SUPPLEMENTARY DATA

AON-induced splice-switching and *DMPK* pre-mRNA degradation as potential therapeutic approaches for Myotonic Dystrophy type 1

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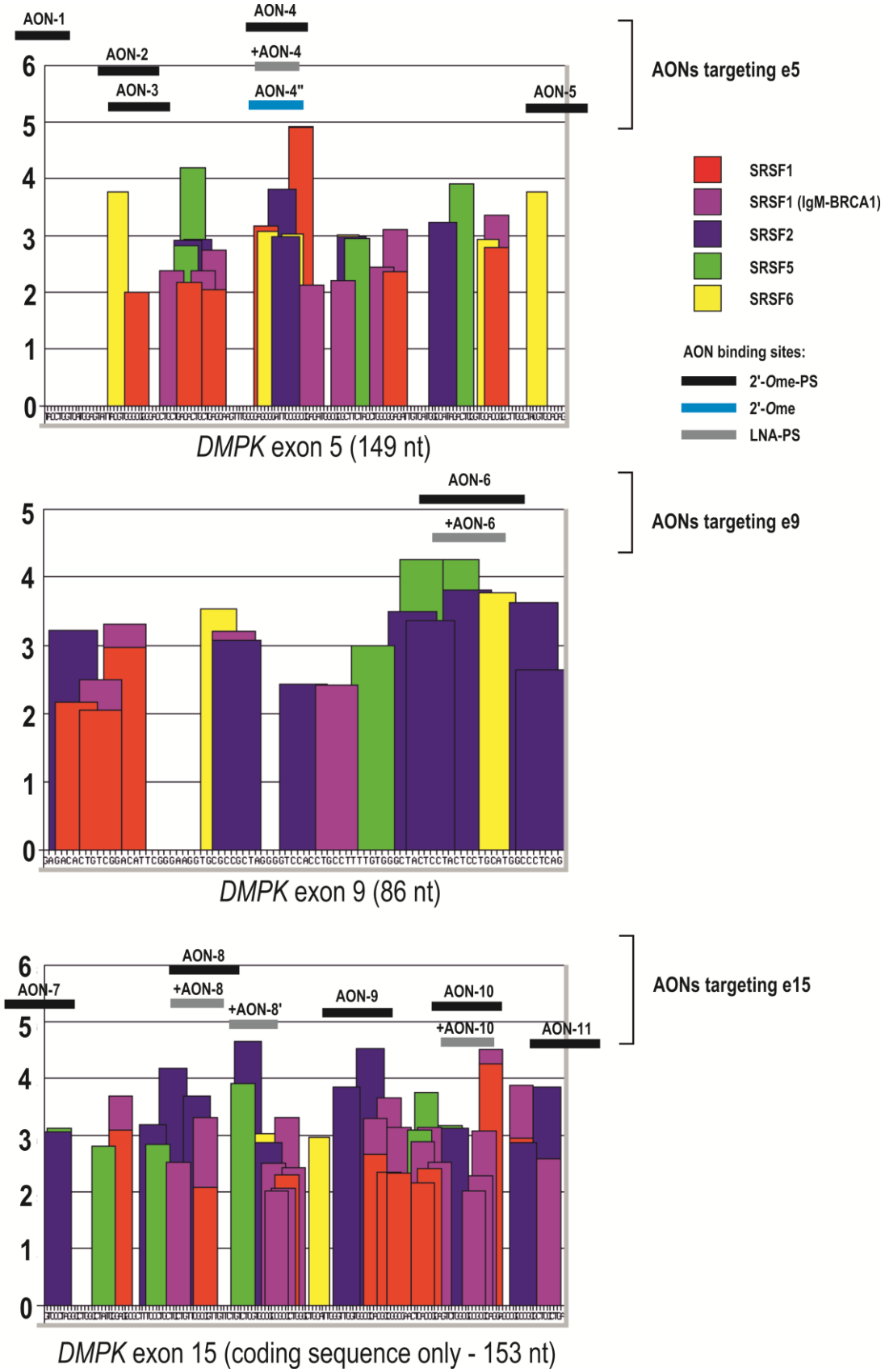
SUPPLEMENTARY MATERIALS AND METHODS

Assessment of cell viability and AONs toxicity

Viability of cells upon experimental manipulation and dosing with AONs was performed using CellToxTM Green Cytotoxicity Assay (Promega) per manufacturer's recommended protocol. Assay was performed in non-DM1 fibroblasts 48h post transfection with 75 nM of distinct AONs targeting the same core sequence within e5, but differing in backbone chemistries (Supplementary Figure S3C).

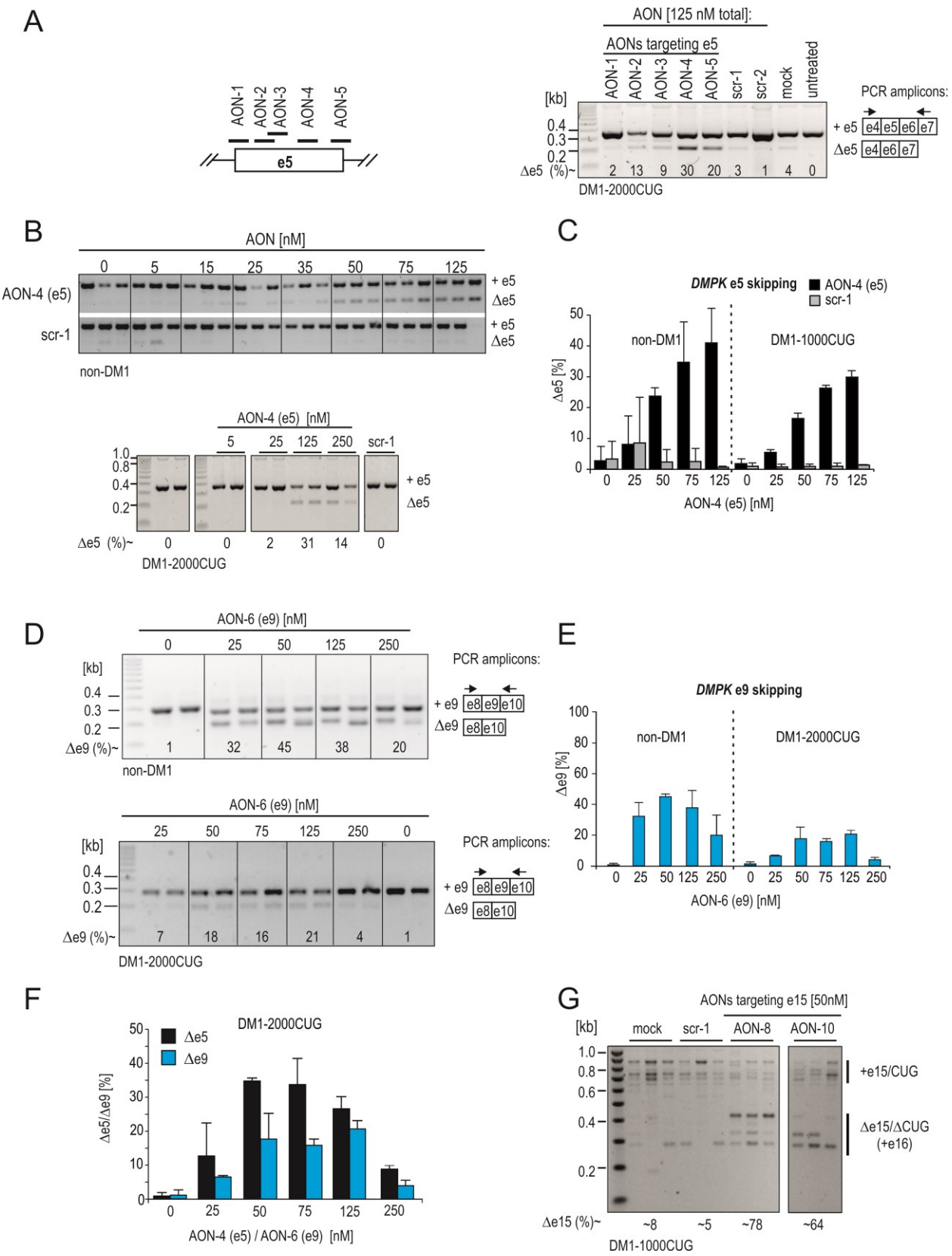
SUPPLEMENTARY FIGURES



Supplementary Figure S1.

