Selective silencing of mutated mRNAs in DM1 by using modified hU7-snRNAs

Virginie Francois^{1-4*}, Arnaud F. Klein^{1-4*}, Cyriaque Beley¹⁻⁴, Arnaud Jollet¹⁻⁴, Camille Lemerrier¹⁻⁴, Luis Garcia^{1-4§} & Denis Furling^{1-4§}

1- Université Pierre et Marie Curie-Paris6, Umr76, Paris, F-75013, France

2- CNRS, Umr7215, Paris, F-75013, France

3- INSERM, U974, Paris, F-75013, France

4- Institut de Myologie, Paris, F-75013, France

*Both authors should be considered as co-first authors

Correspondence should be addressed to D.F. (denis.furling@upmc.fr)

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We describe a novel function for modified human U7-snRNAs distinct from modification of pre-mRNA splicing events. Engineered hU7-snRNAs harboring a poly-CAG antisense sequence targeting the expanded CUG repeats of mutant *DMPK* transcripts in myotonic dystrophy cause specific degradation of pathogenic *DMPK* mRNAs without affecting wild-type *DMPK* allele products. Abolition of the RNA-gain-of-function toxicity responsible for pathogenesis supports use of hU7-snRNAs for gene silencing in RNA-dominant disorders expressing expanded repeats.

Myotonic dystrophy type 1 (DM1), the most prevalent form of adult muscular dystrophy¹ is caused by expanded CTG repeats in the 3'untranslated region of the DM protein kinase (*DMPK*) gene². This autosomal dominant genetic disease belongs to a group of RNA gain-of-function disorders³ expressing pathogenic RNAs. Mutant *DMPK* transcripts containing up to thousands of expanded CUG repeats (CUG^{exp}) are entrapped in the nucleus⁴. Such an accumulation alters the regulation of alternative splicing, which subsequently leads to mis-splicing of several mRNA transcripts and neuromuscular dysfunction⁵. Indeed, CUG^{exp} RNAs are folded in such a way that they aberrantly interact with proteins in the nucleus and bind muscleblind-like 1 (MBNL1) with high affinity to form stable ribonucleoprotein aggregates or foci⁶. Sequestration of MBNL1 in these complexes leads to a loss-of-function of this splicing regulator and alternative splicing defects⁷.

Strategies for phenotype rescue in DM1 using synthetic antisense oligonucleotides that target CUG expansions have been evaluated in mouse DM1 models^{8, 9}. Local delivery of either morpholinos or 2'-*O*-methyl phosphorothioate oligonucleotides was performed with the aim to interfere and unfold CUG^{exp} RNAs to release MBNL1 from foci, thus making it available for its overall splicing function. However the use of synthetic oligonucleotides requires repeated treatments to counteract continuous expression of toxic CUG^{exp} RNAs. To circumvent this limitation and trigger a long lasting effect, we have developed an optimized human U7-snRNA harboring a poly-CAG antisense sequence to target the CUG^{exp} RNAs (**Fig.1a**). A fragment of about 0.5kb containing the human *U7* gene was amplified from human genomic DNA and the U7-snRNA transcript was optimized¹⁰. First, its Sm binding domain was replaced by a canonical Sm sequence derived from the major snRNA (Sm-Opt) to bind appropriate Sm proteins that allow efficient snRNP assembly and increase its nuclear accumulation¹¹. Subsequently, the natural histone pre-mRNA complementary sequence of U7-snRNA was replaced by a poly-CAG. The engineered human U7-snRNA-(CAG)_n was kept under the control of its natural promoter and 3' downstream elements (**Supplementary**

Fig.1). This construct was further cloned into a lentiviral backbone for high efficiency gene transfer and long lasting expression in human cells.

