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Major physiological changes are governed by alternative splicing of RNA, and its misregulation may lead to specific diseases. With the use of a genome-wide approach, we show here that this splicing step can be modified by medication and demonstrate the effects of the biguanide metformin, on alternative splicing. The mechanism of action involves AMPK activation and downregulation of the RBM3 RNA-binding protein. The effects of metformin treatment were tested on myotonic dystrophy type I (DM1), a multisystemic disease considered to be a spliceopathy. We show that this drug promotes a corrective effect on several splicing defects associated with DM1 in derivatives of human embryonic stem cells carrying the causal mutation of DM1 as well as in primary myoblasts derived from patients. The biological effects of metformin were shown to be compatible with typical therapeutic dosages in a clinical investigation involving diabetic patients. The drug appears to act as a modifier of alternative splicing of a subset of genes and may therefore have novel therapeutic potential for many more diseases besides those directly linked to defective alternative splicing.

Introduction

Alternative splicing of RNA is a key mechanism in increasing complexity of mRNA and protein metabolism. Imbalances in this splicing process may thus affect the progression of various human diseases. Identifying compounds capable of modulating this imbalance therefore provides new and interesting therapeutic perspectives.[1] Splicing is a conserved mechanism controlled by the spliceosome, a complex composed of five small nuclear RNAs (U1, U2, U4, U5, and U6) that assemble with proteins to form small nuclear ribonucleoproteins (snRNPs).[2] The production of alternatively spliced mRNAs is regulated by a system of trans-acting proteins that bind to cis-acting sites on the primary transcript itself. Each of these RNA-binding proteins has quite widespread effects on a number of genes. This sheds doubt on the ability of chemical compounds to target these factors in such a way as to have beneficial effects, without inducing concomitant deleterious consequences. One way to address this potential problem would be to focus on the repositioning of FDA-approved compounds that may affect alternative RNA splicing. It has already been demonstrated that marketed drugs such as clotrimazole, flunarizine, digitoxin, pentamidine, and manumycin A can also affect the alternative splicing machinery, raising the exciting prospect that the efficacy of disease-specific therapies may be enhanced by medications that target alternative splicing machinery.[3–6]

Within this framework, we were interested in the hypothesis that metformin, one of the most commonly prescribed antidiabetic drugs, downregulates the expression of a small subset of ribonucleic acid-binding proteins.[7] The effect of metformin on splicing machinery was explored in the pathological context of myotonic dystrophy type 1 (DM1), a model of spliceopathy where metformin is used to treat insulin resistance in DM1 patients.[1,8] DM1 is characterized by a defect in the alternative RNA splicing machinery, where 80% of the splicing alterations are related to the nuclear sequestration of the RNA-binding protein MBNL1 on myotonin protein kinase gene (*DMPK*) transcripts containing an abnormal expansion of CUG repeats in the 3′ UTR.[9–14] These nuclear aggregates also promote CELF1 hyperactivation,[15] and the concomitant deregulation of these two splicing factors promotes the alteration of alternative splicing in various genes that have been linked to symptoms of DM1.[16–18]

We therefore explored the consequences of metformin application with DM1-related abnormalities in alternative RNA splicing, using an *in vitro* model based on a human embryonic stem cell line (hESC) derived from an embryo characterized as a DM1-gene carrier during a preimplantation genetic diagnosis. This cell line was already instrumental in revealing alterations of the expression of several genes associated with functional disturbances in DM1.[19–21] Our results confirmed the ability of metformin to modify alternative splicing events, including some that are defective in DM1.

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Results

Effects of metformin treatment on RNA-binding protein expression

The downregulation of transcripts encoding five ribonucleic acid-binding proteins (RBM3, SRSF1, SFPQ, SRSF6, and RBM45) by metformin in the millimolar range was identified by Larsson et al.[7] Concordant with this study, we confirmed that metformin induced a statistically significant decrease of RBM3 in DM1 and wild-type mesodermal precursor cells (MPCs) differentiated from DM1 and control hESCs at a dose of 25 mmol/l (**Figure 1** and **Supplementary Figure S1a**). In contrast, metformin treatment did not result in a statistically significant alteration of expression of SRSF1, SFPQ, SRSF6, and RBM45 (**Supplementary Figure S1b**) or MBNL1[22] and CELF1, both of which are involved in the pathological mechanisms of DM1 (**Figure 1**).