Supplementary Figure S1.

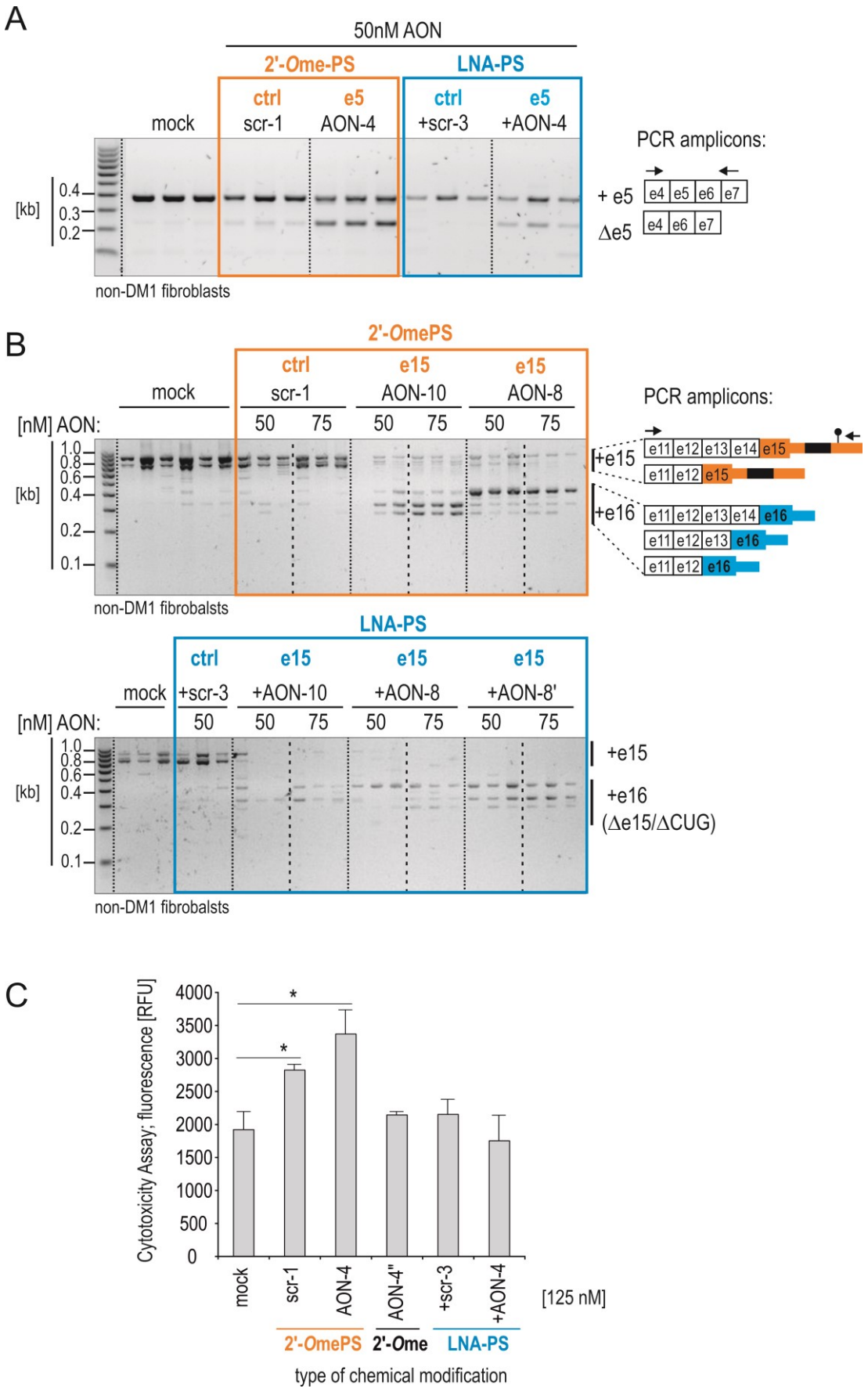
Graphical output of exonic splicing enhancer (ESE) analyses within *DMPK* e5, e9 and e15. The analyzed exonic sequence is reproduced along the X-axis. Putative binding sites for distinct serine/arginine-rich proteins (SRSF) are represented by bars. The height of the bars represents the motif scores, and the width of the bars indicates the length of the motif (6, 7 or 8 nucleotides). Different SRSF protein high-scores are color-coded as illustrated in the legend. Approximate AON binding sites within targeted exons are represented as horizontal lines indicated in the legend.



Supplementary Figure S2.

AONs complementary to sequences within *DMPK* e5, e9 and e15 induce efficient skipping of targeted exons in non-DM1 and DM1 fibroblasts. (A) Schematic representation of AON binding sites within e5 and representative RT-PCR gel showing e5 skipping in DM1-2000CUG fibroblasts transfected