To evaluate the potential of engineered hU7-siRNA-(CAG)_n to target the CUG^{exp} RNAs, skeletal muscle cells isolated from DM1 patients with various sizes of CTG expansions were transduced with lentiviral vectors expressing an optimized hU7-(CAG)₁₅ (hU7-siRNA with 15 CAG repeats). In these cells, mutant alleles exhibited CTG expansions ranging from 800 to 2000 repeats while normal-sized alleles contained less than 37 repeats. Transduced cells were grown for several divisions before assessing *DMPK* mRNA stability. Northern blot analysis showed that the steady-state levels of CUG^{exp} *DMPK* transcripts were significantly ($P<0.001$) reduced by 71 to 82% in DM1 cells expressing hU7-(CAG)₁₅ (**Fig.1b**). Disappearance of expanded *DMPK* mRNAs occurred in a vector dose-dependent manner (**Supplementary Fig.2a**), which correlated with hU7-(CAG)₁₅ expression in transduced DM1 cells (**Supplementary Fig.2b**). The hU7-(CAG)₁₅ system allowed continuous and permanent targeted destruction of the deleterious transcripts over subsequent cell divisions (**Supplementary Fig.2c**) as well as in non-mitotic muscle cells (**Supplementary Fig.2d,e**). Importantly, the normal-sized *DMPK* mRNA was preserved in treated DM1 cells (**Fig.1b**). This phenomenon was also confirmed in transduced wild-type myoblasts containing normal-sized *DMPK* alleles (**Supplementary Fig.3**). In addition, expression of a modified hU7-control (without poly-CAG repeats) in DM1 cells did not induce degradation of CUG^{exp} *DMPK* transcripts demonstrating the specificity of the poly-CAG antisense sequence (**Supplementary Fig.4**). Furthermore, no specific occurrence of alternative modes of *DMPK* transcripts was induced by hU7-(CAG)₁₅ in DM1 cells (**Supplementary Fig.5a** and data not shown). In particular, the alternative splicing event in the 3'*DMPK* region leading to the *DMPK* isoform exon14/exon16 (lacking both exon15 and CUG tract due to the presence of a putative 3'*DMPK* exon16)¹² was not promoted by hU7-(CAG)₁₅, and no change in *DMPK* protein levels was observed in treated-DM1 cells (**Supplementary Fig.5b**), establishing the selective silencing of CUG^{exp} *DMPK* transcripts by engineered hU7-(CAG)₁₅.

To further assess the disappearance of mutated *DMPK* mRNAs, we examined the CUG^{exp}-ribonucleoprotein complexes, which accumulate as foci in nuclei of DM1 cells. Fluorescence in situ hybridization (FISH) analysis showed a dramatic dose-dependent loss of these nuclear structures in treated DM1 myoblasts (**Fig. 1c and Supplementary Fig.6**). Up to 60% of the hU7-(CAG)₁₅ treated DM1 myoblasts displayed no foci ($P<0.001$). In addition, 25% of them displayed only a single faint focus and no remaining foci were observed in the cytoplasmic compartment. Nuclear and cytoplasmic RNA fractionation of DM1 cells containing a

polymorphic restriction site on the expanded *DMPK* allele¹³ confirmed that mutated transcripts are retained in the nucleus and are not exported to the cytoplasm (**Fig. 1d**). In the presence of hU7-(CAG)₁₅, no transcripts from the expanded allele were detected in the cytoplasmic fraction suggesting a selective CUG^{exp} *DMPK* mRNAs nuclear degradation mechanism. Strikingly, this mechanism did not affect *DMPK* pre-mRNAs, indicating that hU7-(CAG)₁₅ acted at mRNAs or foci levels rather than at the RNA genesis stage.

