A

>MBNL-AB (280 amino acids)

ME**YPYDVPDYA**GSAVSVTPI***RDTKWLTLEVCREFQRGTCSRPDTECKFAHPSKSCQVENGRVIACFDSLKGRCSRENCKYLHPPPHLKTQLEINGRNNLIQQKNMAMLAQQMQ***
LANAMMPGAPLQPVPMFSVAPSLATNASAAAFNPYLGPVSPSLVPAEILPTAPMLVTGNPGVPVPAAAAAAAQKLM***RTDRLEVCREYQRGNCNRGENDCRFAHPADSTMIDTNDN***
TVTVCMDYIKGRCSREKCKYFHPPAHLQAKIKAAQYQVNQAAAA**PKKKRKVE**

>MBNL-AA (296 amino acids)

ME**YPYDVPDYA**MAVSVTPI***RDTKWLTLEVCREFQRGTCSRPDTECKFAHPSKSCQVENGRVIACFDSLKGRCSRENCKYLHPPPHLKTQLEINGRNNLIQQKNMAMLAQQMQ***
LANAMMPGAPLQPVPMFSVAPSLATNASAAAFNPYLGPVSPSLVPAEILPTAPMLVTGNPGVPVPAAAAAAAQKLM***RTDKWLTLEVCREFQRGTCSRPDTECKFAHPSKSCQVE***
NGRVIACFDSLKGRCSRENCKYLHPPPHLKTQLEINGRNNLIQQKNMAMLAQQMQVNQAAAA**PKKKRKVE**

>MBNL-BB (262 amino acids)

ME**YPYDVPDYA**MAVSVTPI***RTDRLEVCREYQRGNCNRGENDCRFAHPADSTMIDTNDNTVTVCMDYIKGRCSREKCKYFHPPAHLQAKIKAAQYQ***LANAMMPGAPLQPVPMFS
VAPSLATNASAAAFNPYLGPVSPSLVPAEILPTAPMLVTGNPGVPVPAAAAAAAQKLM***RTDRLEVCREYQRGNCNRGENDCRFAHPADSTMIDTNDNTVTVCMDYIKGRCSREKC***
KYFHPPAHLQAKIKAAQYQVNQAAAA**PKKKRKVE**

Key

HA Tag

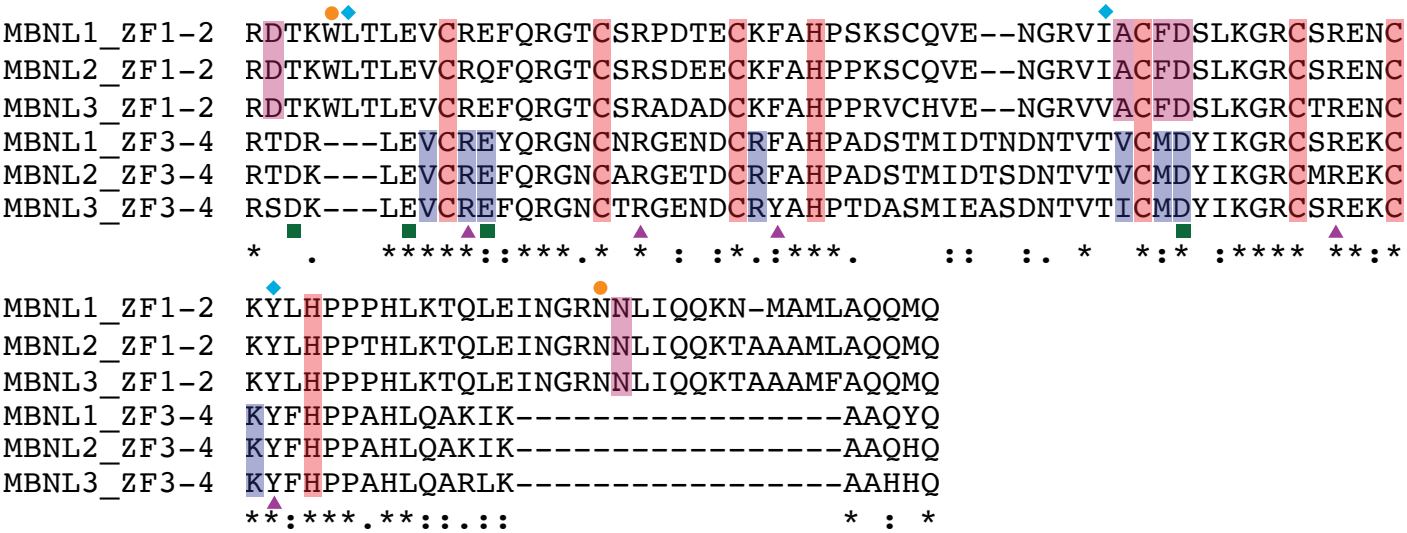
Nuclear Localization Sequence

ZF1-2 domain (93 amino acids)

ZF3-4 doamin (76 amino acids)

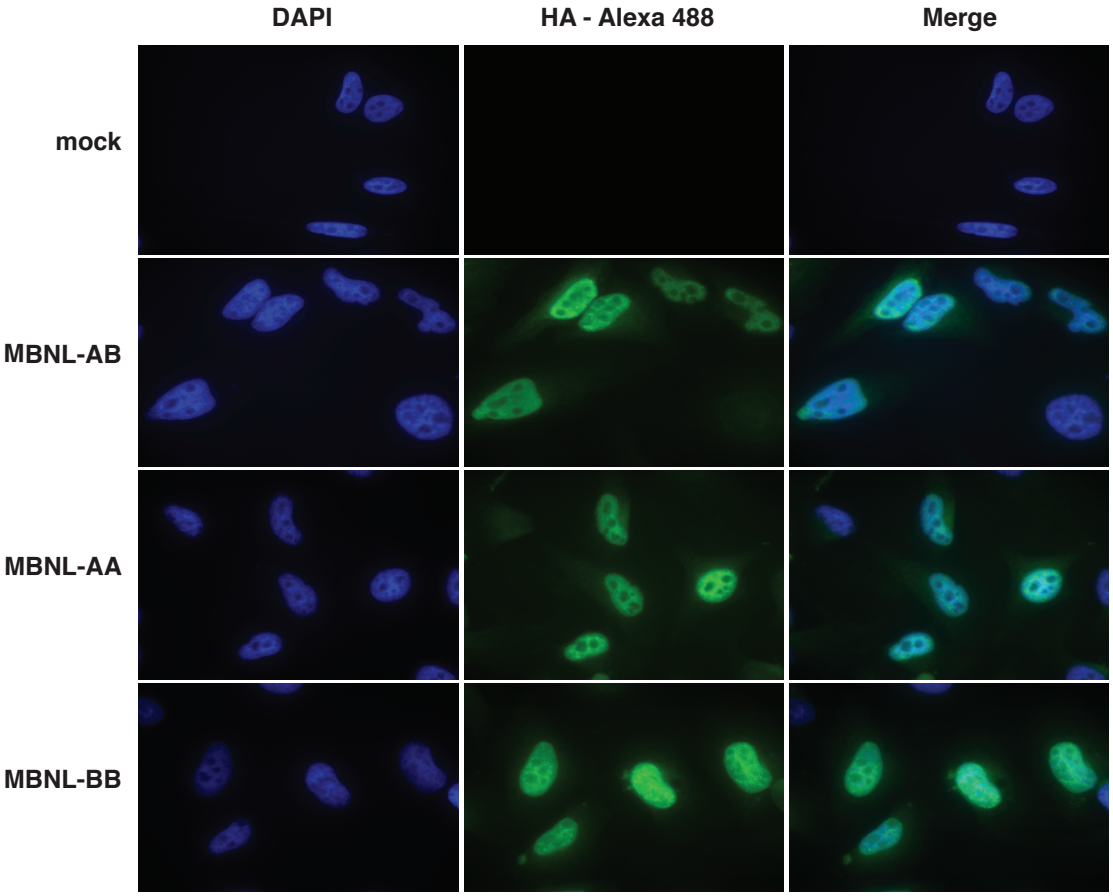
B

C



Supplemental Figure S1 *Synthetic MBNL protein amino acid sequences and MBNL1-3 zinc-finger domain alignments.* (A) Sequences of both MBNL-AB, MBNL-AA, and MBNL-BB proteins are shown. The ZF1-2 domain, ZF3-4 domain, HA tag, and nuclear localization signal (NLS) are highlighted. (B) Sequence alignment of MBNL1 ZF1-2 and ZF3-4 derived using MUSCLE (1). Amino acid residues shown to contact RNA in the crystal structure of ZF3-4 and NMR solution structure of ZF1-2 are highlighted (2, 3). Numbering above and below sequnces cooresponds to ZF1-2 and ZF3-4, respectively. (C) Sequence alignment of the ZF1-2 and ZF3-4 domains within the three human MBNL1 homologs using MUSCLE(1). Amino acid residues shown to contact RNA in the crystal structure of ZF3-4 and NMR solution structure of ZF1-2 are highlighted (2, 3). The protein amino acid sequences used were from the following NCBI accession numbers: NP_066368, NP_659002, and NP_060858 for MBNL1, MBNL2, and MBNL3, respectively.