The effect of metformin treatment on cell viability, toxicity, apoptosis, and proliferation was monitored in DM1 MPCs exposed to a range of metformin doses. Treatments with the drug for 48 hours did not affect viability, cytotoxicity, or apoptosis up to doses of 35 mmol/l. Treatments for 24 hours with increasing doses of ionomycin and staurosporine, used as positive controls, induced as expected a rapid increase
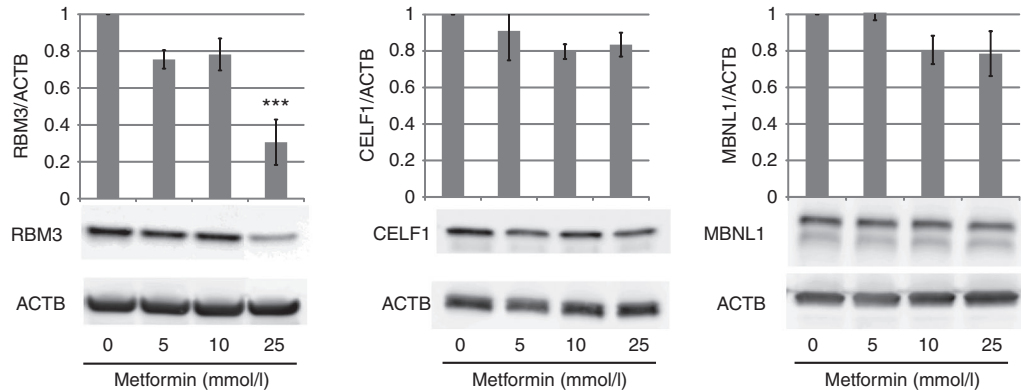

Figure 1 Metformin treatment modulates expression of the RBM3 RNA-binding protein. Western blot analysis of myotonic dystrophy type I mesodermal precursor cells treated for 48 hours with different doses of metformin demonstrated selective downregulation of RBM3, but not of CELF1 and MBNL1. Data (mean + SD) were analyzed with analysis of variance and the Steel-Dwass all pairs *post hoc* test. ***P < 0.001.
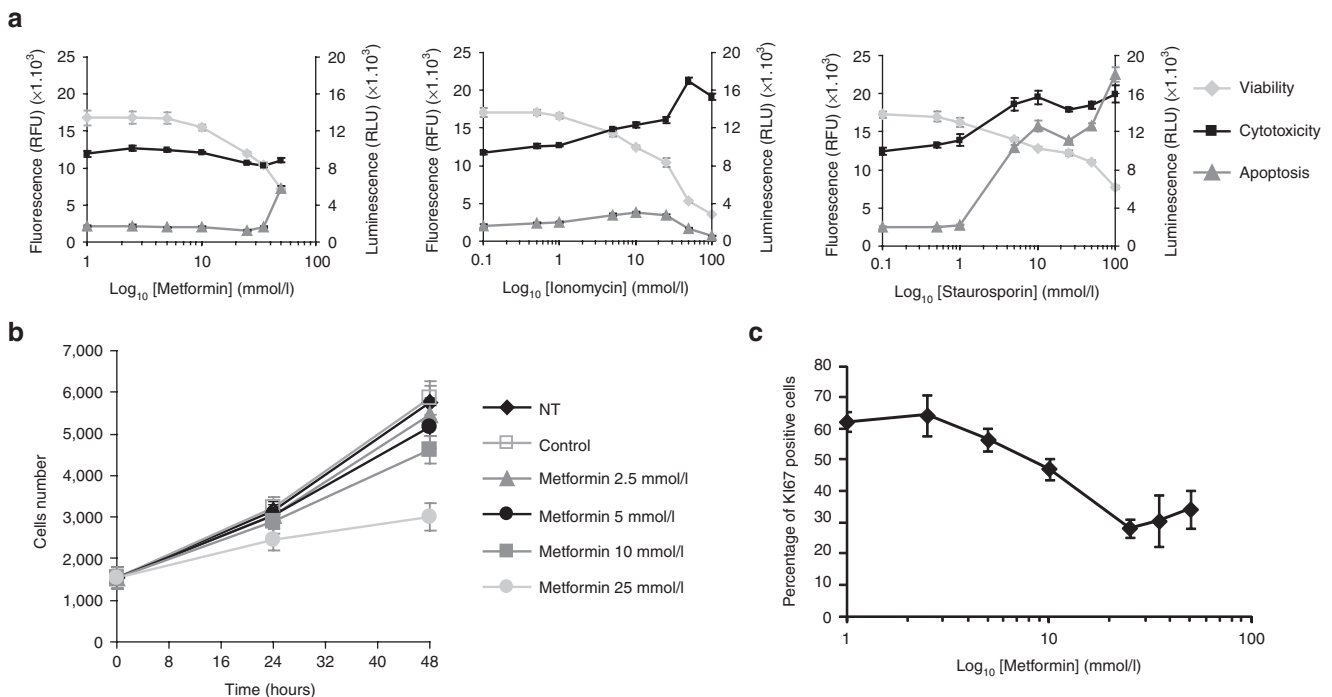
Figure 2 Effect of metformin treatment on myotonic dystrophy type I (DM1) mesodermal precursor cell (MPC) proliferation, apoptosis, and cytotoxicity. (a) DM1 MPCs were incubated with vehicle or increasing concentrations of metformin for 48 hours or staurosporine and ionomycin for 24 hours before measurement of viability, apoptosis, and toxicity with the ApoTox reagent. **(b,c)** DM1-mutated MPCs were treated with a range of metformin doses. Total cell numbers were determined at 0, 24, and 48 hours. Percentage of cells expressing the Ki-67 proliferation marker was analyzed after 48 hours of treatment. NT, not treated; RFU, relative fluorescence unit; RLU, relative luminescence unit.