In order to evaluate the effect of the length of the CAG antisense sequence, we designed additional hU7-siRNA constructs harboring shortened CAG sequences (7 and 11 repeats). All hU7-(CAG)_n targeted and silenced efficiently CUG^{exp} *DMPK* mRNAs ($P<0.001$) in DM1 cells (**Fig.2a**), when expressed at similar level in DM1 cells (**Supplementary Fig.7a**). However the shortened constructs also affected ($P<0.01$) the products of the normal-sized *DMPK* allele bearing 13 repeats. Such a loss of specificity prompted us to analyze six non-related human transcripts bearing CUG tracts ranging from 7 to 16 repeats (**Supplementary Fig.7b**). Four of these gene products were unaffected in DM1 cells expressing hU7-(CAG)₇either11or15. Nevertheless, altered expressions of *CPA6*(7CUG) and *LRP8*(11CUG) transcripts were observed in the presence of hU7-(CAG)_{7or11} while the larger (CAG)₁₅ construct did not affect any of the selected transcripts.

To determine whether hU7-(CAG)₁₅ can reverse pathophysiological consequences of toxic CUG^{exp} RNAs, we focused on DM1 hallmarks such as MBNL1 sequestration and abnormal regulation of alternative splicing. Even if MBNL1 distribution is not homogenous in primary DM1 cell cultures (**Supplementary Fig.8a,b**), combined FISH-immunofluorescence analysis showed that silencing CUG^{exp} mRNAs leads to the release of sequestered MBNL1 from the nuclear CUG^{exp}-aggregates and a redistribution of MBNL1 in treated DM1 cells (**Fig.2b**) as confirmed by the reduced number of DM1 cells containing MBNL1 foci (**Supplementary Fig.8c**). The outcome on DM1 splicing misregulation was examined on *DMD*, *BIN1* and *LDB3* genes, which are abnormally spliced in differentiated DM1 muscle cells. Splicing profiles of these genes were significantly normalized ($P<0.05$) in the presence of hU7-(CAG)₁₅, while hU7-(CAG)₁₅ did not affect the splicing of these genes in wild-type cells (**Fig.2c**). In addition, hU7-(CAG)₁₅ did not induce a general shift in splicing since alternative splice modes that are unaltered in DM1 samples⁷ were not affected (**Supplementary Fig.9**). Moreover, because large CUG expansions altered the myogenic differentiation of DM1 muscle cells¹⁴, we also tested whether hU7-(CAG)₁₅ can overcome this deficiency. In the presence of hU7-(CAG)₁₅, the fusion index of treated-DM1 myoblasts was appreciably restored ($P<0.01$) to a level similar to that of wild-type myoblasts (**Fig.2d**).

In conclusion, our data show that the hU7-(CAG)_n system allowed long-lasting selective destruction of deleterious CUG^{exp} RNAs in DM1 cells. MBNL1 and possibly other mRNA binding factors are subsequently released from foci, thereby improving splicing abnormalities and differentiation defects. The length of the CAG antisense sequence seems critical because hU7-(CAG)_n harboring less than 15 CAG repeats affects the normal-size *DMPK* allele products and perturbs the expression of some non-related transcripts bearing short (CUG)_n tract. The precise mechanism by which the hU7-(CAG)₁₅ triggers selective and robust silencing of CUG^{exp} RNAs is not fully determined but seems to require the association of multiple hU7-sRNA molecules. Engineered hU7-sRNA-(CAG)_n described in this study as well as morpholinos⁸ or 2'-*O*-methyl phosphorothioates⁹, which have been successfully applied in DM1 models, are not supposed to trigger RNase activities. On the contrary, these compounds are commonly used to force alternative splicing in both exon skipping and exon inclusion strategies¹⁵. It seems unlikely that the selective destruction of CUG^{exp} *DMPK* mRNAs in the presence of these (CAG)_n antisense molecules is based on canonical RNA interference mechanisms. Furthermore, small 19-22 nucleotides RNA fragments composed of CUG or CAG repeats were not detected in DM1 cells expressing or not hU7-(CAG)₁₅ (**Supplementary Fig.2b**), strongly suggesting that the RNAi machinery is not involved in hU7-(CAG)₁₅ activity. Finally, specific hU7-(CAG)₁₅-mediated silencing that appears to take place in the nuclear compartment, is not a splicing-related mechanism. Rather, disappearance or accelerated decay of CUG^{exp} RNA/ hU7-(CAG)₁₅ heteroduplexes may be due to natural instability of the mutant *DMPK* mRNAs that is counteracted by MBNL1 binding in DM1 pathological context, or to a novel RNA degradation mechanism. Taken together, abolition of the RNA toxicity associated with DM1 pathogenesis supports the use of hU7-sRNAs for gene silencing in RNA-dominant disorders expressing expanded repeats.