A



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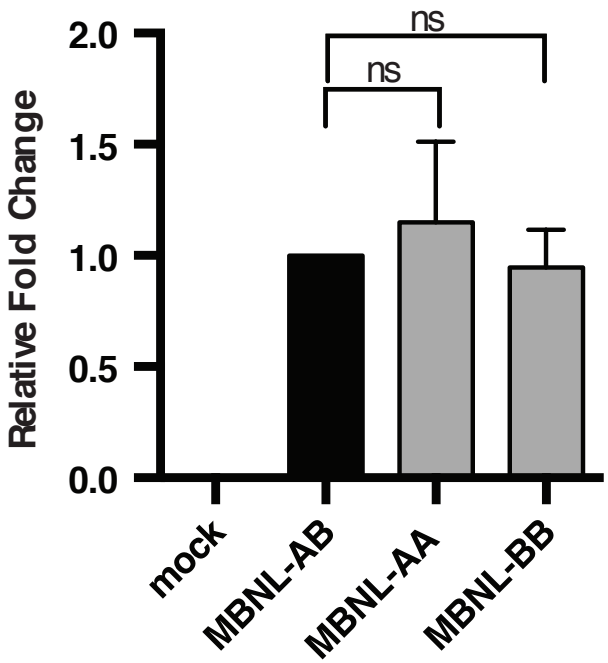
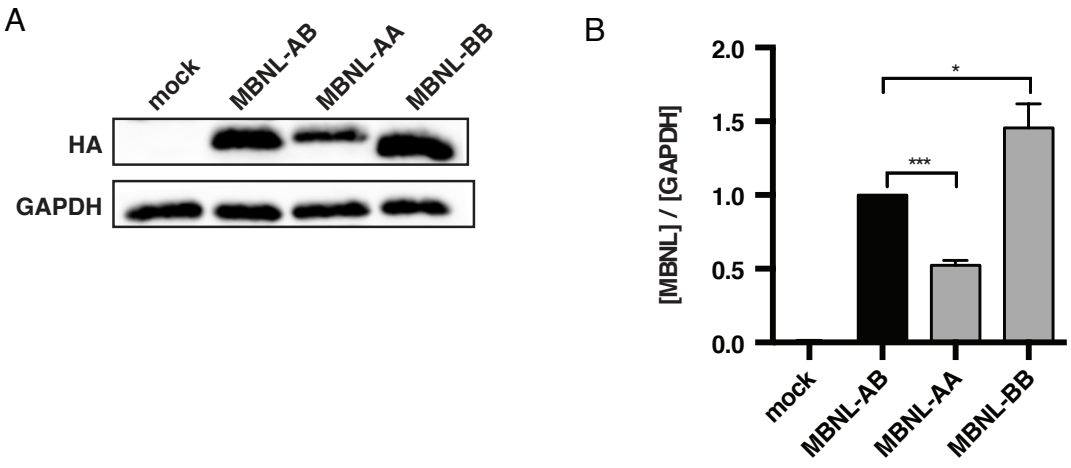
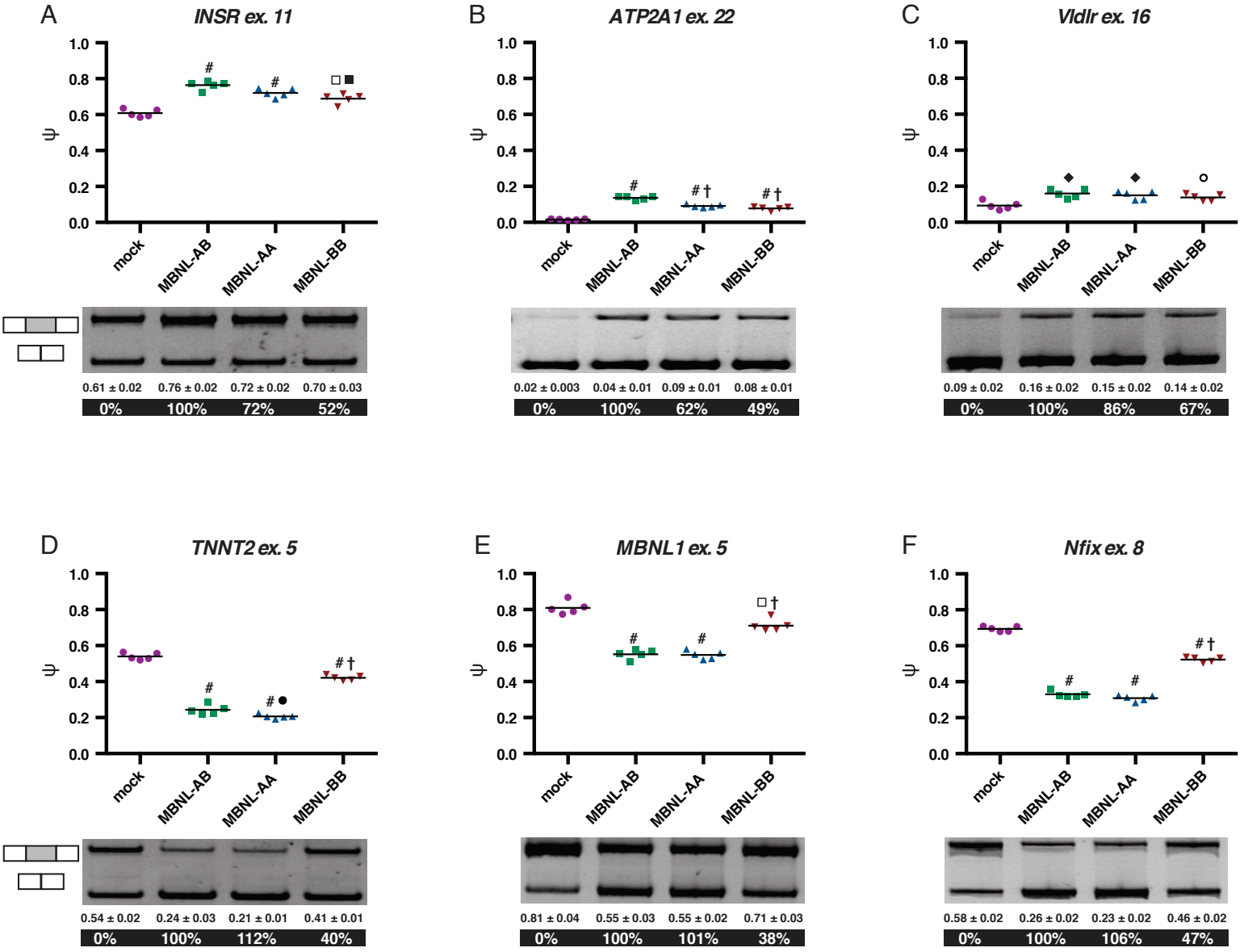


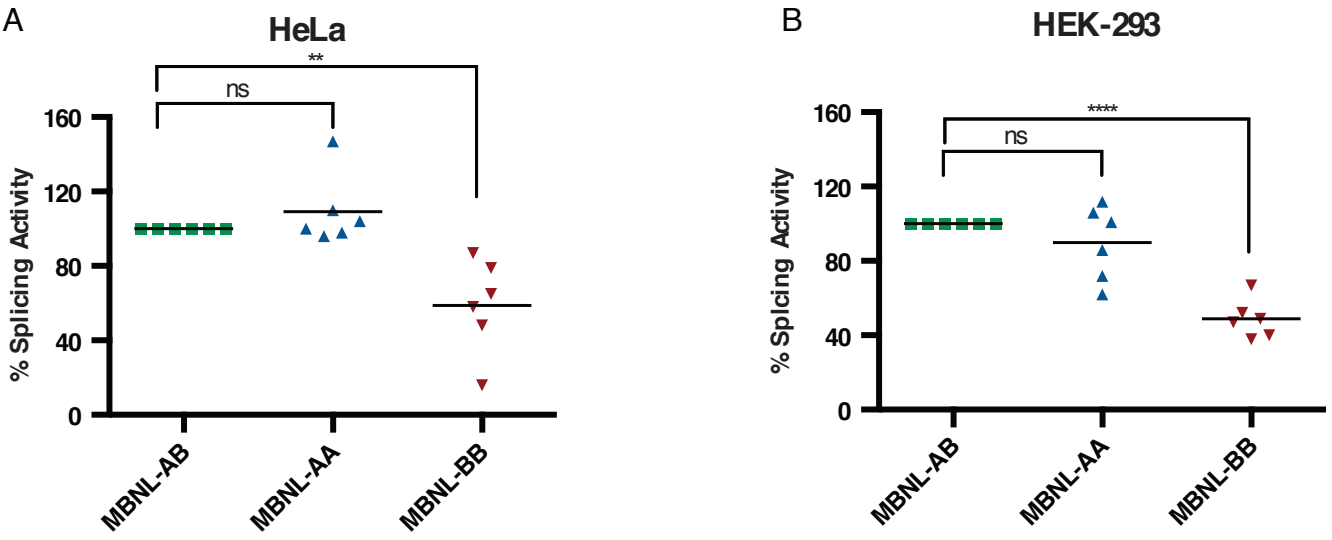
Supplemental Figure S2 *Subcellular localization and mRNA expression levels are not impacted by ZF domain rearrangement in transfected HeLa cells.* (A) Subcellular protein localization of synthetic proteins was determined using immunofluorescence against the HA tag. No significant differences in subcellular localization were detected between synthetic MBNL proteins. (B) Real-time qPCR analysis of synthetic MBNL RNA levels normalized to GAPDH in transfected HeLa cells. Determination of fold change in expression showed no differences in RNA levels of MBNL-AA and MBNL-BB compared to MBNL-AB (data represented as mean ± standard error).