of cytotoxicity and apoptosis, respectively[23,24] (**Figure 2a–c**). Proliferation analysis of DM1 MPCs showed that metformin treatment tended to promote a cytostatic effect at a dose of 10 mmol/l, with a greater effect at 25 mmol/l (**Figure 2b**). This was correlated with a progressive decrease in the number of cells expressing the Ki-67 proliferation marker (**Figure 2c**).

Effects of metformin on DM1-associated splicing defects
The consequences of metformin treatment were next analyzed on DM1-associated splicing defects in MPCs derived from DM1 hESCs. Changes in *INSR* exon 11 were studied using reverse transcription–PCR (RT–PCR), as the two isoforms of this gene were readily expressed in MPCs. Metformin corrected *INSR* exon 11 splicing defects in DM1 MPCs, increasing the inclusion from 18% (±2.3) to 34% (±1.8) at a dose of 25 mmol/l (**Figure 3a**). A similar shift towards an increased proportion of *INSR* exon 11 inclusion was also observed in wild-type MPCs, demonstrating the ability of metformin to regulate alternative splicing independently of the presence of the DM1 mutation. This analysis was extended to two other well-described alternative RNA splicing events associated with DM1, *TNNT2* exon 5 and *Clcn1* exon 7a.[17,25] This was based on the use of minigenes because the two isoforms of these genes are only expressed in heart and skeletal muscle.[25,26] The transient transfection of the minigenes in DM1 MPCs treated with 25 mmol/l metformin showed a statistical lowering of the percentage of inclusion of *TNNT2* exon 5 inclusion (from 51 ± 0.5% to 17 ± 0.4%) and *Clcn1* exon 7a inclusion (from 36% to 23 ± 2.03%) to levels similar to those quantified in wild-type MPCs (22 ± 1.5% and 20 ± 1.5%, respectively) (**Figure 3b**).

Overall effects of metformin treatment on alternative RNA splicing
To better understand the overall effects of metformin on gene expression and alternative RNA splicing, DM1 MPCs treated with 25 mmol/l metformin were analyzed using deep RNA sequencing. Treatment with 10 mmol/l of metformin

was also included in this analysis as this dose induced a slight effect on *INSR* splicing (**Figure 3a**). A total of 63 and 1,171 genes in DM1 MPCs were regulated with an absolute log2-fold change of ≥1 ($P ≤ 0.05$) in response to 10 and 25 mmol/l metformin, respectively. In these sets of genes, biological processes corresponding to cell cycle, response to DNA damage, cytoskeleton and ATP binding were enriched (**Table 1** and **Supplementary Tables S1** and **S2**). Downregulation of *RBM3* transcript was also detected in DM1 MPCs treated with 25 mmol/l metformin with fold changes of 0.33 (adjusted P value: 1.28×10^{-7}) confirming our previous observations (**Figure 1**). Analysis at the exonic level identified variations in 95 and 416 exons regulated above 10% ($P ≤ 0.05$) at drug concentrations of 10 and 25 mmol/l, respectively (**Figure 4a**; **Supplementary Figure S2a** and **Supplementary Table S3**). Eighty-nine common splicing events were found to be deregulated at both concentrations. Of the 20 splicing events most highly regulated (>20%) by 25 mmol/l metformin, 19 were confirmed by RT–PCR in DM1 MPCs