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AUTHOR CONTRIBUTIONS

V.F. and A.K. conducted most of the experiments. C.B. performed the constructs. A.J. produced the lentiviral vectors. C.L. supported some experiments. L.G. engineered the modified hU7-snRNA. D.F. supervised the entire project. L.G. and D.F wrote the manuscript.

[§]L.G and [§]D.F should be considered as equal contributors.

COMPETING FINANCIAL INTERESTS

The method described in this paper is the subject of a patent application (inventors L.G, D.F, C.B and T.V)

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FIGURE LEGENDS

Figure 1 CUG^{exp} RNAs silencing by hU7-(CAG)₁₅. **(a)** Structure of the hU7-snRNA-(CAG)₁₅ indicating the loop, the Sm-Opt and the CAG antisense sequences. **(b)** Representative Northern blot and analysis (n=5) of *DMPK* mRNA expression in DM1 muscle cells (13/800CTG) transduced with hU7-(CAG)₁₅ lentiviral vector ($4\text{-}8 \times 10^6$ vg ml⁻¹). **(c)** FISH analysis (n=4) of the number of CUG^{exp}-mRNA foci (red spots) into the nuclei (blue) of hU7-(CAG)₁₅ transduced (8×10^6 vg ml⁻¹) DM1 cells (800CTG). **(d)** RT-PCR assay of normal and CUG^{exp}-*DMPK* mRNA, *GAPDH* mRNA, U6 snRNA and *DMPK* pre-RNA in nuclear and cytoplasmic fractions of DM1 converted muscle cells (11/1300CTG). BpmI restriction site polymorphism located within exon 10 of expanded *DMPK* allele identifies normal and CUG^{exp} allele products.

Figure 2 Consequences of hU7-(CAG)_n expression in DM1 muscle cells **(a)** Expression of normal and CUG^{exp}-*DMPK* mRNAs in DM1 cells (13/800CTG) transduced with hU7-(CAG)_n vectors (4×10^6 vg ml⁻¹) harboring antisense sequences of 7, 11 or 15 CAG (Northern blot, n=3). **(b)** Localization of the splicing regulator MBNL1 in DM1 cells. **(c)** Correction of alternative splicing misregulation of *BIN1*, *DMD* and *LDB3* transcripts in differentiated DM1 muscle cells (2000CTG) transduced with hU7-(CAG)₁₅ vectors (8×10^6 vg ml⁻¹)(RT-PCR, n=3). **(d)** Effect of hU7-(CAG)₁₅ expression on myogenic differentiation of DM1 muscle cells (2000CTG) quantified as fusion index (n=6).

a **b** **c** **d**

Supplementary information

Selective silencing of mutated mRNAs in DM1 by using modified hU7-snRNAs

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TACTGCCGAATCCAGGTCTCCGGGCT
TAACAACAACGAAGGGGCTGTGACTG
GCTGCTTTCTCAACCAATCAGCACCGA
ACTCATTTGCATGGGCTGAGAACAAT
GTTGCGAACTCTAGAAATGAATGACT
TAAGTAAGTTCCTTAGAATATTATTTTC
CTACTGAAAGTTACCACATGCGTCGTT
GTTTATACAGTAATAGGAACAAGAAAAA
AGTCACCTAAGCTCACCCATCAATT
GTGGAGTTCCTTTATATCCCATCTTCTC
TCCAAACACATACGCA CAGCAGCAGC
AGCAGCAGCAGCAGCAGCAGCAGCA
GCAGCAGCAGAATTTTGGAGTAGGC
TTTCTGGCTTTTACCGGAAAGCCCCT
CTTATGATGTTTGTTGCCAATGATAGAT
TGTTTTCACTGTGCAAAAATTATGGGTA
GTTTTGGTGGTCTTGATGCAGTTGTAA
GCTTGGAG

