean 1.5 ml tube.

5. T1 RNase digestion.

- Resuspend beads in 50 µl of PTMCa buffer (PBS/0.05% Tween-20/1 mM $MgCl_2$/1 mM $CaCl_2$*) with 0.1 U/µl RNase T1 (Sigma, cat no. R1003). Add 5 µl of DNase RQ1. * RQ1 requires Ca^{2+}.
- Incubate for 20 min. at 25°C in Thermomixer (Eppendorf) with 500 rpm shaking.
- Separate the beads on a magnet, remove buffer, wash the beads with PTE buffer (PBS/0.05% Tween-20/5 mM EDTA pH 8.0), 1 min rotate on Stuart rotator, short spin on centrifuge.

6. BAP dephosphorylating of 3´-RNA edns.

- Prepare 50 µl of dephosphorylation solution: 36 µl H_2O, 5 µl Dephosphorylation Buffer (100 mM Tris-HCl pH 8.0), 4 µl BAP (150 U/µl, Invitrogen cat no. 18011-015), 2 µl Rnasin.
- Add dephosphorylation solution to beads (step 5). Incubate 20 min at 25°C in a Thermomixer with 500 rpm shaking.
- Separate beads on a magnet, remove buffer.
- Wash beads three times with PTE buffer, transfer them to a clear 1.5 ml tube.

7. Radioactive labeling of 5´-RNA ends.

- Prepare the 50 µl reaction solution for radioactive labeling: 32.5 µl H_2O, 5 µl 10× OptiKinase Reaction Buffer (500 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, 50 mM DTT), 2 µl Rnasin , 1 µl [γ-^{32}P]ATP (Hartman Analytics, 5000 Ci/mmol, 10 mCi/ml), 2 µl Opti Kinase (10 U/µl, USB 78334Y). The volume of beads is about 7.5 µl.
- Separate beads on a magnet, remove buffer (step 6).

- Add radioactive labeling reaction solution, incubate 20 min at 25°C in Thermomixer with 500 rpm shaking.
- Add 5 µl of cold ATP (10 mM Invitrogen cat no. 18330-019), incubate additional 5 min.
- Separate beads on a magnet, wash beads two times with PTE buffer, transfer beads to a tube.

8. RNA-protein complexes denaturation.

- Separate beads on a magnet, remove radioactive solution, short spin on centrifuge, again separate beads on a magnet, remove the rest of radioactive solution, and short spin to sit beads on the bottom of the tube.
- Add 31 µl of Sample Loading Buffer (1× NuPAGE MOPS-SDS buffer (cat no. NP0001), 20% glycerol, 2% β-mercaptoethanol, 2% SDS, 1 mM EDTA pH 8.0, 0.002% Briliant Blue), pipet 6-8 times.
- Heat sample at 95°C for 1 min.
- Cool down sample on ice, short spin, separate on a magnet, transfer Sample Loading Buffer that contain denatured RNA-Mbnl1 complexes to a clean tube.

9. Electrophoresis and elektrotransfer.

- Prepare 800 ml 1× MOPS-SDS buffer: 40 ml 20× NuPAGE MOPS-SDS, 760 ml H2O.
- Run the sample on gradient 4-12% NuPAGE gel (cat no. NP0321) in XCell SureLock MiniCell. Use 200 ml of 1× MOPS running buffer with 0.5 ml NuPAGE Antioxidant (NP0005) in upper chamber and 600 ml in lower chamber. Load 3 µl of the PageRuler pre-stained protein size marker (Fermentas, cat no.SM0671).
- Run the gel for 15 min 50V and then ~1h at 200 V.
- Transfer the protein-RNA complexes from the gel to a Whatmann OPTITRAN BA-S 85 nitrocellulose membrane (Sigma cat no. 8418878) using the XCell II Blot Module wet transfer apparatus. Transfer 1 h at 30 V, 300 ml buffer:15 ml NuPAGE Transfer Buffer (20×, cat no. NP0006), 255 ml H_2O, 30 ml methanol, 0.3 ml Antioxidant.
- After the transfer, two times rinse the membrane in PBS buffer, then wrap it in saran wrap and expose it to the screen O/N.