Table 1 Biological process enriched in genes and splicings regulated by metformin

Biological process	Nb of genes	P value[a]	Fold enrichment
1,171 genes regulated by 25 mmol/l metformin (absolute log2-fold change > 1)			
Cell cycle	140	5.0 E-40	33.09
ATP binding	115	3.3 E-05	6.38
Cytoskeleton	112	1.1 E-05	8.51
Response to DNA damage stimulus	55	1.6 E-10	10.37
416 splicing events regulated by 25 mmol/l metformin (>10%)			
Cytoskeleton	53	3.3 E-04	4.26
Nuclear lumen	48	3.1 E-02	2.32
Kinase	30	1.6 E-02	1.76
RNA binding	24	3.2 E-02	1.87

[a]One tail Fischer exact probability value used for gene-enrichment analysis, using DAVID software and the human genome as reference.
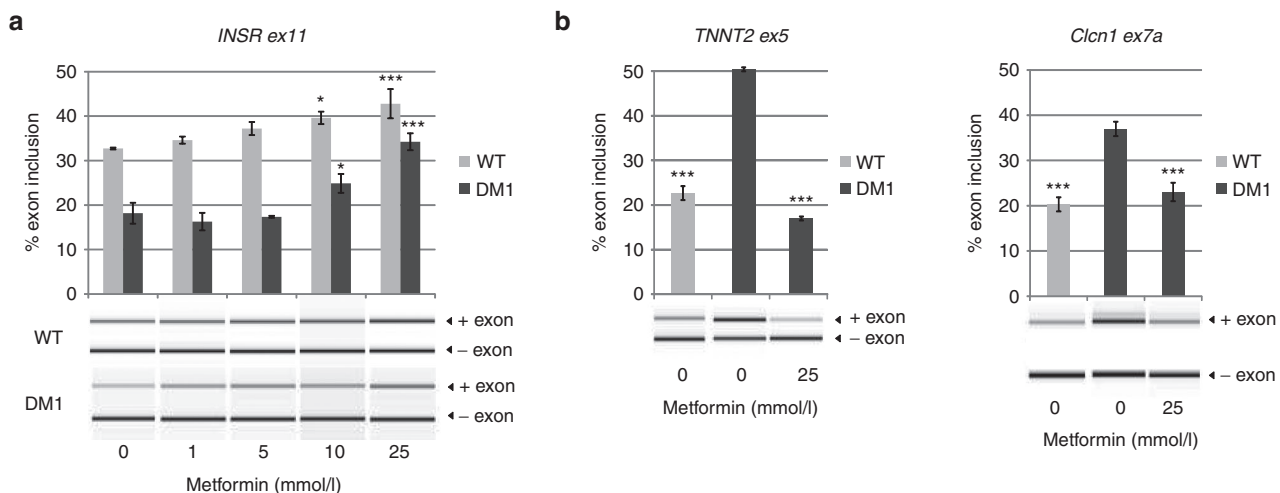
Figure 3 Characterization of metformin treatment on DM1-associated splicing defects in DM1-mutated mesodermal precursor cells (MPCs). (**a**) Alternative splicing of *INSR* exon 11 was analyzed with reverse transcription–PCR (RT–PCR) in wild type and DM1 MPCs treated with a range of metformin doses for 48 hours. (**b**) Alternative splicing of *TNNT2* exon 5 and *Clcn1* exon 7a were analyzed with RT–PCR in wild type and DM1 MPCs. Transfected DM1 MPCs were also treated for 48 hours with vehicle or 25 mmol/l metformin. Data (mean + SD) were analyzed with analysis of variance and the Kruskal–Wallis *post hoc* test. *$P < 0.05$, ***$P < 0.001$. DM1, myotonic dystrophy type I; WT, wild type.

a

Metformin	Splicing events modulated >10%
10 mmol/l	95
25 mmol/l	416
10 and 25 mmol/l	89