Supplementary Fig. 1 Sequence of the modified human U7 gene containing its natural promoter, the (CAG)₁₅ antisense sequence (in red), the Sm binding domain (Sm-Opt in blue), the loop (in green) and its 3' downstream elements.

Supplementary Fig. 2 (a) *DMPK* mRNA expression was analyzed by Northern blot in DM1 muscle cells (800CTG) transduced with increasing concentrations of hU7-(CAG)₁₅ lentiviral vector. (b) Expression of both hU7-snrRNA-(CAG)₁₅ and endogenous hU7-snrRNA was analyzed by Northern blot using a human U7 probe in DM1 myoblasts (left panels). Expression of hU7-(CAG)₁₅ (87 nt) and small 19-22 nt RNA fragments composed of CAG or CUG repeats was assessed by Northern blot using (CTG)₇ or (CAG)₇ in DM1 myoblasts (right panels). Detection of miR-24 was used as control. (c) Silencing of the CUG^{exp} *DMPK* transcripts was measured by Northern blot in proliferating hU7-(CAG)₁₅-transduced DM1 myoblasts (8x10⁶ vg ml⁻¹) over a period of 16 days. (d) Silencing of CUG^{exp} *DMPK* transcripts was measured by Northern blot in non-proliferating hU7-(CAG)₁₅-transduced DM1 myoblasts (8x10⁶ vg ml⁻¹), blocked by mitomycin C (n=3). (e) Silencing of the CUG^{exp} *DMPK* transcripts was measured by Northern blot in non-mitotic/differentiated hU7-(CAG)₁₅-transduced DM1 muscle cells (8x10⁶ vg ml⁻¹) (n=3).

Supplementary Fig. 3 (a) *DMPK* mRNA expression in wild-type myoblasts (13/18CTG) transduced with hU7-(CAG)₁₅ lentiviral vector (8×10^6 vg ml⁻¹) was analyzed by RT-PCR (exon 9-exon 10). *GAPDH* was used as loading control (n=4). (b) *DMPK* mRNA expression in wild-type myoblasts (13/18 CTG) transduced with hU7-(CAG)₁₅ lentiviral vector (8×10^6 vg ml⁻¹) was analyzed by Northern blot (n=3).

Supplementary Fig. 4 (a) *DMPK* mRNA expression was analyzed in DM1 muscle cells (800CTG) transduced with either hU7-(CAG)₁₅ or hU7-control lentiviral vectors (8×10^6 vg ml⁻¹). (b) *DMD* mRNA expression in DM1 myoblasts transduced with hU7-control lentiviral vector containing an antisense sequence complementary to the 3' *DMD* region.

Supplementary Fig. 5 (a) Expression of the spliced *DMPK* mRNA isoform exon14/exon16 (E14/16, lacking exon 15 as well as CUG tract) in hU7-(CAG)₁₅-transduced DM1 myoblasts (8×10^6 vg ml⁻¹) was analyzed by RT-PCR (n=3). *GADPH* was used as loading control. (b) *DMPK* protein levels in hU7-(CAG)₁₅-transduced DM1 myoblasts (8×10^6 vg ml⁻¹) were examined by Western blot using the MANDM1 antibody that detects *DMPK* but also *CRP* proteins (n=7). The level of *CRP* was not altered in DM1 cells and was used as internal control for equal loading.