10. Proteinase K digestion.

- Isolate the Mbnl1-RNA complexes of the appropriate size using the autoradiograph as a mask (40-70 kDa). Cut this piece of membrane into several small slices and place them into the 2.0 ml tube.
- Prepare 600 µl Proteinase K (Sigma, cat no. P2308) solution (2 mg/ml). Mix 120 µl Proteinase K stock solution (10 mg/ml) with 180 µl H_2O and 300 µl 2x buffer (2 mM $CaCl_2$, 1% SDS,

200 mM Tris-HCl pH 7.5, 100 mM NaCl). Incubate at 37°C for 30 min at 500 rpm (Thermomixer) to remove RNases.

- Add EDTA pH 8.0 to final 5 mM concentration. Incubate for 3 min.
- Add 300 µl of such prepared 2 mg/ml Proteinase K solution to the nitrocellulose slices. Incubate at 25°C for 30 min at 1000 rpm (Thermomixer).

10. RNA isolation.

- Transfer the solution (without the nitrocellulose) to a new 1.5 ml tube and add 1 ml of Tri Reagent (Sigma, cat no. 93289).
- Add 0.2 ml chloroform and mix.
- Store for 15 min at RT.
- Centrifuge 20,000 × g for 15 min at 4°C.
- Transfer aqueous phase to a clean tube.
- Add 0.5 ml isopropanol and 1 µl glycogen (USB, cat no. 16445) and mix.
- Store at -20°C O/N.
- Centrifuge 20,000 × g for 8 min at 4°C.
- Wash the pellet twice with 1 ml 75% ethanol.
- Centrifuge 7,500 × g for 5 min at 4°C.
- Wash the pellet twice with 0.1 ml 75% ethanol.
- Centrifuge 7,500 × g for 5 min at 4°C.
- Air dry the pellet.
- Dissolve the RNA pellet in 5.5 µl.

11. Library preparation.

Proceed with TruSeq Small RNA Preparation Guide with the modifications below:

- Reduced the volume of PCR to 25 µl.
- Applied 20 cycles.
- From the 6% PAA gel select part from 145 to about 200 bp.

(E) Step-by-step Protocol for MBNL1-CLIP-seq in C2C12 myoblasts.

Procedure was developed based on original iCLIP protocol (3).

1. C2C12 cell transfection.

- Split C2C12 cells on 10 cm culture dish 24 hrs before transfection.
- Transfect cells with using X-tremeGENE HP DNA Transfection Reagent (Roche). Prepare 800 ul of serum- and antibiotic-free DMEM with 16 μg of DNA. Add 48 μl of transfection reagent (1:3 ratio). Incubate 30 min at RT.
- Add the DNA/ transfection reagent mix to 80% confluent C2C12 cells.
- Visualize cells after 24-30 h.

2. UV cross-linking of tissue culture cells.

- Remove the media and add 1 ml ice-cold PBS to cells grown in a 10 cm plate.
- Remove lid and place the dish on a tray with ice. UV cross-link cells with 150 mJ/cm^2 at 254 nm UV Crosslinker (UVP CL-1000).
- Harvest the cells by scraping with a cell lifter.
- Transfer the cell suspension to a 1.5 ml tube. Spin at top speed for 10 sec at 4°C to pellet cells then remove the supernatant.
- Freeze the cell pellet at -80°C until use.

3. Magnetic beads preparation.

- Add 5 μl of protein G Dynabeads per experiment to a clean 1.5 ml tube.
- Wash beads two times with lysis buffer: 50 mM Tris-HCl pH 7.4, 100 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate.
- Resuspend beads in 100 μl of lysis buffer with 1 μl (μg) of FLAG-M2 antibody (Sigma, cat no. F1804).
- Rotate tubes at RT for 60 min.
- Wash three times with 500 μl of lysis buffer and leave in the last wash until ready to proceed to the next step.

4. Cell lysis.

- Resuspend the cell pellet in 0.5 ml of lysis buffer supplemented with fresh 1 mM PMSF and 2 mM benzamidine.
- Spin at 4°C and 15,000 × g for 15 min to clear the lysate. Carefully collect the supernatant (leave about 50 μl lysate with the pellet).