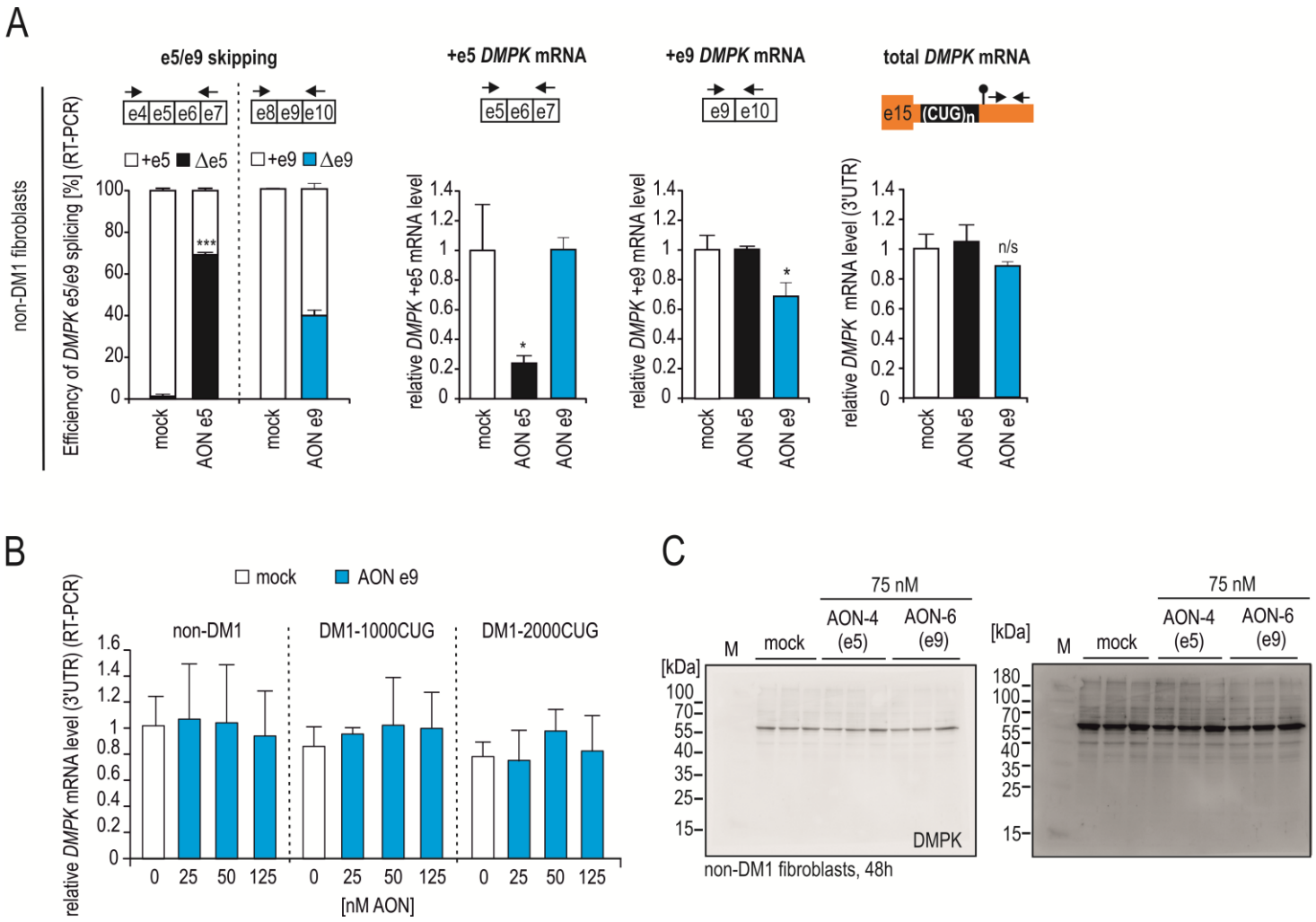
with 125 nM of indicated AONs. ∆e5 (%) indicates percent e5 spliced out. **(B)** Representative RT-PCR analysis of e5 skipping in non-DM1 (top) vs DM1-2000CUG fibroblasts (bottom) transfected with indicated amounts of AON-4 targeting e5, or control scrambled sequence AON (scr-1). **(C)** Quantitation of e5 skipping efficiency in non-DM1 vs DM1-1000CUG fibroblasts transfected with increasing amounts of indicated AONs. **(D)** Representative RT-PCR analyses of AON-6-induced e9 skipping in control (top) and DM1 fibroblasts (bottom). PCR amplicons are indicated and ∆e9 (%) refers to the percentage of e9 spliced out. **(E)** Quantitation of results shown in (D). **(F)** RT-PCR-based quantitation of e5 and e9 skipping in DM1 fibroblasts treated with AON-4 and AON-6 at the same time. **(G)** Representative RT-PCR gel image showing e15/CUG skipping in DM1-1000CUG fibroblasts dosed with indicated AONs.



Supplementary Figure S3.

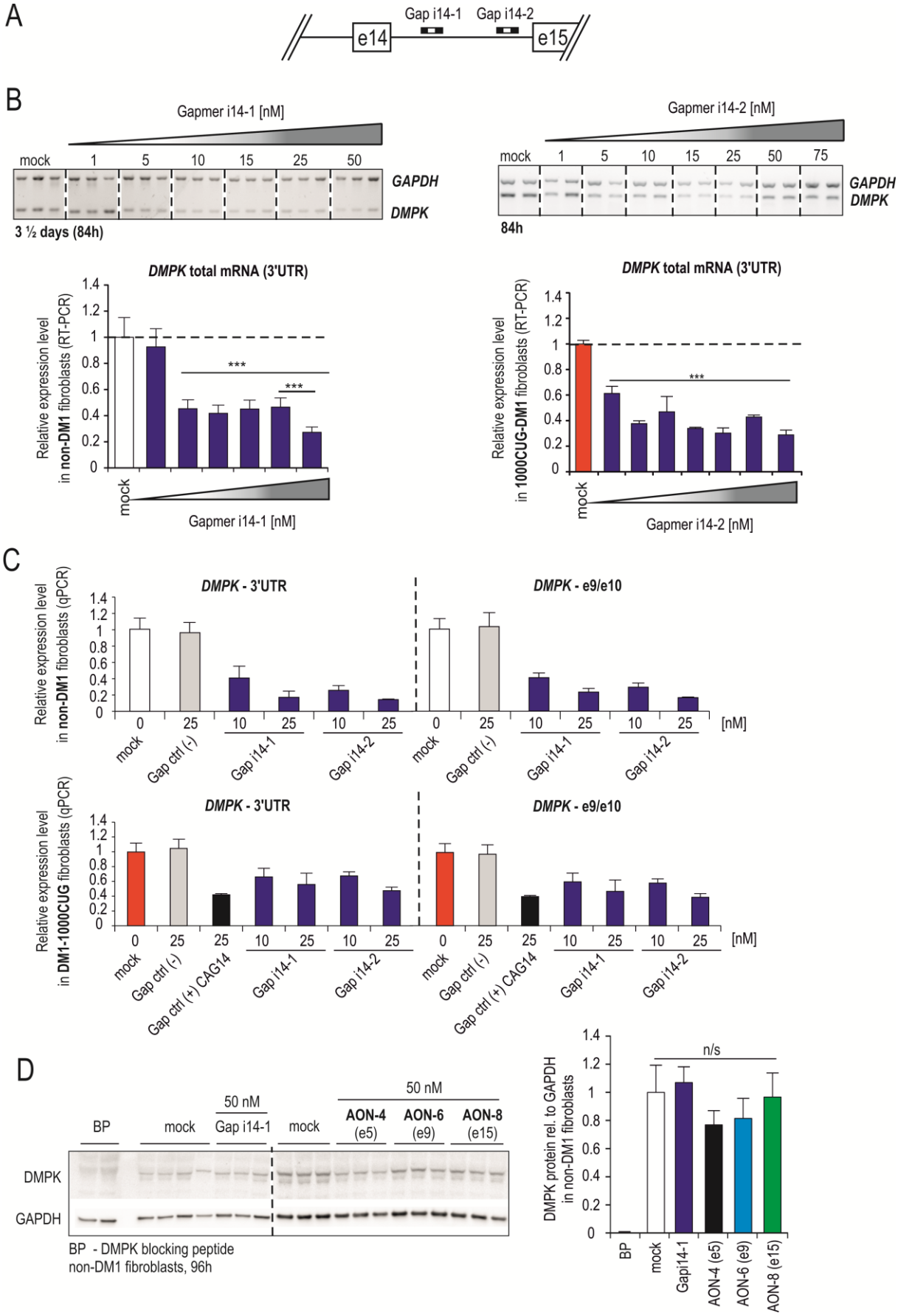
Supplementary Figure S3.