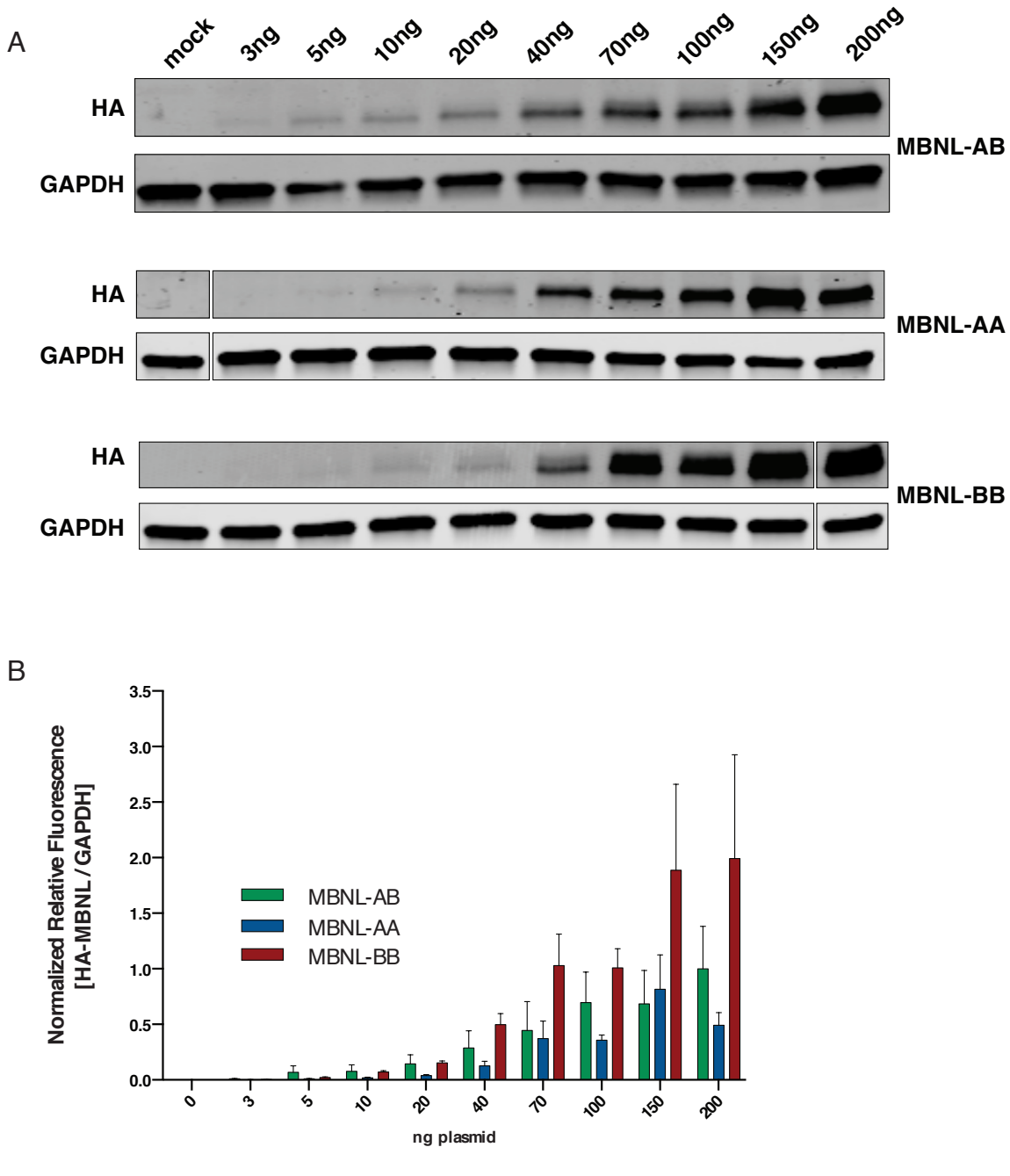
Supplemental Figure S3 *Synthetic MBNL proteins are expressed at different levels in transfected HEK-293 cells.* (A) Representative immunoblot comparing relative protein levels of synthetic MBNL proteins in transfected HEK-293 cells. (B) Quantification of protein levels in HEK-293 cells (n = 3) via Western blot against the HA tag. Relative levels of each protein were normalized to GAPDH. MBNL-AB levels were then set equal to 1 and MBNL-AA and MBNL-BB protein levels normalized (data represented as mean ± standard error; *$p < 0.05$, ***$p < 0.001$, Student's t-test).



Supplemental Figure S4 *Synthetic MBNL proteins regulate splicing of minigenes in HEK-293 cells with different activities.* (A-F) Jitter plot representations of splicing assays using *INSR*, *ATP2A1*, *Vldlr*, *TNNT2*, *MBNL1*, and *Nfix* minigenes, respectively. HEK-293 cells were transfected with empty vector (mock), MBNL-AB, MBNL-AA, or MBNL-BB protein-expression plasmids and a single minigene reporter. RNA was isolated from cells, RT-PCR performed, and the cDNA products resolved on a native gel. Percent spliced in (PSI, ψ) (i.e. percent exon inclusion) for each protein treatment was then quantified. Each point is from a single experiment and the line represents the average of all experiments for that condition (n = 5 for each protein treatment). Average ψ (± standard deviation) and percent splicing activity (displayed in white) are listed below representative splicing gels. (○p < 0.05 vs. mock, ◆p <0.01 vs. mock, ✶p < 0.001 vs. mock, #p < 0.0001 vs. mock, •p < 0.05 vs. MBNL-AB, ■p < 0.001 vs. MBNL-AB, †p < 0.0001 vs. MBNL-AB, Student's t-test)

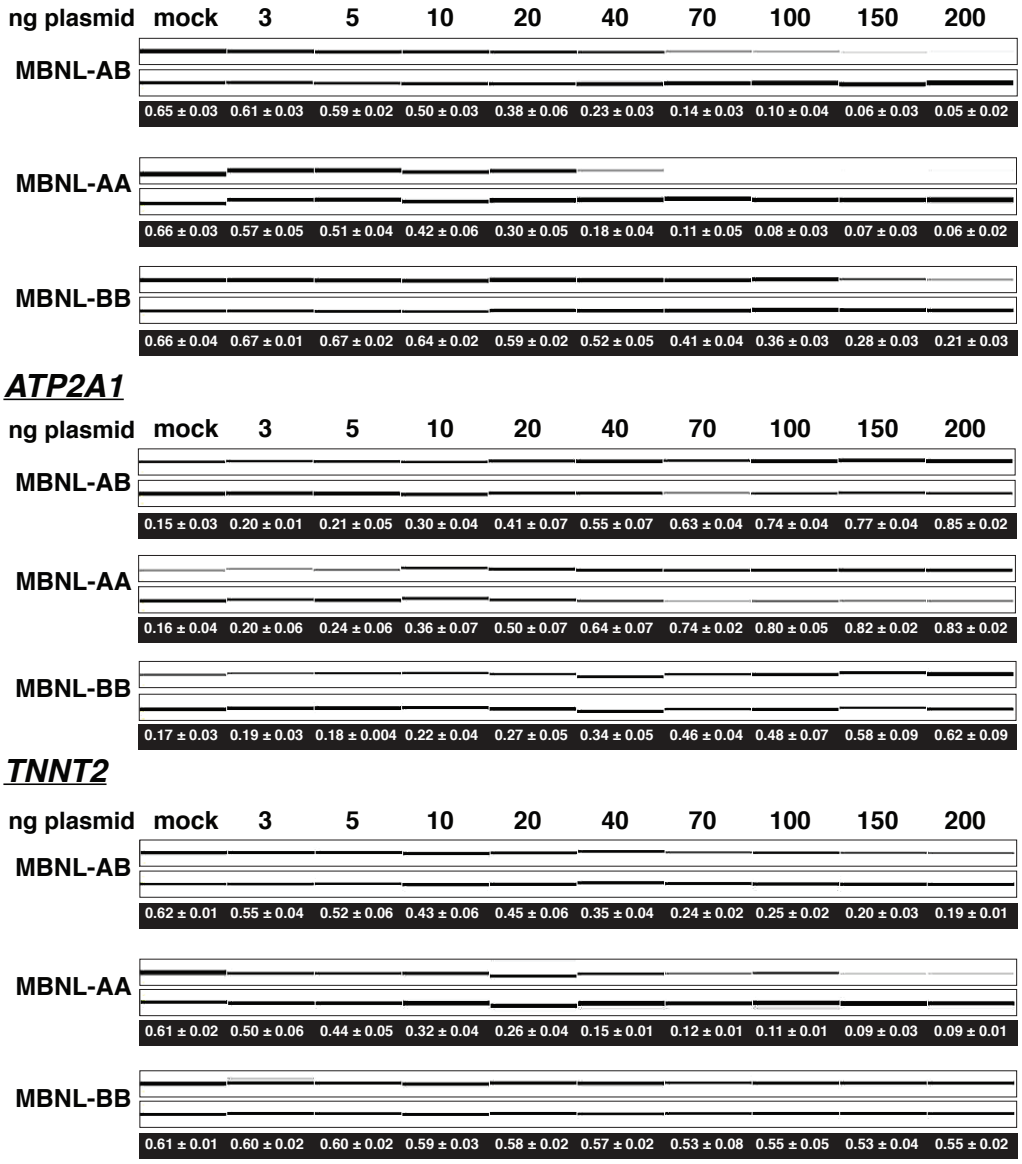


Supplemental Figure S5 *Average splicing activity of synthetic MBNL proteins across six minigene events.* (A-B) Jitter plot representation of average splicing activities for MBNL-AB and synthetic proteins in HeLa and HEK-293 cells, respectively. Each point is the splicing activity of each protein for a single minigene event tested (see Figure 2 and Supplemental Figure S4) and the line represents the average of all splicing activities. MBNL-AB was considered to have 100% splicing activity for each event and the values for MBNL-AA and MBNL-BB were calculated accordingly (**$p < 0.01$, ****$p < 0.001$, Student's t-test).



Supplemental Figure S6 *Representative immunoblots used to create dose-response curves in plasmid dosing system.* (A) Representative immunoblots used determine relative MBNL1 protein levels in plasmid dosing assay. HeLa cells were transfected with increasing concentrations of MBNL1 expression-plasmid and MBNL1 levels detected using an anti-HA antibody. Protein levels at each plasmid dose were normalized to a GAPDH loading control. Please note that breaks in the MBNL-AA and MBNL-BB blots are due to removal of lanes in immunoblot that were not used for quantification of protein levels. All lanes are from the same blot. (B) Normalized quantification of protein expression levels at each plasmid dose for all synthetic MBNL proteins (n=3 at each dose, data represented as mean ± standard error).