Supplementary Fig. 6 (a) The distribution of CUG^{exp} *DMPK* foci was determined in the nuclei of DM1 myoblasts (800CTG) transduced with increasing concentrations of hU7-(CAG)₁₅ lentiviral vector. (b) Representative fields of DM1 myoblasts transduced or not with increasing concentrations of hU7-(CAG)₁₅ lentiviral vector. CUG^{exp} *DMPK* foci were revealed by FISH with Cy3-labelled (CAG)₇ probe (red) and the nuclei were revealed with Hoechst staining (blue).

Supplementary Fig. 7 (a) Levels of hU7-(CAG)_n and endogenous hU7-snRNA in DM1 myoblasts transduced with hU7-(CAG)_n lentiviral vectors expressing antisense sequences of 7, 11 or 15 CAG were analyzed by RT-PCR. **(b)** Fold-change of non-related transcripts bearing short (CUG)_n tract was analyzed by RT-PCR in DM1 myoblasts expressing hU7-(CAG)_{7, 11 or 15}.

Supplementary Fig. 8 (a) Combined FISH-immunofluorescence to detect CUG^{exp} *DMPK* foci (Cy3-labeled probe, red) and MBNL1 (green) in primary DM1 muscle cells cultures. (b) Inserts (1-3) showed heterogeneity of MBNL1 distribution and intensity in DM1 cells. (c) Evaluation of the number of MBNL1 foci in DM1 myoblasts transduced or not with hU7-(CAG)₁₅ lentiviral vectors (3.4×10^6 vg ml⁻¹).

Supplementary Fig. 9 Effects of hU7-(CAG)₁₅ on alternative splice modes that were described as unaltered in DM1 samples (Lin et al., 2006) were analyzed by RT-PCR in transduced DM1 muscle cells (8×10^6 vg ml⁻¹)(n=3). Numbers below the gels indicate either ratio between major/minor bands when a splice event is detectable or ratio between targeted transcripts and *GAPDH* mRNAs.

SUPPLEMENTARY METHODS

Cell culture

Human muscle cells were isolated from skeletal muscle biopsies or autopsies as described¹, in accordance with French legislation on ethical rules. Wild-type (WT) and DM1 myoblasts were grown in HAM's F10 medium supplemented with 20 % FCS and 5 $\mu\text{g ml}^{-1}$ gentamycin (Invitrogen), at 5% CO₂ and 37°C. To trigger differentiation, growth medium was removed from subconfluent cultures and replaced by DMEM medium supplemented with 10 $\mu\text{g ml}^{-1}$ insulin and 100 $\mu\text{g ml}^{-1}$ transferrin (Sigma). To block cell division, the cultures were treated with 30 $\mu\text{g ml}^{-1}$ of mitomycin C (Sigma) for three days. DM1 fibroblasts containing a BpmI polymorphic restriction site on the expanded *DMPK* allele² were immortalized and converted into muscle cells using an inducible MyoD system as previously described³.

Lentivirus production and transduction

A self-inactivated HIV⁻¹-based lentiviral vector, pRRL-hU7-(CAG)_n and pRRL-hU7-control (containing the GAAGTCTTATCTTTAATATG antisense sequence) were generated from the previously described pRRL-cPPT-hPGK-EGFP-WPRE vector⁴. VSV-G-pseudotyped vectors were produced by transient transfection of 293T cells⁵. The conditioned medium containing viral particles was collected and concentrated by ultracentrifugation. Vector titers (vector genome vg ml⁻¹) were determined by quantitative PCR on genomic DNA of infected cells as described⁵. 1x10⁶ to 1x10⁷ vg ml⁻¹ were used to transduce 1.5x10⁵ human muscle cells. Vector transduction was performed overnight in the presence of 4 $\mu\text{g/ml}$ of polybrene (Sigma) and the transduced cells were grown and amplified at least one week before analyses.