LNA-PS AONs targeting e5 and e15 induce weaker exon skipping compared to their 2'-*O*me-PS equivalents. (A-B) Representative images of RT-PCR comparing e5 (A) and e15 (B) skipping in non-DM1 fibroblasts transfected with indicated amounts of 2'-*O*me-PS (orange) or equivalent LNA-PS (blue) AONs targeting the same core sequences within *DMPK* e5 (A) or e15 (B). Scrambled AONs scr-1 and +scr-3 with appropriate chemical modifications were used as controls. *DMPK* splice isoforms and primer locations are indicated to the right of gel images. AON sequences are listed in Supplementary Table 1, and schematic of AON binding sites within targeted exon is shown in Supplementary Figure 1. **(C)** Quantitation of cell viability/cytotoxicity in non-DM1 fibroblasts treated with 75nM of e5 targeting AON-4. Chemical modifications are indicated. AON-4, AON-4'' and +AON-4 target the same core sequence within e5.



Supplementary Figure S4.

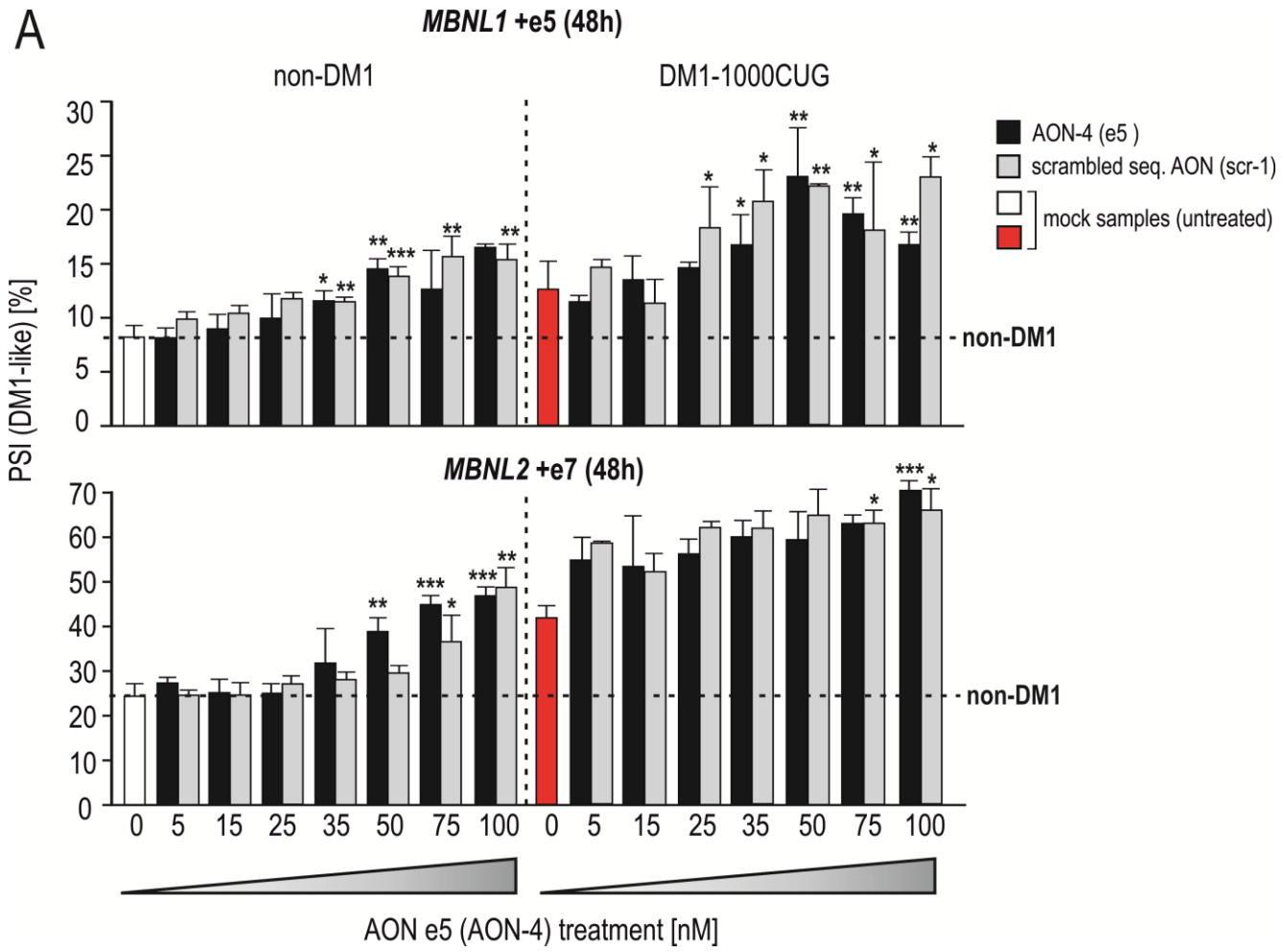
AON-mediated exclusion of e5 and e9 fails to reduce *DMPK* mRNA. (A) RT-PCR-based quantitation of e5 or e9 skipping efficiency, e5-containing *DMPK* mRNA (+e5 *DMPK* mRNA), e9-containing *DMPK* mRNA (+e9 *DMPK* mRNA) and total *DMPK* mRNA (3'UTR) in control non-DM1 primary fibroblasts treated with indicated amounts of AONs directed at e5 (AON-4) and e9 (AON-6). Schematics of PCR amplicons are indicated. **(B)** RT-PCR quantitation of total *DMPK* mRNA (amplicon within 3'UTR) demonstrates lack of *DMPK* transcript reduction upon e9 skipping in control and indicated DM1 fibroblasts. *DMPK* expression is shown relative to *GAPDH* and increasing doses of AON e9 (AON-6) are indicated. **(C)** Whole-membrane scan of western blot analysis showing unaffected *DMPK* protein level in non-DM1 fibroblasts dosed with 75 nM of indicated AONs directed at e5 (AON-4) and e9 (AON-6). Low exposition (left) and high exposition (right) images of the same western blot analysis are shown to visualize protein ladder (M) and lack of detectable truncated protein isoforms. Three lanes indicate individual samples.