A



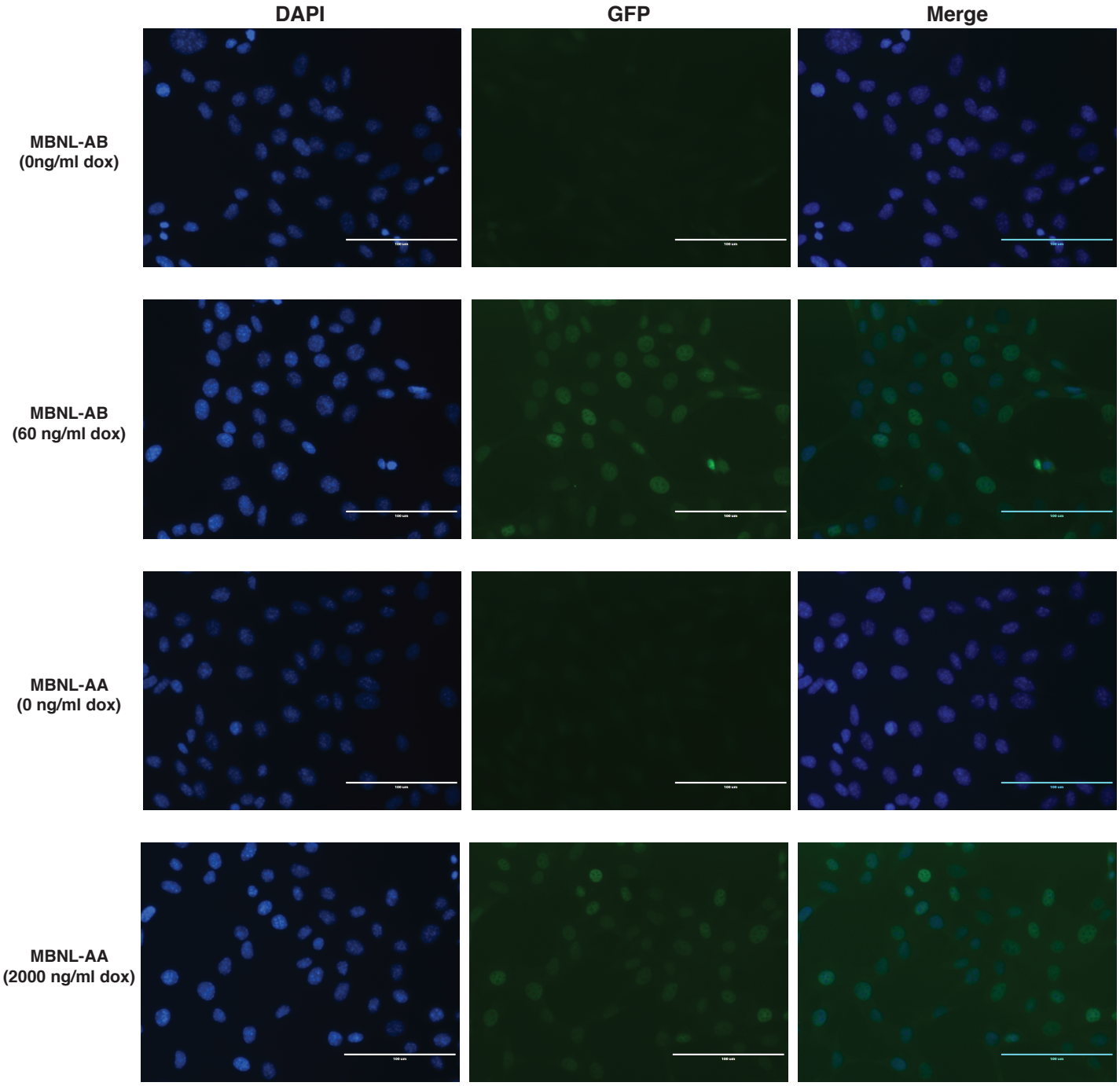
B

	Parameter	MBNL-AB	MBNL-AA	MBNL-BB
MBNL1	Log(EC_{50})	-0.71 ± 0.04	-1.44 ± 0.07	0.06 ± 0.02
	Hill Slope	-1.84 ± 0.24	-1.06 ± 0.21	-1.35 ± 0.13
	R^2	0.97	0.96	0.96

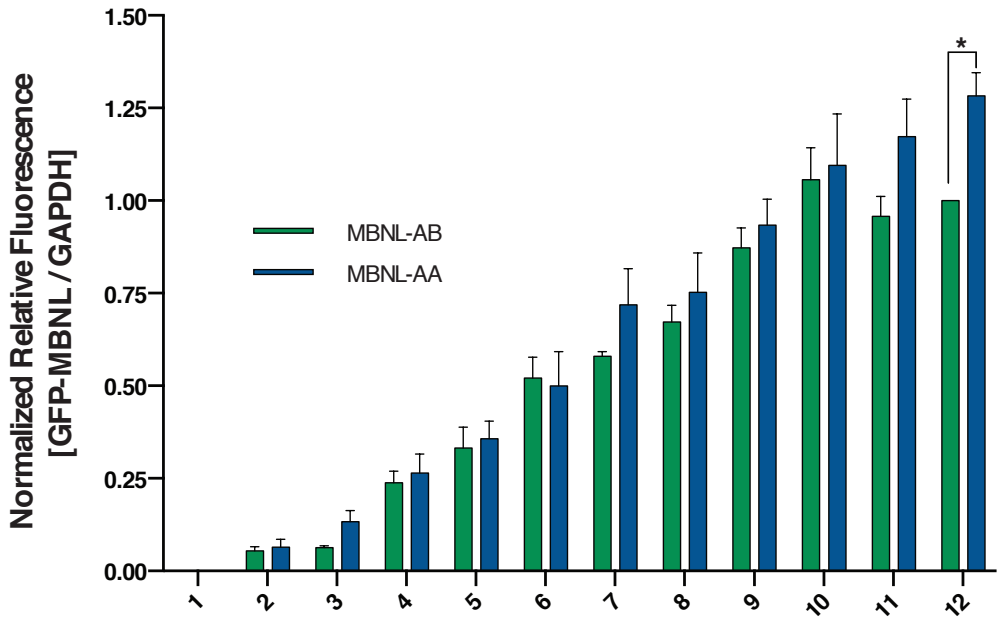
	Parameter	MBNL-AB	MBNL-AA	MBNL-BB
ATP2A1	Log(EC_{50})	-0.48 ± 0.11	-1.28 ± 0.10	0.10 ± 0.04
	Hill Slope	1.34 ± 0.29	0.93 ± 0.23	1.25 ± 0.20
	R^2	0.96	0.95	0.90

	Parameter	MBNL-AB	MBNL-AA	MBNL-BB
TNNT2	Log(EC_{50})	-0.54 ± 0.16	-1.62 ± 0.08	1.67 ± 1.18
	Hill Slope	-1.35 ± 0.48	-1.14 ± 0.26	-0.58 ± 0.54
	R^2	0.90	0.96	0.34

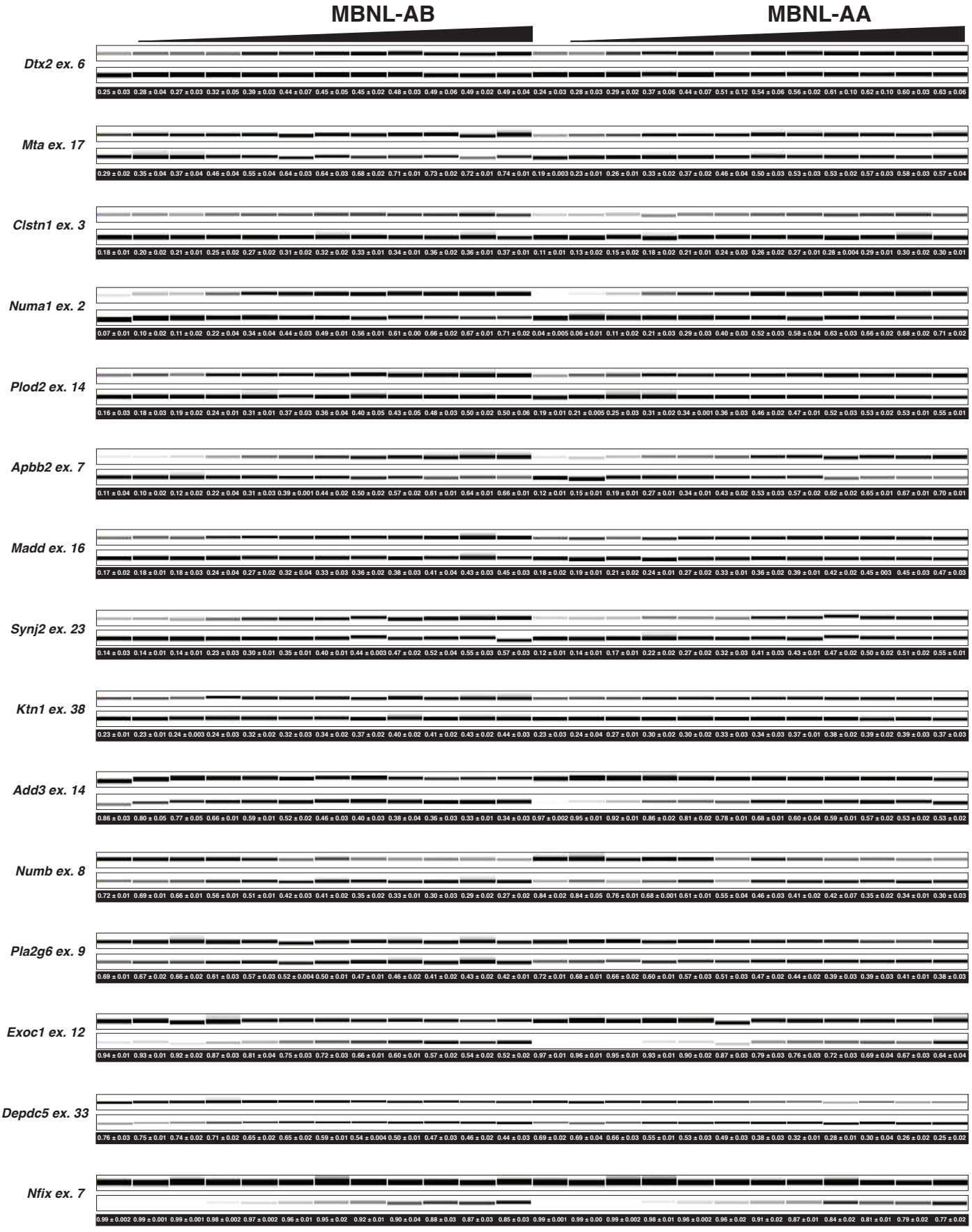
Supplemental Figure S7 *Representative splicing gels used to calculate changes in exon inclusion across the gradient of protein expression produced within the plasmid dosing system and quantitative parameters derived to describe dose-response behavior.* (A) Representative splicing gels for three minigenes tested acquired using the Advanced Analytical Fragment Analyzer. Bands are representative of relative fluorescence units (RFU) for each cDNA product and were used to calculate ψ for each plasmid dose. Average ψ ± standard deviation at each plasmid dose are listed below each gel (n = 3 – 5 for each). (B) Table of quantitative parameters (log(EC_{50}) and Hill Slope, ± standard error) generated from the dose-response curves. Overall quality of the fit, as represented by R^2 values, are also listed.