b

c

Figure 4 Genome-wide analysis of alternative splicing regulation by metformin. (**a**) Number of splice events modified by metformin treatment at 10 or 25 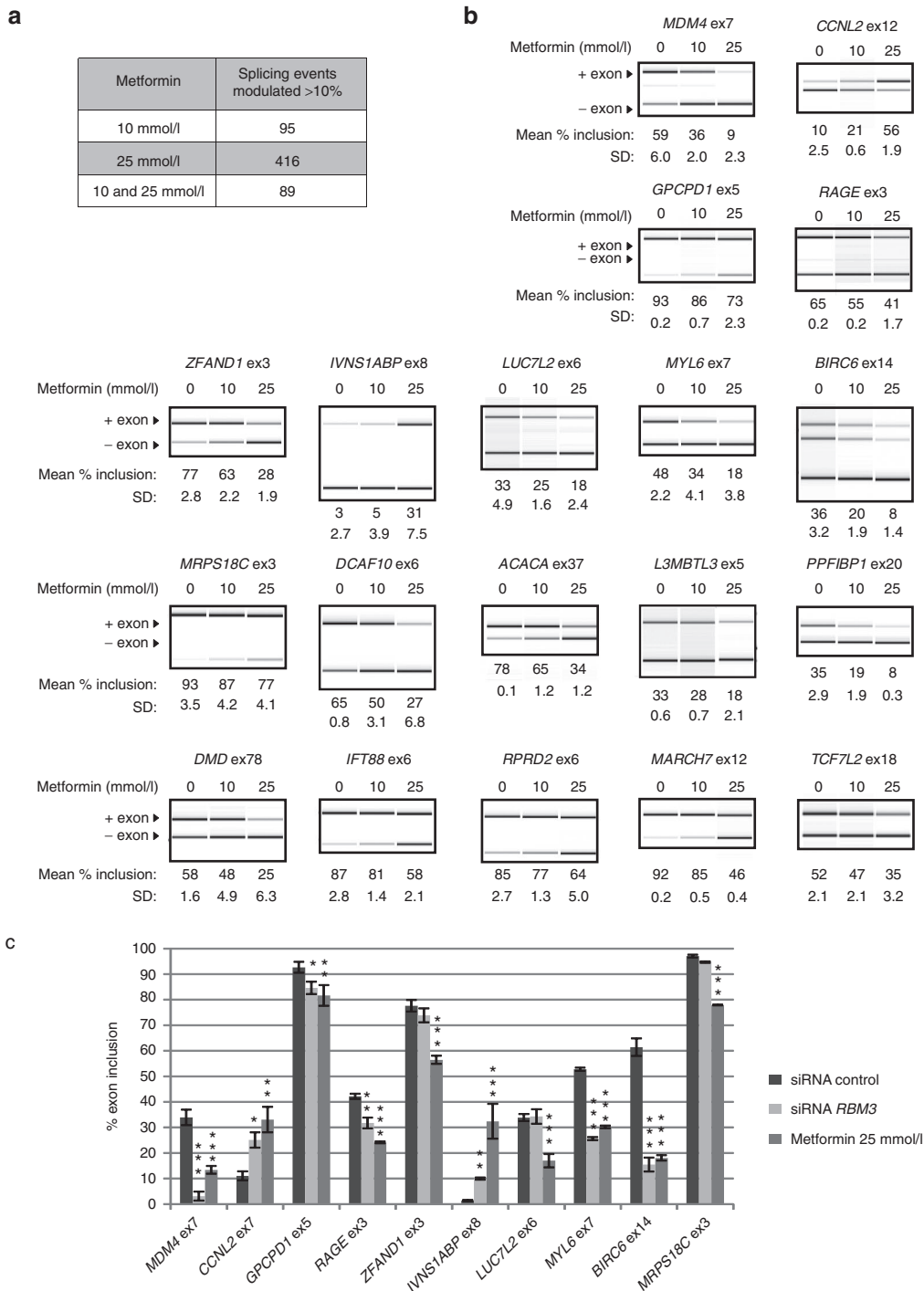mmol/l. The number of common splice events modified at both concentrations is also indicated. (**b**) Reverse transcription–PCR (RT–PCR) validation of alternative splicing events identified by RNA sequencing in myotonic dystrophy type I (DM1) mesodermal precursor cells (MPCs) treated for 48 hours using 10 and 25 mmol/l of metformin. (**c**) Ten of the splicing events most regulated by metformin were analyzed by RT–PCR in DM1 MPCs transfected for 48 hours with a siRNA specific for *RBM3* or exposed for 48 hours to treatment with 25 mmol/l metformin. Data (mean + SD) were analyzed with analysis of variance and the Kruskal–Wallis *post hoc* test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

(**Figure 4b**). Gene set enrichment analysis of the 416 splicing events regulated at 25 mmol/l identified sets of genes involved with the cytoskeleton, nuclear lumen, RNA binding, or with kinase activity (**Table 1**). Interestingly, most of the deregulated genes are also enriched in genes involved with the cytoskeleton (**Table 1**). None of the deregulated genes are associated with an alternative splicing modulation.