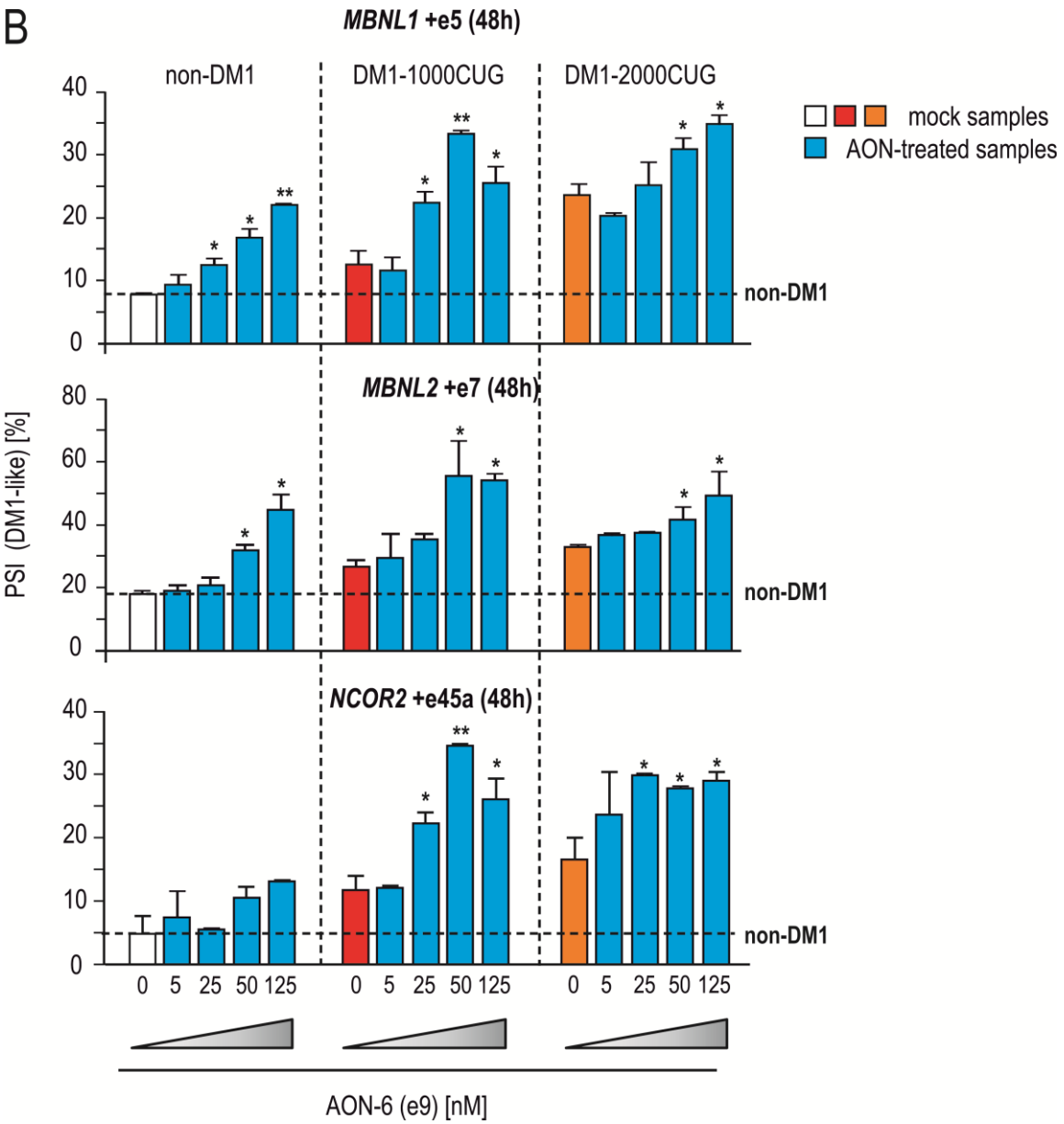
Supplementary Figure S5.

Supplementary Figure S5.

Antisense gapmers complementary to *DMPK* intron 14 efficiently reduce *DMPK* RNA. (A) Schematic representation of distinct regions within *DMPK* intron 14 (i14) targeted by antisense gapmers Gap i14-1 and Gap i14-2. White boxes indicate exons, horizontal line indicates i14, approximate gapmer binding sites are depicted. **(B)** Representative RT-PCR gels (top) and quantitations (bottom) of total *DMPK* mRNA in unaffected (left panel) vs DM1-affected (right panel) human fibroblasts treated with indicated amounts of Gap i14-1 (left panel) and Gap i14-2 (right panel) for 3 ½ days. Gapmer dose increment is indicated with triangles. **(C)** qPCR (real-time PCR) – based quantitation of *DMPK* transcript level in non-DM1 (top) vs DM1-affected (bottom) human fibroblasts 72h after dosing the cells with indicated gapmer-AONs. Quantitation is based on analyses of two distinct amplicons across *DMPK* transcript: 3'UTR (left) or internal region spanning e9 through e10 (right). Grey bars indicate negative control – scrambled sequence gapmer-AON, black bars indicated positive control – gapmer-AON targeting CUG-repeat region. **(D)** Representative immunoblot showing lack of DMPK protein reduction in non-DM1 fibroblasts 96h post transfection with indicated AONs. Note that no protein reduction is detectable even after dosing the cells with gapmer AON. Antibody blocking peptide (BP) was used as a negative control.

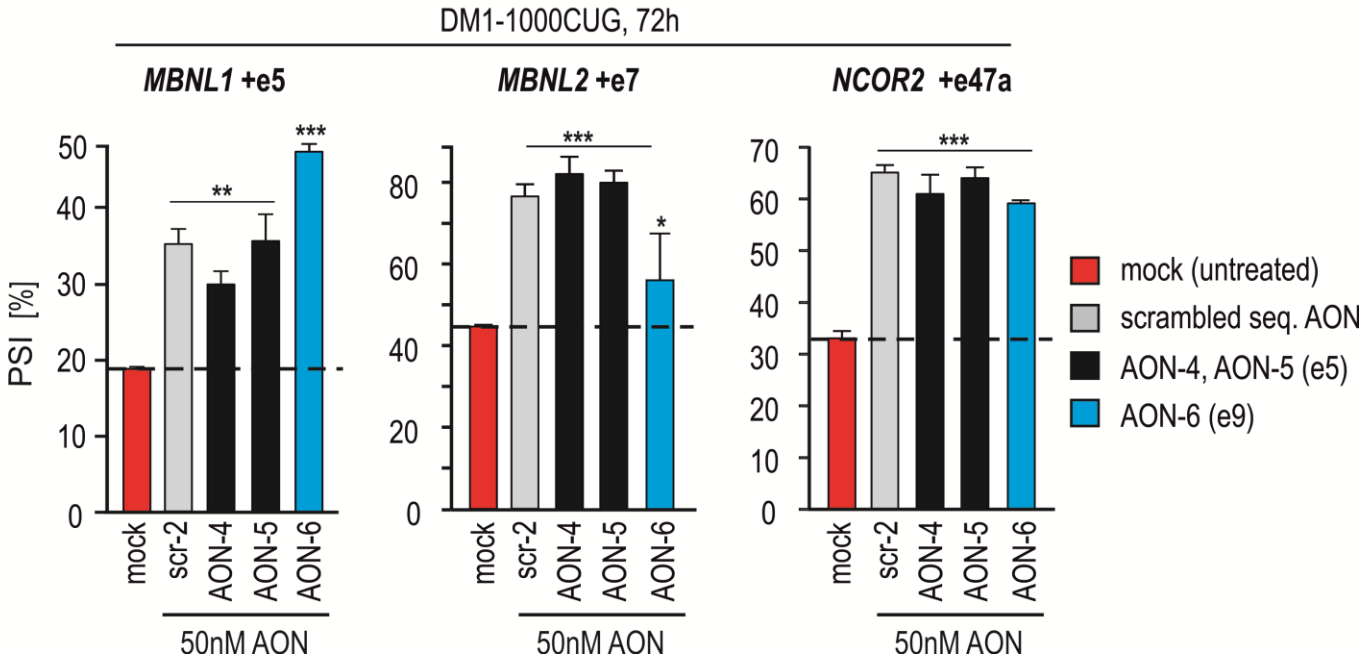


Supplementary Figure S6.