RNA isolation and Northern blot

Cells were lysed in a proteinase K buffer (500 mM NaCl, 10 mM Tris-HCl, pH 7.2, 1.5 mM MgCl₂, 10 mM EDTA, 2% SDS and 0.5 mg ml⁻¹ of proteinase K) for 45 min at 55°C. Then, RNAs were isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNAs were also isolated from nuclear and cytoplasmic fractions prepared as described previously² by hypotonic lysis in the presence of NP-40. For Northern blot analysis, 8-10 μg of RNA was separated on 1.3 % agarose MOPS-gels containing 0.66 M formaldehyde and transferred onto Hybond-N+ membrane (Amersham Pharmacia Biotech) by capillary transfer

with 10x SSC. Blots were hybridized with random-primed ^{32}P -labeled (Bgl II-Sac I fragment of *DMPK* cDNA) probe in a hybridization buffer (2 % SDS, 10 % dextran sulfate, 1 x SSPE, 100 $\mu\text{g ml}^{-1}$ salmon sperm DNA, 2 % Denhart's) at 68°C overnight. Signals were analyzed on a phospho-imager (Molecular Imager FX, Bio-Rad) and quantified using Quantity One (Bio-Rad). All values were normalized to *18S* rRNA signal after hybridization with 5'-end ^{32}P -labeled *18S* rRNA-oligonucleotide probes. To detect the small hU7-snRNAs and miR-24, RNA was isolated using TRIzol reagent (Invitrogen). For Northern blot analysis, 15-20 μg of RNA was separated on 12 % polyacrylamide urea gels and transferred to Hybond-N+ membrane. Blots were hybridized with a hU7-snRNA ^{32}P -labeled probe (AGGGGCTTTCCGGTAAAA) or miR24 probe (TGTTCTGCTGAACTGAGCCA) in hybridization buffer (6 X SSPE, 2 % Denhart's, 0,1 % SDS) at 42°C overnight.

RT-PCR analysis

1 μg of RNA was reverse transcribed using M-MLV first-strand synthesis system according to the manufacturer's instructions (Invitrogen) in a total volume of 20 μl . One μL of cDNA preparation was subsequently used in a semiquantitative PCR analysis according to standard procedures (ReddyMix, Thermo Scientific). PCR amplification was carried out for 20-35 cycles, within the linear range of amplification for each gene. The signal of *GAPDH* was used for normalization. PCR products were analyzed on 1-3% agarose gels, stained by ethidium bromide. Quantification was done using the Quantity One software (Bio-Rad). For alternative splicing analysis, the genes and exons selected had been previously described as being altered in muscle from DM1 patients: exon 78 for *DMD*⁶ (dystrophin), exon 7 for *LDB3*⁷ (cypher) and exon 11 for *BINI*⁸ (bridging integrator1). The ratios of exon inclusion were quantified and expressed as percentage of inclusion relative to total intensities of isoform signals. For *DMPK* analysis, to distinguish the two alleles of *DMPK*, 6 μl of the PCR mixture was put into a 25 μl digestion mixture containing 2.5 units of BpmI (New England Biolabs) and incubated overnight at 37°C as described². For hU7-snRNA analysis, addition of a poly(A) tail to 2 μg of total RNA was made using poly(A) plus polymerase tailing kit (Epicentre technologies, Madison, WI) according to manufacturer protocol. Then 1 μg of the resulting poly(A)-RNAs was reverse transcribed using a specific dT-UPR primer. hU7-snRNA-(CAG)_n or endogenous hU7-snRNA were amplified using forward specific primers and reverse UPR primers.