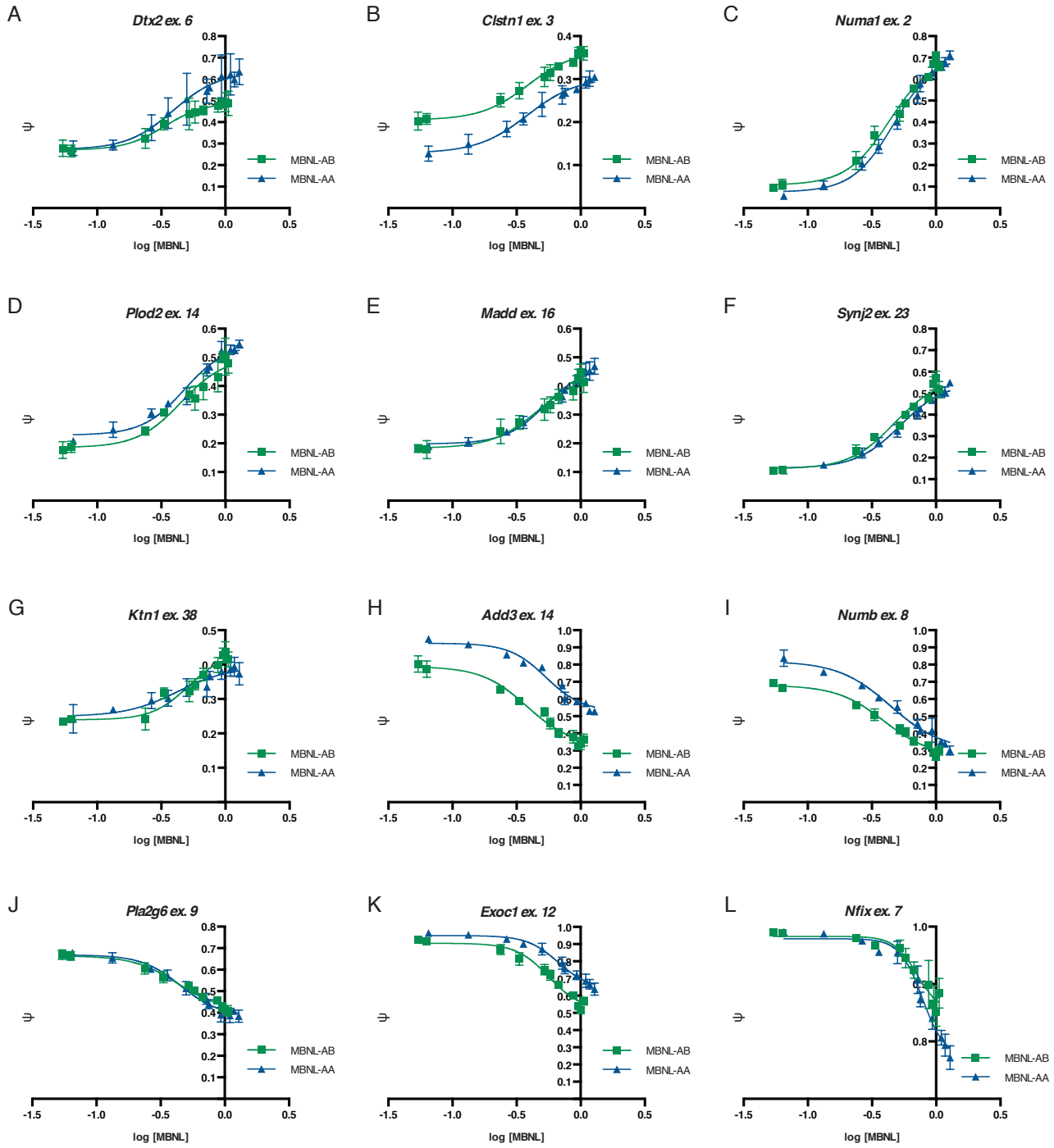
Supplemental Figure S8 *N-terminal GFP tagged MBNL-AB and MBNL-AA localize with the nucleus in response in doxycycline treatment in inducible MEFs.* Subcellular protein localization of synthetic MBNL proteins was determined using fluorescence of the GFP tag in the presence and absence of doxycycline. In the absence of doxycycline treatment, there was no visible protein expression. In the presence of doxycycline, no significant differences in subcellular localization were detected between synthetic MBNL proteins.



Supplemental Figure S9 *Quantification of relative MBNL-AB and MBNL-AA protein levels across a gradient of doxycycline treatment in inducible MEF system.* Normalized quantification of protein expression levels at each matched doxycycline dose for MBNL-AB (0 – 60 ng/ml) and MBNL-AA (0 – 2000 ng/ml) (n=3 at each dose, data represented as mean ± standard error). Relative MBNL expression levels for each protein was determined via immunoblot at each doxycycline dose and normalized to GAPDH. MBNL-AB levels at the highest dose were then set equal to 1 and all other values for MBNL-AB and MBNL-AA normalized accordingly. No statistical significance for relative protein levels was detected expect at the highest dose in each cell line (*$p < 0.05$, Student's t-test).



Supplemental Figure S10 *Representative splicing gels used to calculate changes in exon inclusion across the gradient of protein expression produced within the inducible MEF system.* Representative splicing gels for the 15 endogenous events tested acquired using the Advanced Analytical Fragment Analyzer. Bands are representative of relative fluorescence units (RFU) for each cDNA product and were used to calculate ψ for each doxycycline dose. Average ψ ± standard deviation at each plasmid dose are listed below each gel (n = 3).

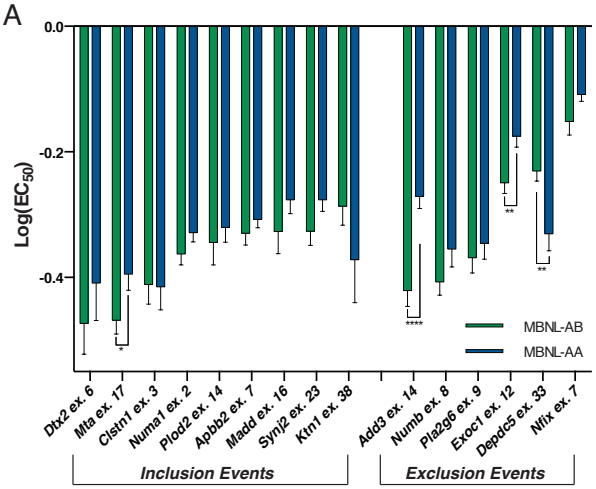


M

R^2 Values		
Event	**MBNL-AB**	**MBNL-AA**
Dtx2 ex. 6	0.84	0.82
Mta ex. 17	0.97	0.96
Clstn1 ex. 3	0.93	0.94
Numa1 ex. 2	0.97	0.98
Plod2 ex. 14	0.90	0.96
Apbb2 ex. 7	0.97	0.99
Madd ex. 16	0.90	0.96
Synj2 ex. 23	0.96	0.97
Ktn1 ex. 38	0.90	0.80
Add3 ex. 14	0.96	0.96
Numb ex. 8	0.97	0.96
Pla2g6 ex. 9	0.95	0.95
Exoc1 ex. 12	0.95	0.95
Depdc5 ex. 33	0.94	0.95
Nfix ex. 7	0.85	0.96

Supplemental Figure S11 *MBNL-AB and MBNL-AA produce similar dose-response curves for an additional 12 endogenous splicing events assayed in the inducible MEF system.* (A-L) Dose-response curves generated for 12 endogenous splicing events in MBNL-AB and MBNL-AA inducible MEFs. MEFs were treated with increasing amounts of doxycycline (n = 3) and ψ values quantified at each dose (representative splicing gels used to quantify ψ values can be found in Supplemental Figure S10). These ψ values (data represented as mean ± standard deviation) were then plotted against log [MBNL] levels (quantified via immunoblot, see Figure 4A and supplemental Figure S9) and fit to a four-parameter dose-curve. Due to ambiguous curve fitting in some cases, for all events the top or bottom (i.e. inclusion or exclusion event, respectively) of the curve was constrained to match the average ψ at the highest doxycycline dose. (M) Overall quality of fit of the four-parameter dose-curve, as represented by R^2 values, is listed for all 12 events in this figure and in Figure 4B-D.

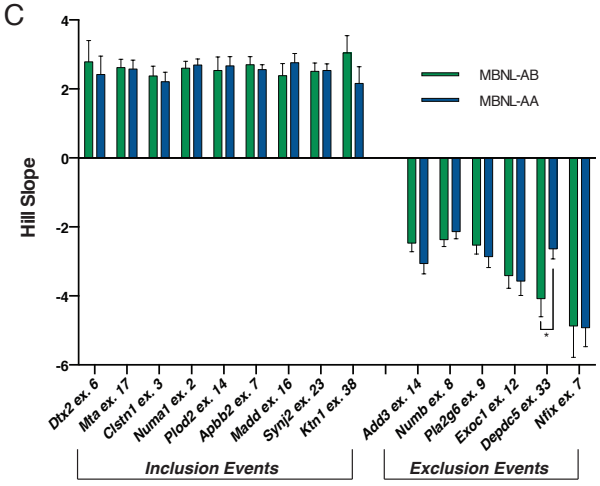
A



B

Log(EC_{50})			
Event	**MBNL-AB**	**MBNL-AA**	**Statistical Significance**
Dtx2 ex. 6	-0.47 ± 0.05	-0.41 ± 0.06	n.s
Mta ex. 17	-0.47 ± 0.02	-0.39 ± 0.03	* (p < 0.05)
Clstn1 ex. 3	-0.41 ± 0.03	-0.42 ± 0.04	n.s
Numa1 ex. 2	-0.36 ± 0.02	-0.33 ± 0.01	n.s
Plod2 ex. 14	-0.34 ± 0.04	-0.32 ± 0.02	n.s
Apbb2 ex. 7	-0.33 ± 0.02	-0.31 ± 0.01	n.s
Madd ex. 16	-0.33 ± 0.04	-0.28 ± 0.02	n.s
Synj2 ex. 23	-0.33 ± 0.02	-0.28 ± 0.02	n.s
Ktn1 ex. 38	-0.29 ± 0.03	-0.37 ± 0.07	n.s
Add3 ex. 14	-0.42 ± 0.03	-0.27 ± 0.02	**** (p < 0.0001)
Numb ex. 8	-0.41 ± 0.02	-0.35 ± 0.03	n.s
Pla2g6 ex. 9	-0.37 ± 0.02	-0.35 ± 0.03	n.s
Exoc1 ex. 12	-0.25 ± 0.02	-0.17 ± 0.02	** (p < 0.01)
Depdc5 ex. 33	-0.23 ± 0.02	-0.33 ± 0.03	** (p < 0.01)
Nfix ex. 7	-0.15 ± 0.02	-0.11 ± 0.01	n.s