- Add 1 µl TURBO DNase (Ambion, cat no. AM2238) and 12.5 µl Rnasin (for 0.5 ml).

5. Immunoprecipitation.

- Remove the wash buffer from the beads then add the cell lysate.
- Rotate the samples for 2 h at 4°C.
- Discard the supernatant and wash the beads two times with 500 µl of high-salt buffer: 50 mM Tris-HCl pH 7.4, 1 M NaCl, 1 mM EDTA, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate.
- Wash three times with 900 µl wash buffer: 20 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 0.2% Tween-20.

6. Next steps perform according to protocol of Mbnl1 CLIP-seq on mouse tissues.

(F) Step-by-step protocol for Mbnl1-RIP-seq.

1. **Mouse tissue preparation.**
   - Dissect skeletal muscle (quadriceps) from 3-mounth old C57BL/6J buck mouse. Immediately freeze the sample at liquid nitrogen.

2. **Magnetic bead preparation.**
   - Resuspend Dynabeads Protein G (Invitrogen, cat no. 10003D) and transfer 75 µl of solution to a fresh 1.5 ml tube.
   - Wash beads three times with 100 mM Na-citrate pH 5.0. To separate beads keep the tube 1 minute on a magnet (Dynal MPC).
   - Resuspend beads in 75 µl of 100 mM Na-citrate with 3.75 µl A2764 anti-Mbnl1 antibody (gift from Charles Thornton, University of Rochester).
   - Mix gently at 4°C, O/N.

3. **Tissue lysis.**
   - Muscle tissue triturate with liquid nitrogen in chilled mortar.
   - Tissue powder transfer to glass Dounce homogenizer. Add 0.5 ml ice-cold lysis & immunoprecypitation buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM EDTA pH 8.0, 0.1% NP-40, 0.05% Tween-20, 1 mM PMSF*, 2 mM benzamidine*). * add fresh. Homogenize tissue using loose (20 moves) and tight (35 moves) pestles respectively.
   - Transfer the solution to a clean 1.5 ml tube and centrifuge 20,000 × g for 10 min at 4°C.
   - Transfer supernatant to a clear 1.5 ml tube and again centrifuge 20,000 × g for 5 min at 4°C.
   - Add add 10 µl of DNase RQ1 (Promega, cat no. M6101) and 10 µl of RNasin (Promega cat no. N2511).

4. **Immunoprecypitation.**
   - Perform according to protocol of Mbnl1 CLIP-seq on mouse tissues.
   - Separate equal amount of beads to three clear 1.5 tubes during last washing step.

5. **T1 RNase digestion.**
   - Perform according to protocol of Mbnl1 CLIP-seq on mouse tissues with the modification below.
   - Use three different concentrations of T1 RNase: 0.002, 0.01, 0.5 U/µl.

6. RNA isolation.

Add 1 ml of TRI Reagent (Sigma, cat no. 93289) to each tube. Isolate RNA according to protocol of Mbnl1 CLIP-seq on mouse tissues.

7. Library preparation

Proceed with TruSeq Small RNA Preparation Guide with the modifications below.

- Reduced the volume of PCR to 25 μl.
- Applied 25 cycles.
- From the 6% PAA gel select part from 145 to about 300 bp.

(G) CLIP-seq and RIP-seq data analysis.

FASTX Toolkit (link) was used for quality filtering and the removal of redundant sequences in analyzed datasets. Only one copy of each unique read sequence was kept. Filtered read sequences were aligned to the mouse reference genome (mm10, UCSC) using Bowtie (4), allowing for maximum 5 mappings per read. Overlapping reads were merged into clusters and only clusters consisting of at least 3 reads and spanning at least 30 bp were kept. Clusters were annotated by comparing them to Ensembl 73 annotations (5) using custom Python scripts.

In order to generate Mbnl Interactome Browser (MIB), we pooled our sequencing data with public Mbnl1-CLIP-seq dataset (6-8). From MIB, we excluded C2C12 samples with MBNL1 overexpression. MIB was supplemented in CLIP-seq data for Mbnl2 (8-10), Mbnl3 (8, 11) and other non-Mbnl splicing factors, Celf1 (7) and Nova (12).

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