The following primers were used:

GAPDH-F, TGAAGGTCGGAGTCAACGGATTTGGT

GAPDH-R, GATGACAAGCTTCCCGTTCTCAGCC

U6 snRNA-F, CTCGCTTCGGCAGCACA

U6 snRNA-R, AACGCTTCACGAATTTGCGT

DMPK exon 9-exon 10-F, CACTGTCGGACATTCGGGAAGGTGC

DMPK exon 9-exon 10-R, GCTTGCACGTGTGGCTCAAGCAGCTG

DMPK intron 9-intron 10-F, CTACCCACAGGCCAGAAGTT

DMPK intron 9-intron 10-R, GGAAGCCCTCACCTTTTCTC

DMPK intron 2-intron 4-F, CCCCAATCCTAGAGCTTCCT

DMPK intron 2-intron 4-R, CCGTCTCCACTCTGTCTCACT

DMPK splice junction exon 14/16-exon 16-F, CTGCTCCCTGCCAGGGCTGA

DMPK splice junction exon 14/16-exon 16-R, TGTCGGGGTCTCAGTGCATCCA

CPA6-F, ACTGATGTCCATATCCCCCA

CPA6-R, TTTGAGTCGTGATCGTCTGC

LTBP3-F, GAGAAGAGCCTGTGTTTCCG

LTBP3-R, GAAAAGTCACTCTCGCCCTG

LRP8-F, CTCCACTGACTTCCTGAGCC

LRP8-R, GTGCTCGGTAGCACCTCTTC

TMCC1-F, GAGCAAAGGTGACTGGCTTC

TMCC1-R, CGCTCCTCCTGTAAGGTCTG

CASK-F, CAGAGTTCGGCTGGTACAGT

CASK-R, ACAGGACGAAGACTGAGTGC

MAP3K4-F, AAGGGCACGTATAGCATTGG

MAP3K4-R, TGGTTCTCCAGCAGGTCTCT

BIN1-exon 11-F, AGAACCTCAATGATGTGCTGG

BIN1-exon 11-R, TCGTGGTTGACTCTGATCTCGG

DMD-exon 78-F, TTAGAGGAGGTGATGGAGCA

DMD-exon 78-R, GATACTAAGGACTCCATCGC

LDB3-exon 7-F, GCAAGACCCTGATGAAGAAGCTC

LDB3-exon 7-R, GACAGAAGGCCGGATGCTG

Endogenous hU7-snRNA-F, CAGTGTTACAGCTCTTTTAGA

hU7-snRNA-CAGn-F, AGCAGCAGCAGAATTTTTTGG

dT-UPR : TTTTTTTTTTTTTTTTTTTTTTTTGCAAAATGGCGTATTACCGACAGCCGTA

UPR-R, AATGGCGTATTACCGACAGC

FISH and immunofluorescence

Fluorescent in situ hybridization (FISH) was done as described⁹ using a Cy3-labeled peptide nucleic acid (CAG)₇ probe. To determine the number of foci per nucleus, more than 500 DM1 cells were counted in at least three independent experiments. Combined FISH-immunofluorescence (IF) experiment was done as described¹⁰ using a monoclonal MBNL1 antibody (MB1a developed by G. Morris¹¹) followed by a secondary Alexa 488-conjugated goat anti-mouse (Invitrogen) antibody. Pictures were captured using a Leica confocal microscope and software (Leica microsystems), and processed with Adobe Photoshop software (Adobe System Inc.). For fusion index analysis, IF was performed on differentiated muscle cell cultures as described¹² using a desmin (D33, DAKO) antibody and the nuclei were counterstained with Hoechst 33258 (Sigma). More than 1500 nuclei were counted and the fusion index was determined by the number of nuclei in differentiated myotubes (>2 myonuclei) as a percentage of the total number of nuclei in desmin-positive cells.

Western blotting

Western blotting was performed with standard methods using a DMPK antibody (MANDM1) as described previously¹³.

Statistical analyses

Group data are expressed as mean $\pm$ SEM. Between group comparison was performed by unpaired Student's t test (Fig. 1b, 1c, S3, S4, S5 and S7) and Newman-Keuls test (Fig. 2a, 2b, 2c) using GraphPad Prism 4 software. Differences between groups were considered significant when $P < 0.05$ (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

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