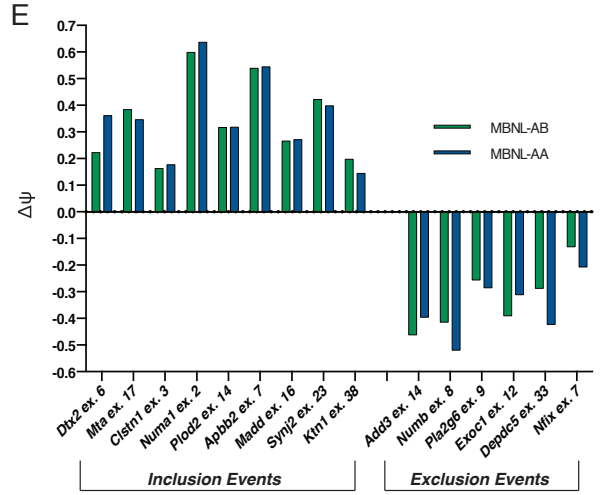
C



D

Hill Slope			
Event	**MBNL-AB**	**MBNL-AA**	**Statistical Significance**
Dtx2 ex. 6	2.79 ± 0.61	2.42 ± 0.54	n.s
Mta ex. 17	2.62 ± 0.24	2.57 ± 0.26	n.s
Clstn1 ex. 3	2.38 ± 0.29	2.22 ± 0.27	n.s
Numa1 ex. 2	2.61 ± 0.19	2.70 ± 0.17	n.s
Plod2 ex. 14	2.54 ± 0.39	2.67 ± 0.26	n.s
Apbb2 ex. 7	2.70 ± 0.23	2.56 ± 0.14	n.s
Madd ex. 16	2.38 ± 0.35	2.76 ± 0.27	n.s
Synj2 ex. 23	2.51 ± 0.24	2.54 ± 0.19	n.s
Ktn1 ex. 38	3.05 ± 0.50	2.16 ± 0.49	n.s
Add3 ex. 14	-2.47 ± 0.25	-3.06 ± 0.30	n.s
Numb ex. 8	-2.37 ± 0.20	-2.14 ± 0.20	n.s
Pla2g6 ex. 9	-2.52 ± 0.26	-2.86 ± 0.32	n.s
Exoc1 ex. 12	-3.41 ± 0.37	-3.57 ± 0.41	n.s
Depdc5 ex. 33	-4.08 ± 0.53	-2.64 ± 0.29	* (p < 0.05)
Nfix ex. 7	-4.87 ± 0.91	-4.92 ± 0.55	n.s

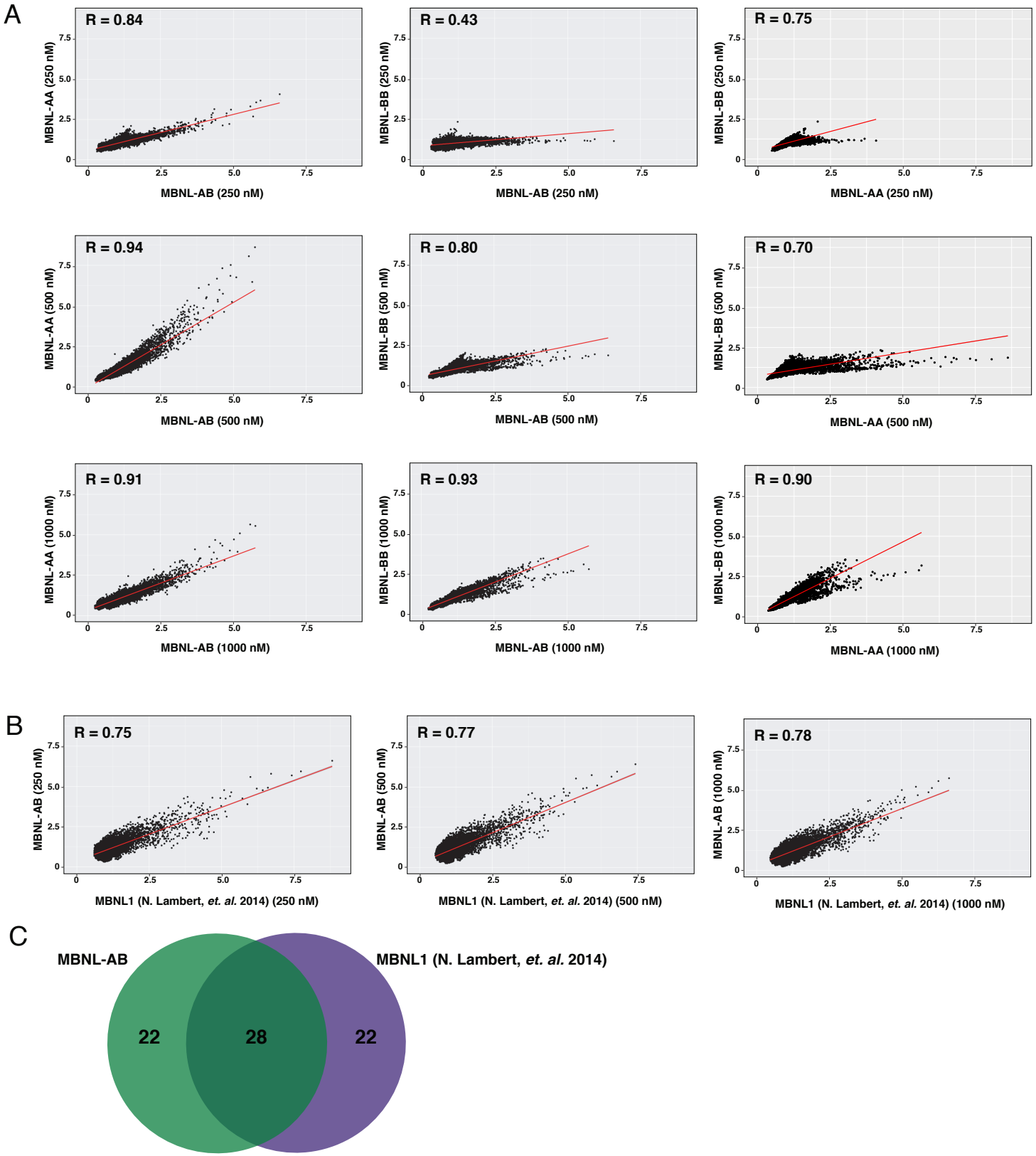
E



F

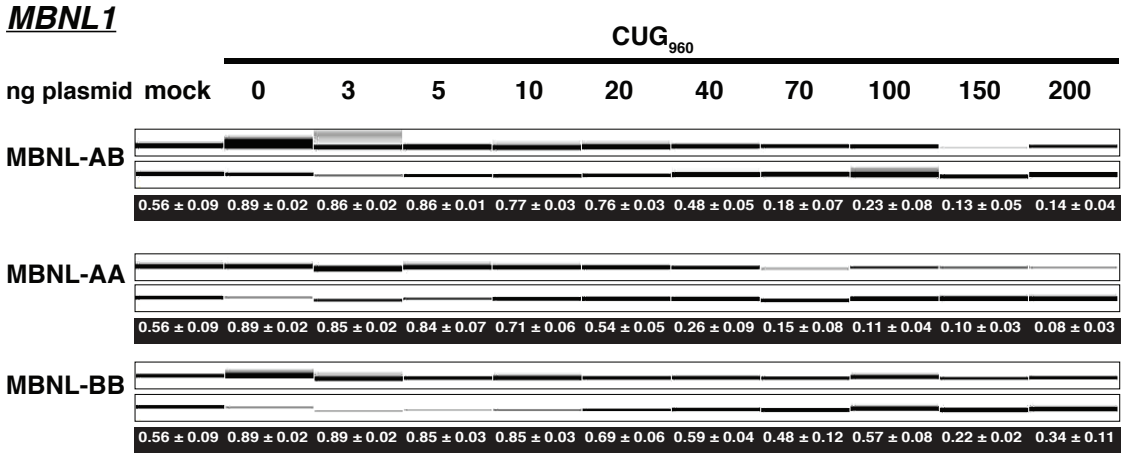
ΔΨ		
Event	**MBNL-AB**	**MBNL-AA**
Dtx2 ex. 6	0.22	0.36
Mta ex. 17	0.38	0.35
Clstn1 ex. 3	0.16	0.18
Numa1 ex. 2	0.60	0.64
Plod2 ex. 14	0.32	0.32
Apbb2 ex. 7	0.54	0.54
Madd ex. 16	0.27	0.27
Synj2 ex. 23	0.42	0.40
Ktn1 ex. 38	0.20	0.14
Add3 ex. 14	-0.46	-0.40
Numb ex. 8	-0.41	-0.52
Pla2g6 ex. 9	-0.26	-0.28
Exoc1 ex. 12	-0.39	-0.31
Depdc5 ex. 33	-0.29	-0.42
Nfix ex. 7	-0.13	-0.21

Supplemental Figure S12 *Quantitative parameters used to describe dose-response behavior of 15 endogenous splicing assayed in inducible MEF system.* Bar plots and tables of log (EC_{50}) (A and B), Hill slopes (C-D) and Δψ (E-F) values derived from dose-response curves (Figure 4B-D and Supplemental Figure S11A-L) to describe the dose-response behavior of MBNL-AB and MBNL-AA. Data represented as mean ± standard error for all values except for Δψ in which only the mean is reported due to the dose-curve fitting constraints.