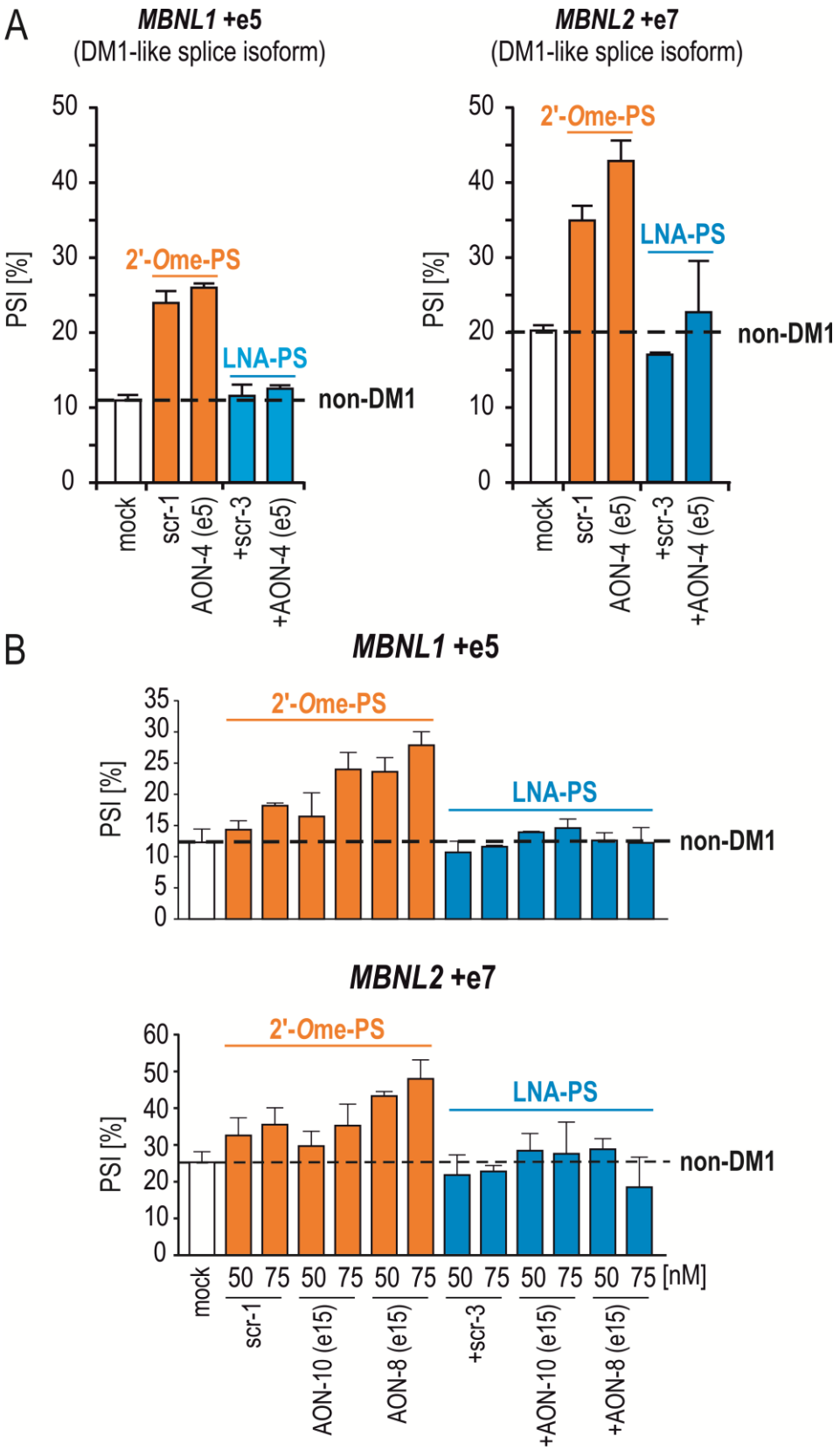
Supplementary Figure S6.

C



Supplementary Figure S6.

2'-*O*me-PS-modified AONs aggravate pre-existing DM1-spliceopathy. RT-PCR-based quantitation of splicing changes in the alternative exons of established DM1-biomarker transcripts: *MBNL1*, *MBNL2*, and *NCOR2* in control (non-DM1) and DM1 fibroblasts (DM1-1000CUG and DM1-2000CUG) dosed with increasing amounts of indicated AONs targeting e5 **(A, C)** or e9 **(B, C)**. Dose increment is depicted with triangles, and exact AON amounts are indicated. Increasing percentages of alternative exons inclusion (PSI) observed in (A-C) upon increasing doses of AONs directed at e5 and e9 indicate a DM1-like splicing shift of a given alternative exon (compare mock samples in non-DM1 vs DM1 cells). Time-points post AON treatment are indicated. Horizontal dashed lines mark the percentage of alternative exon inclusion in mock (lipofectamine) controls. Statistical significance compared to respective mock controls is indicated.



Supplementary Figure S7.

LNA-PS equivalents of 2'-*O*me-PS splice-switching AONs do not affect alternative splicing events in non-DM1 fibroblasts. (A-B) Quantitation of DM1-like splicing shift (alternative exon inclusion; PSI) in

the alternative exons of indicated DM1 biomarkers in non-DM1 fibroblasts 48h post transfection with indicated amounts of AONs targeting the same core sequence within e5 (A) or e15 (B), but harboring distinct chemical modification – either 2'-*O*me-PS (orange) or LNA-PS (blue). 2'-*O*me-PS- and LNA-PS-modified scrambled sequence AONs were used as chemistry controls (scr-1 and +scr-3, respectively).

SUPPLEMENTARY TABLES

2'-*O*me-PS AONs

AON name	target exon (e) / intron (i) ‡	sequence 5'-3'	length (nt)
SPLICE-SWITCHING AONs TARGETING e5			
AON-1	e5 -15/+10	mU* mG* mA* mC* mC* mA* mG* mG* mU* mA* mC* mU* mG* mA* mG* mA* mA* mG* mG* mG* mG*	25
AON-2	e5 +20+41	mG* mU* mC* mA* mG* mC* mA* mG* mG* mU* mC* mC* mC* mC* mG* mC* mC* mC* mA* mC* mG* mU	22
AON-3	e5 +22+43	mG* mU* mG* mU* mC* mA* mG* mC* mA* mG* mG* mU* mC* mC* mC* mC* mG* mC* mC* mC* mA* mC	22
AON-4	e5 +60+81	mC* mA* mU* mC* mU* mC* mG* mG* mC* mC* mG* mG* mA* mA* mU* mC* mC* mG* mC* mU* mC* mC	22
AON-5	e5 +135+149/-7	mC* mA* mC* mC* mC* mA* mC* mC* mU* mG* mU* mG* mC* mA* mC* mG* mU* mA* mG* mC* mC* mA	22
SPLICE-SWITCHING AONs TARGETING e9			
AON-6	e9 +63+80	mG* mC* mC* mA* mU* mG* mC* mA* mG* mG* mA* mG* mU* mA* mG* mG* mA* mG	18
SPLICE-SWITCHING AONs TARGETING e15			
AON-7	e15 -15/+10	mG* mC* mC* mU* mA* mG* mG* mG* mA* mC* mC* mU* mG* mC* mG* mG* mG* mG* mA* mG* mA*	25
AON-8	e15 +39+59	mG* mA* mC* mA* mG* mA* mA* mC* mA* mA* mC* mG* mG* mC* mG* mA* mA* mC* mA* mG* mG	21
AON-9	e15 +92+112	mU* mG* mA* mG* mU* mU* mG* mG* mC* mC* mG* mG* mC* mG* mU* mG* mG* mG* mC* mC* mA	21
AON-10	e15 +121+141	mG* mG* mC* mG* mG* mC* mU* mC* mC* mU* mG* mG* mG* mC* mG* mG* mC* mG* mC* mC* mA	21
AON-11	e15 +145+153/-12	mC* mA* mG* mU* mU* mC* mU* mA* mG* mG* mG* mU* mU* mC* mA* mG* mG* mG* mA* mG* mC	21
SCRAMBLED SEQUENCE CONTROL AONs			
scr-1	none	mG* mC* mC* mU* mC* mG* mA* mC* mC* mU* mA* mU* mC* mG* mC* mG* mC* mC* mU* mC* mA* mG	22
scr-2	none	mG* mA* mG* mA* mA* mC* mG* mA* mA* mC* mA* mA* mC* mG* mA* mG* mC* mG* mG* mC* mA	21