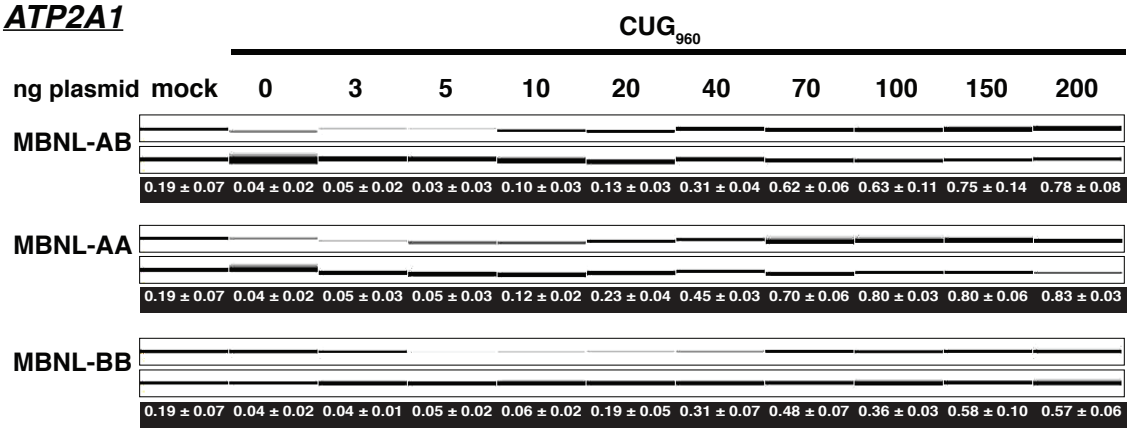


Supplemental Figure S13 *Comparison of R values and kmers derived from this and other RBNS studies with MBNL1.* (A) Scatterplots comparing R values for all kmers (k=7) between MBNL-AB, MBNL-AA and MBNL-BB at three different protein concentrations. Correlation coefficients indicate strong similarities between MBNL-AA and MBNL-AB, although some kmers for MBNL-AA display increased R values. MBNL-BB and MBNL-AB are not as strongly correlated and the scatterplots display the overall low R values of MBNL-BB across all protein concentrations. Comparison of MBNL-AA and MBNL-BB magnifies these differences in RNA binding specificity between the two proteins. (B) Scatterplots comparing R values for all kmers between MBNL-AB and those identified with MBNL1 in a previous RBNS study at three protein concentrations (4). Correlation coefficients indicate a strong correlation of R values between each MBNL1 protein indicating a similarity in motif recognition and RNA binding activity in each protein population (C) Area-proportional venn diagram showing overlap in top 50 kmers for MBNL-AB and those identified using MBNL1 in a previous RBNS study (4). Values listed represent number of kmers within each sub-population.

A

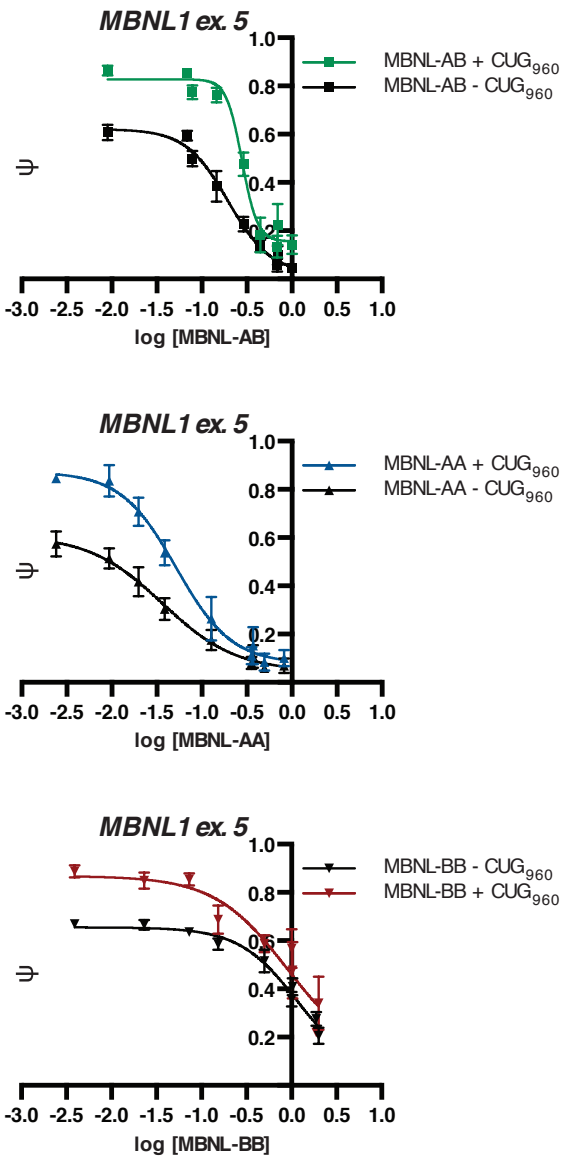


B



Supplemental Figure S14 *Representative splicing gels used to create dose response curves with plasmid dosing system in the presence of toxic RNA.* (A-B) Representative splicing gels from plasmid dosing assay with the *MBNL1* and *ATP2A1* minigene reporters, respectively, in the presence of CUG repeat RNA. Images were acquired using the Advanced Analytical Fragment Analyzer. Bands are representative of relative fluorescence units (RFU) for each cDNA product and were used to calculate ψ for each plasmid dose. Average ψ ± standard deviation at each plasmid dose are listed below each gel (n = 3 – 5 for each).

A



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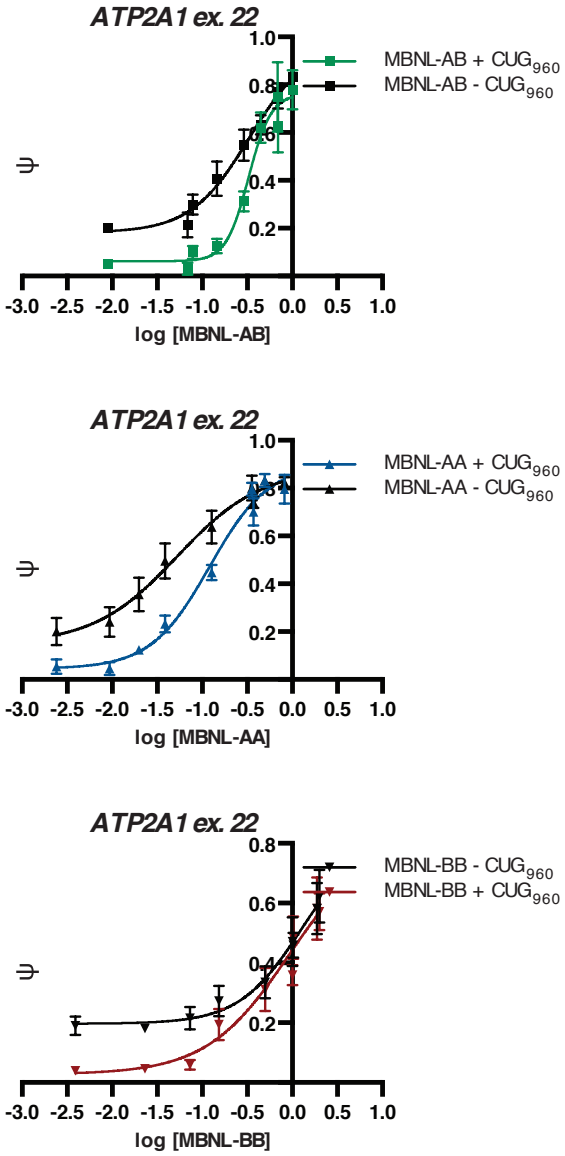

C

MBNL1	Log(EC_{50})	Hill Slope	R^2
MBNL-AB - CUG_{960}	-0.71 ± 0.04	-1.84 ± 0.24	0.97
MBNL-AB + CUG_{960}	-0.56 ± 0.02	-5.06 ± 1.40	0.97
MBNL-AA - CUG_{960}	-1.44 ± 0.07	-1.06 ± 0.21	0.96
MBNL-AA + CUG_{960}	-1.29 ± 0.05	-1.41 ± 0.21	0.97
MBNL-BB - CUG_{960}	0.06 ± 0.02	-1.35 ± 0.13	0.96
MBNL-BB + CUG_{960}	-0.05 ± 0.06	-1.08 ± 0.20	0.86

ATP2A1	Log(EC_{50})	Hill Slope	R^2
MBNL-AB - CUG_{960}	-0.48 ± 0.11	1.34 ± 0.29	0.96
MBNL-AB + CUG_{960}	-0.49 ± 0.03	3.48 ± 0.92	0.94
MBNL-AA - CUG_{960}	-1.28 ± 0.10	0.93 ± 0.23	0.95
MBNL-AA + CUG_{960}	-0.92 ± 0.06	1.33 ± 0.20	0.98
MBNL-BB - CUG_{960}	0.10 ± 0.04	1.25 ± 0.20	0.90
MBNL-BB + CUG_{960}	-0.03 ± 0.05	0.95 ± 0.13	0.92

Supplemental Figure S15 *Expression of CUG repeat toxic RNA alters the MBNL dose-response curve.* (A-B) Comparison of dose curves with the *MBNL1* and *ATP2A1* minigene in presence and absence of *DMPK*- CTG_{960} transfection for all three MBNL1 proteins tested (data represented as mean ± standard deviation) (C) Table of quantitative parameters (log(EC_{50}) and Hill Slope, mean ± standard error) generated from the dose-response curves in the presence and absence of toxic repeat RNA expression. Overall quality of the fit, as represented by R^2 values, is also listed.