2'-*O*me AONs

AON name	target exon (e) / intron (i) ‡	sequence 5'-3'	length (nt)
SPLICE-SWITCHING AONs TARGETING e5			
AON-4"	e5 +60+81	mC mA mU mC mU mC mG mG mC mC mG mG mA mA mU mC mC mG mC mU mC mC	22

LNA-PS AONs

AON name	target exon (e) / intron (i) ‡	sequence 5'-3'	length (nt)
SPLICE-SWITCHING AONs TARGETING e5			
+AON-4	e5 +64+77	+T*+C*+G*+G*+C*+C*+G*+G*+A*+A*+T*+C*+C*+G	14
SPLICE-SWITCHING AONs TARGETING e9			
+AON-6	e9 +63+76	+T*+G*+C*+A*+G*+G*+A*+G*+T*+A*+G*+G*+A*+G	14
SPLICE-SWITCHING AONs TARGETING e15			
+AON-8	e15 +39+52	+C*+A*+A*+C*+G*+G*+C*+G*+A*+A*+C*+A*+G*+G	14
+AON-8'	e15 +57+70	+C*+G*+G*+C*+G*+G*+C*+A*+C*+G*+A*+G*+A*+C	14
+AON-10	e15 +125+138	+G*+G*+C*+T*+C*+C*+T*+G*+G*+G*+C*+G*+G*+C	14
SCRAMBLED SEQUENCE CONTROL AON			
+scr-3	none	+A*+G*+C*+A*+G*+G*+G*+A*+C*+G*+C*+A*+A*+C	14

LNA-PS/DNA-PS AONs

RNase H-COMPATIBLE GAPMER AONs			
Gap i14-1	i14 +67+82	A*A*A*T*G*C*G*C*A*G*C*T*A*A*G*C	16
Gap i14-2	i14 +183+197	G*T*T*A*G*T*C*C*A*C*T*C*G*C*A	15
Gap ctrl (+) CAG14	CUG repeat region; (+) control	+A*+G*+C*A*G*C*A*G*C*A*G*+C*+A*+G	14

NEGATIVE CONTROL GAPMER AON			
Gap ctrl (-)	none; (-) control	G*C*T*C*C*C*T*T*C*A*A*T*C*C*A*A	16

Supplementary Table 1.

Summary of AONs used in this study. 5'-3' sequences are shown including 2'-*O*methyl (m) and phosphorothioate modifications (*) as well as locked nucleic acid modifications (+). (‡) Numbers correspond to complementary region within targeted exon relative to the first nucleotide (+1). (/) indicates AONs targeting an intron-exon (-/+) or exon-intron (+/-) boundary, respectively.

Note: the position of LNA modifications in Gapmer AONs is proprietary to Exiqon. CAG14 gapmer used as a positive control was described previously (14).

A

***DMPK* e5:**
TACCTGGTCATGGAGTATTACGTGGGCGGGGACCTGCTGACACTGCTGAGCAAGTTTGGGGAGCGGATTCCGGCCGAGATGGCGCGCTTCTACCTGGCGGAGAT
TGTCATGGCCATAGACTCGGTGCACCGGCTTGGCTACGTGCACAG

SRSF1 **threshold: 1.956**			**SRSF1 (IgM-BRCA1)** **threshold: 1.867**			**SRSF2** **threshold: 2.383**			**SRSF5** **threshold: 2.67**			**SRSF6** **threshold: 2.676**		
Position*/Site/Score			Position*/Site/Score			Position*/Site/Score			Position*/Site/Score			Position*/Site/Score		
24 (-126)	GGGCGGG	1.99388	34 (-116)	CTGCTGA	2.37079	38 (-112)	TGACACTG	2.91892	38 (-112)	TGACACT	2.81550	19 (-131)	TACGTG	3.76391
39 (-111)	GACACTG	2.16497	43 (-107)	CTGCTGA	2.37079	41 (-109)	CACTGCTG	2.93377	40 (-110)	ACACTGC	4.18642	62 (-88)	AGCGGA	3.06999
46 (-104)	CTGAGCA	2.03848	46 (-104)	CTGAGCA	2.73315	65 (-85)	GGATTCCG	3.80645	87 (-63)	CTTCTAC	2.95219	69 (-81)	TCCGGC	3.01803
61 (-89)	GAGCGGA	3.16789	71 (-79)	CGGCCGA	4.91206	66 (-84)	GATTCCGG	2.97103	117 (-33)	AGACTCG	3.90189	85 (-65)	CGCTTC	3.00116
71 (-79)	CGGCCGA	4.90209	74 (-76)	CCGAGAT	2.12433	85 (-65)	CGCTTCTA	2.98317				125 (-25)	TGCACC	2.92201
98 (-52)	CGGAGAT	2.36054	83 (-67)	CGCGCTT	2.20838	111 (-39)	GGCCATAG	3.22712				139 (-11)	TACGTG	3.76391
127 (-23)	CACCGGC	2.78479	94 (-56)	CTGGCGG	2.43327									
			98 (-52)	CGGAGAT	3.10259									
			127 (-23)	CACCG	3.36278									

B

***DMPK* e9:** GAGACACTGTCGGACATTCGGGAAGGTGCGCCGCTAGGGGTCCACCTGCCTTTTGTGGGCTACTCCTACTCCTGCATGGCCCTCAG

SRSF1 **threshold: 1.956**			**SRSF1 (IgM-BRCA1)** **threshold: 1.867**			**SRSF2** **threshold: 2.383**			**SRSF5** **threshold: 2.67**			**SRSF6** **threshold: 2.676**		
Position*/Site/Score			Position*/Site/Score			Position*/Site/Score			Position*/Site/Score			Position*/Site/Score		
3 (-84)	GACACTG	2.16497	7 (-80)	CTGTCGG	2.49246	2 (-85)	AGACACTG	3.21649	52 (-35)	TTTGTGG	2.99502	27 (-60)	TGCGCC	3.53067
7 (-80)	CTGTCGG	2.04268	11 (-76)	CGGACAT	3.31052	29 (-58)	CGCCGCTA	3.07557	60 (-27)	CTACTCC	4.26167	73 (-14)	TGCATG	3.76391
11 (-76)	CGGACAT	2.96466	29 (-58)	CGCCGCT	3.21040	40 (-47)	GTCCACCT	2.42696	66 (-21)	CTACTCC	4.26167			
			46 (-41)	CTGCCTT	2.41158	58 (-29)	GGCTACTC	3.48823						
						61 (-26)	TACTCCTA	3.36043						
						67 (-20)	TACTCCTG	3.81032						
						78 (-9)	GGCCCTCA	3.62831						
						79 (-8)	GCCCTCA	2.63323						

Supplementary Table 2.