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Event	Exon	Fwd Primer (5' --> 3')	Rev Primer (5' --> 3')	Inclusion (bp)	Exclusion (bp)	Tm (ºC)	Cycle Number
Add3	14	GCAGTTTGACGATGACGATCAGG	GACATCATGCATCTCGTCCTTGC	301	205	55	26
Apbb2	7	TCATTTCAGACAGATCCCGATTTGC	GTTTCAAGGATGCATAGCGTAGC	334	271	55	26
Clstn1	3	CATGGGATAGTCACCGAGAACG	CCTCACCCGTGGACTTATCC	196	166	57	26
Exoc1	12	TGACTGGCACCTCTAAAGAAAGC	GGTCAGAGGCAGACATGTTCC	210	165	57	28
Madd	16	TCACACTGCCCACCAAAGG	GAAGGTTCTCTTTTCACGGTTGG	190	130	55	28
Nfix	7	CCATCGACGACAGTGAGATGG	CTGGATGATGGACGTGGAAGG	296	173	51	27
Numa1	2	CGACAAGAAGCACAGAGTACTAGC	CTTCTGTTGCTGCACCTTGC	231	189	55	26
Numb	8	AGCATCAGCTCCTTGTGTTCC	GCAGCACCAGAAGACTGACC	302	155	57	25
Pla2g6	9	AGAAGTGGACACCCCAAACG	CTCATGGAGCTCAGGATGAACG	294	129	57	28
Plod2	14	AGGAATCTGGAATGTCCCATATATGG	TCTGCCAGAAGTCATTGTTAAGATGG	302	239	55	26
Synj2	23	CTCGGTGGAGACAACTCTTCC	GTGCTCCTGGGAGAAGTTTCG	335	200	55	28
Mta1	17	ACCCCGTGAAGAGTTCATCC	GTGCCTGGTCTGTCCATGG	191	155	57	28
Ktn1	38	AGATGGAGCGATCGACTTACG	AATCATCAGCTACCTTCTTTCTCTCC	198	114	51	27
Depdc5	33	CGACTGTGCACGGAAAAAGC	CCAGAGTTTGCAGAGGGAAAGG	295	229	57	28
Dtx2	6	CCGTGCAGATGCCAAAGG	TCAGGGGCCACTTTCAGC	253	115	51	32

Supplemental Table S1 *Sequences of primers used for RT-PCR of endogenous splicing events in MEFs.* Table includes sequences of primers targeting flanking exons of MBNL regulated splicing events as well as the cycle number and annealing temperature utilized with each primer pair. The overall size in base pairs of inclusion and exclusion products for each event are also listed.

Protein	Concentration [nM]	Index Primer	Sequences (5' --> 3')
MBNL-AB	0	PEIndex_8	CAAGCAGAAGACGGCATACGAGAT CACTGT GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
MBNL-AB	16	PEIndex_7	CAAGCAGAAGACGGCATACGAGAT ATTGGC GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
MBNL-AB	32	PEIndex_6	CAAGCAGAAGACGGCATACGAGAT GATCTG GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
MBNL-AB	125	PEIndex_5	CAAGCAGAAGACGGCATACGAGAT TCAAGT GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
MBNL-AB	250	PEIndex_4	CAAGCAGAAGACGGCATACGAGAT CTGATC GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
MBNL-AB	500	PEIndex_3	CAAGCAGAAGACGGCATACGAGAT AAGCTA GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
MBNL-AB	1000	PEIndex_2	CAAGCAGAAGACGGCATACGAGAT GTAGCC GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
MBNL-AB	2000	PEIndex_1	CAAGCAGAAGACGGCATACGAGAT TACAAG GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
MBNL-AA	0	PEIndex_16	CAAGCAGAAGACGGCATACGAGAT GGACGG GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
MBNL-AA	16	PEIndex_15	CAAGCAGAAGACGGCATACGAGAT TGACAT GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
MBNL-AA	32	PEIndex_14	CAAGCAGAAGACGGCATACGAGAT GGAACT GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
MBNL-AA	125	PEIndex_13	CAAGCAGAAGACGGCATACGAGAT TTGACT GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
MBNL-AA	250	PEIndex_12	CAAGCAGAAGACGGCATACGAGAT CGTGAT GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
MBNL-AA	500	PEIndex_11	CAAGCAGAAGACGGCATACGAGAT ACATCG GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
MBNL-AA	1000	PEIndex_10	CAAGCAGAAGACGGCATACGAGAT GCCTAA GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
MBNL-AA	2000	PEIndex_9	CAAGCAGAAGACGGCATACGAGAT TGGTCA GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
MBNL-BB	0	PEIndex_24	CAAGCAGAAGACGGCATACGAGAT CTCTAC GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
MBNL-BB	16	PEIndex_23	CAAGCAGAAGACGGCATACGAGAT GCGGAC GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
MBNL-BB	32	PEIndex_22	CAAGCAGAAGACGGCATACGAGAT TTTCAC GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
MBNL-BB	125	PEIndex_21	CAAGCAGAAGACGGCATACGAGAT GGCCAC GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
MBNL-BB	250	PEIndex_20	CAAGCAGAAGACGGCATACGAGAT CGAAAC GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
MBNL-BB	500	PEIndex_19	CAAGCAGAAGACGGCATACGAGAT CGTACG GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
MBNL-BB	1000	PEIndex_18	CAAGCAGAAGACGGCATACGAGAT CCACTC GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
MBNL-BB	2000	PEIndex_17	CAAGCAGAAGACGGCATACGAGAT GCTACC GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
Input RNA	N/A	PEIndex_25	CAAGCAGAAGACGGCATACGAGAT ATCAGT GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC

Supplemental Table S2 *Index primers used to identify each RBNS library within the multiplexed sequencing reads.* All cDNA libraries were labelled with PEUniversal (5' – AATGATACGGCGACCACCGAGATCTA- CACTCTTTCCCTACACGACGCTCTTCCGATCT – 3') and an index primer as listed in the table below to designate which protein at a specific concentration was utilized to isolate the bound RNA in the RBNS protocol.

Supplemental Materials and Methods

Immunofluorescence and Microscopy

To acquire immuofluorescence images in transfected HeLa cells, eight-well culture slides were treated with poly-lysine solution (Sigma) for 30 minutes at 37 °C. HeLa cells were then plated at 2 x 10^4 cells/chamber. Cells were transfected 24 hours post-plating with 200 ng total plasmid (100 ng protein expression plasmid and 100 ng of empty pCI vector, 200ng of pCI for mock) using 1µl of Lipofectamine 2000 (Invitrogen) as per the manufacturer's protocol. Cells were placed in Opti-MEM I reduced serum media (Gibco) at the time of transfection. Six hours later the Opti-MEM I was replaced with DMEM-Glutamax (Gibco) supplemented with 10 % fetal bovine serum (FBS) and 1X antibiotic-antimycotic (Gibco). 18 hours post-medium exchange cells were fixed for 10 minutes on ice with 4 % paraformaldehyde. Cells were then permeabilized with 0.1 % Triton X-100 in 1X PBS for 10 minutes at room temperature (RT). Next, cells were treated with Image-iT FX Signal Enhancer (Invitrogen) for 30 minutes at RT. The cells were probed overnight at 4 °C with mouse anti-HA antibody (1:100 dilution, 6E2, Cell Signaling Technology). After 3 washes in 1X PBS for 5 minutes at RT, cells were then probed with goat anti-mouse Alexa 488 (5 µg/ml dilution, Invitrogen) for 1 hour at RT. Finally, cells were mounted using Prolong Diamond Antifade Mountant with DAPI (Invitrogen). After the slides had cured, images were acquired using a Zeiss Axioskop 2 with equal exposures across all samples (Supplemental Figure S2A).

To acquire images of MBNL proteins in doxycycline treated MEFs, glass cover slips were placed in six-well plates and treated with poly-lysine solution for 30 minutes at 37 °C. MEFs were then plated at 1.5 x 10^4 cells/well. 24 hours later, fresh doxycycline (Sigma) was prepared at 1 mg/ml, diluted, and then added to the cells at the appropriate concentrations to induce GFP-MBNL protein expression. 24 hours post-drug treatment, cells were fixed for 10 minutes on ice with 4 % paraformaldehyde. Cells were then permeabilized with 0.1 % Triton X-100 in 1X PBS for 10 minutes at room temperature (RT). Cells were then mounted using Prolong Diamond Antifade Mountant with DAPI. After the slides had cured, images were acquired using the EVOS FL Cell Imaging System (ThermoFisher) (Supplemetal Figure S8).

Real-time PCR

RNA was isolated from HeLa cells transfected with equivalent amounts of empty vector (mock) or plasmid expressing a synthetic MBNL protein using the Aurum Total RNA mini kit (Bio-Rad). 500 ng of RNA was DNAsed using Promega RQ1 RNAse-Free DNAse as per the manufacturer's protocol. The RNA was then reverse transcribed with SuperScript IV with random hexamer priming according to the manufacturer's protocol except that half of the recommended SuperScript IV was utilized. Real-time PCR analysis was then conducted using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) as per the manufacturer's guidelines. Negative controls were

performed in the absence of template or reverse-transcriptase. Primers utilized are as listed: MBNL-AB/MBNL-AA/MBNL-BB (forward: 5'-AGAGAAAGGTCGAATGAGCGG-3', reverse: 5'-TGCATTCTAGTTGTGGTTTGTCC-3') and GAPDH (forward: 5'-AATCCCATCACCATCTTCCA-3', reverse: 5'-TGGACTCCACGACGTACTCA-3'). No differences in amplification efficiency were detected for GAPDH or target primer pairs. Expression levels of MBNL were determined via normalization of the cycle threshold (C_t) to GAPDH. Calculations of fold change relative to MBNL-AB were determined via the formula $2^{-\Delta\Delta Ct}$.

Cell culture and transfection

HEK-293 cells (Flp-In T-Rex 293, Invitrogen) were routinely cultured as a monolayer in DMEM-Glutamax (Gibco) supplemented with 10% fetal bovine serum and 10 µg/ml basticidin / 300 µg/ml zeocin at 37 °C under 5 % CO_2. Prior to transfection, cells were plated in twenty four-well plates at a density of 1.5×10^5 cell/well. Cells were transfected 24 hours later at roughly 80 % confluency. Plasmids (500 ng/well) were transfected using 1.5 µl of Transit-293 (Mirus) as per the manufacturer's protocol. For all overexpression cell-based splicing assays (Supplemental Figure S4) and Western blots (Supplemental Figure S3) 250 ng of protein expressing plasmid or empty pCI vector (mock) were co-transfected with 250 ng of minigene reporter. 24 hours post-transfection cells were harvested using TrypLE (Gibco) and pelleted using centrifugation.

SUPPLEMENTAL MATERIALS REFERENCES

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