C

***DMPK* e15** (coding sequence only):
GTCCCTAGGCCTGGCCTATCGGAGGCGCTTTCCCTGCTCCTGTTCGCCGTTGTTCTGTCTCGTGCCGCCGCCCTGGGCTGCATTGGGTTGGTGGCCCACGCCGGCCAACTCACCGCA
GTCTGGCGCCGCCCAGGAGCCGCCCGCGCTCCCTGA

SRSF1 threshold: 1.956			SRSF1 (IgM-BRCA1) threshold: 1.867			SRSF2 threshold: 2.383			SRSF5 threshold: 2.67			SRSF6 threshold: 2.676		
Position*/Site/Score			Position*/Site/Score			Position*/Site/Score			Position*/Site/Score			Position*/Site/Score		
20 (-134)	CGGAGGC	3.08488	20 (-134)	CGGAGGC	3.68554	1 (-153)	GTCCCTAG	3.05185	2 (-152)	TCCCTAG	3.11530	63 (-91)	TGCCGC	3.01803
45 (-109)	CGCCGTT	2.07093	37 (-117)	CTCCTGT	2.52436	29 (-125)	TTTCCCTG	3.18541	15 (-139)	CCTATCG	2.80006	79 (-75)	TGCATT	2.96614
69 (-85)	CGCCCTG	2.30151	45 (-109)	CGCCGTT	3.31440	35 (-119)	TGCTCCTG	4.17117	31 (-123)	TCCCTGC	2.84049			
95 (-59)	CCCACGC	2.65699	65 (-89)	CCGCCGC	2.50941	42 (-112)	GTTCGCCG	3.69293	56 (-98)	TGTCTCG	3.90423			
99 (-55)	CGCCGGC	2.33756	66 (-88)	CGCCGCC	2.01370	57 (-97)	GTCTCGTG	4.65783	108 (-46)	ACTCACC	3.07925			
102 (-52)	CGGCCAA	2.32435	68 (-86)	CCGCCCT	2.05880	63 (-91)	TGCCGCCG	2.85990	110 (-44)	TCACCGC	3.74479			
109 (-45)	CTCACCG	2.16204	69 (-85)	CGCCCTG	3.31465	86 (-68)	GGTTGGTG	3.83890	117 (-37)	AGTCTGG	3.16024			
111 (-43)	CACCGCA	2.41369	71 (-83)	CCCTGGG	2.41702	93 (-61)	GGCCCACG	4.53042						
129 (-25)	CCCAGGA	4.25952	95 (-59)	CCCACGC	3.29648	118 (-36)	GTCTGGCG	3.11571						
138 (-16)	CGCCCGC	2.94168	99 (-55)	CGCCGGC	3.66101	138 (-16)	CGCCCGCG	2.86339						
			102 (-52)	CGGCCAA	3.13241	145 (-9)	GCTCCCTG	3.84665						
			109 (-45)	CTCACCG	2.88698									
			111 (-43)	CACCGCA	3.13987									
			114 (-40)	CGCAGTC	2.52351									
			124 (-30)	CGCCGCC	2.01370									
			126 (-28)	CCGCCCA	2.28650									
			127 (-27)	CGCCCAG	3.07831									
			129 (-25)	CCCAGGA	4.51294									
			138 (-16)	CGCCCGC	3.86893									
				CTCCCT										

Supplementary Table 2.

Tabular output of ESE analyses within *DMPK* e5 (A), e9 (B) and e15 (C). Putative ESEs were determined using ESEfinder 3.0 (34,35). The score values (relative to default threshold values) of ESEs corresponding to different SRSF are listed. Both positions from 5' end and 3' end (marked with "-") of the analyzed exonic sequence are given.

PCR target	Assay	Primer set	5'-3' sequence
***DMPK* Δe5**	AON-induced e5 skipping; *DMPK* mRNA expression	DMPK_e4F	AGAGGGACGTGTTGGTGAAT
		DMPK_e7R	GATCTCGGGGGACAGGTAGT
***DMPK* Δe9**	AON-induced e9 skipping; *DMPK* mRNA expression	DMPK_e8F	CCTTCTTCTTTGGCCTCGAC
		DMPK_e10R	CTTGCACGTGTGGCTCAAG
***DMPK* Δe15**	AON-induced e15 skipping	DMPK_10F	CCTGGAGGAGGAGGTGCT
		DMPK_558R	CTTTGCGAACCAACGATAGG
***DMPK* 3'UTR**	*DMPK* mRNA expression [qPCR]	DMPK 3'UTR_F	GCGATCTCTGCCTGCTTACT
		DMPK 3'UTR_R	GTCCTAGGTGGGGACAGACA
***DMPK* e9-e10**	*DMPK* total mRNA expression (e9-e10); +e9 mRNA expression	N11	CACTGTCGGACATTCGGGAAGGTGC
		133	GCTTGCACGTGTGGCTCAAGCAGCTG
***DMPK* +e5 mRNA**	*DMPK* total mRNA expression (e5-e7); +e5 mRNA expression	DMPK_e5-F	CTTCTACCTGGCGGAGATTG
		DMPK_e7-R	GATCTCGGGGGACAGGTAGT
***CELF1* 3'UTR**	mRNA expression	CBP1_F	CCAAGGACCTGGTCTGAAAA
		CBP1_R	ATCCCTGGGAGGACTTTCAT
GAPDH	mRNA expression	GAPDH_F	CATCAATGGAAATCCCATCAC
		GAPDH_R	GGTTTTTCTAGACGGCAGGTC
i14/e15/CUG	AON-induced splicing out of *DMPK* pre-mRNA fragment containing i14/e15/CUG [qPCR]	DMPK_i 14F	AGTCCCAGGAGCCAATCAG
		DMPK_e15R	CCCGGAGTCGAAGACAGTTC
DMPK i9-e10	pre-mRNA expression (i9-e10) [qPCR]	DMPK_i9F	CATTCTGTCTCCCTCCCCAC
		DMPK_e10R	CTTGCACGTGTGGCTCAAG
***MBNL1* e1**	alternative splicing	HuEx1_F1	CAGCGACATGCAACAGTCTT
		HuEx1_R1	TGTCAGCAGGATGAGCAAAC
***MBNL1* e5**	alternative splicing	MBNL1_F	ACCAACAGGCTCTAGCCAACATGC
		MBNL1_R	CGTCCTTTACTCTAACCAAGC
***MBNL2* e7**	alternative splicing	MBNL2_F	GCTGCCCAATACCAGGTCAAC
		MBNL2_R	TGGTGGGAGAAATGCTGTATGC
***NCOR2* e45a**	alternative splicing	NCOR2_F	ACACCCACAACCGGAATGAGCCTG
		NCOR2_R	GGACTTGGCTTTTCGGCTGCTG

Supplementary Table 3.

PCR primers used for alternative splicing assays and gene expression analyses. Assay, PCR targets as well as 5'-3' sequences are indicated for each primer pair.