As our initial results indicated that metformin led to a reduced expression of RBM3, possible involvement of this splice factor in the regulation of alternative splicing by metformin was explored

using a siRNA transfection approach on 10 of the newly identified splicing events most altered by drug treatment. Thus, we compared the effects of metformin and transient extinction of *RBM3* in DM1 MPCs. Downregulation of *RBM3* expression induced similar results to metformin treatment for 7 out of 10 splicing events. These results strongly suggested that *RBM3* contributes to the mechanism of action of metformin on alternative splicing (**Figure 4c** and **Supplementary Figure S2b,c**).

As *RBM3* is described to be alternatively spliced, we also tested the possibility that the decreased expression of RBM3 induced by metformin was not associated to an effect of metformin on the expression of one of the alternate splice isoforms of *RBM3*. First, we analyzed, by RT–PCR, the expression profile of the 11 transcripts for *RBM3* that are described in ENSEMBL data bank in the presence and absence of metformin treatment. Among those, we detected six *RBM3* variant transcripts independently of metformin treatment (**Figure 5a**). We have next quantified the expression of each variant by quantitative PCR. Our results indicate that metformin treatment does not modify alternate splicing of *RBM3* transcripts (**Figure 5a**,**b**). In parallel, as two additional transcripts for *RBM3* have been described as candidates for nonsense-mediated mRNA decay process in NCBI data base, we also analyzed their expression after treatment with nonsense-mediated mRNA decay inhibitors, such as cycloheximide.[27] We first validated the effect of different concentrations of cycloheximide on the decay of unstable mRNA such as *c-FOS*. Our results indicate that, independently of the dose of cycloheximide used, the two *RBM3* transcripts are not expressed in our cell cultures in presence or not of metformin (**Supplementary Figure S3**). Altogether, our results indicate that the decreased *RBM3* expression observed after metformin treatment is not correlated to a change in alternative splicing of *RBM3* transcript.

Molecular mechanisms involved in the regulation of alternative RNA splicing by metformin

We next seek to understand the signaling pathways by which metformin treatment leads to a modification of splicing factor expression and consequently a modification of alternative splicing. The well-known antidiabetic action of metformin has been correlated to the inhibition of complex I of the respiratory chain,[28] raising the intracellular AMP/ATP ratio, a signal that finally triggers the downstream activation of AMPK.[28,29] Activity of respiratory chain complex I and AMP intracellular levels were monitored in DM1 MPCs after metformin treatment. Spectrometric measurements confirmed that progressive doses of metformin specifically inhibited the respiratory chain complex I in a dose-dependent manner up to a concentration of 10 mmol/l (**Figure 6a**). At 25 mmol/l, metformin also inhibited complexes II and IV and, to a lesser extent, complex V. This led to an increased AMP/ATP ratio, as determined by high-performance liquid chromatography (**Figure 6b**). The potential role of AMPK activation in alternative RNA splicing induced by metformin was tested by challenging DM1 MPCs with the AMPK activator AICAR (5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside, Acadesine, N¹-(β-D-ribofuranosyl)-5-aminoimidazole-4-carboxamide). Treatment of DM1 MPCs with 2 mmol/l AICAR for 24 hours promoted the downregulation of RBM3 (**Figure 6c**) together with changes in exon inclusions on five of the splicing events most regulated

by metformin (*MDM4* exon 7, *GPCPD1* exon 5, *CCNL2* exon 7, *RAGE* exon 3, and *ZFAND1* exon 3) (**Figure 6d** and **Supplementary Table S3**). Strikingly, neither AICAR treatment nor downregulation of *RBM3* expression seemed to modulate the *INSR* exon 11 splicing in DM1 MPCs, suggesting that metformin might activate additional molecular pathways to mediate its global effect on alternative splicing (**Figure 6e**).

Impact of metformin on DM1-associated splicing defects in myoblasts

The potential interest of metformin in the pathological context of DM1 was evaluated by measuring the effect of metformin treatment on 20 splicing events that showed graded changes correlated with muscle strength in a cohort of 50 DM1 subjects.[30] This was accomplished by using primary cultures of myoblasts derived from two healthy individual and two distinct DM1 patients. In addition, splicing of *INSR* exon 11 and *TNNT2* exon 5, previously studied by the use of minigenes in DM1 MPCs (**Figure 3**), was analyzed as they are also expressed in cultured myoblasts. Metformin promoted changes in alternative splicing of exons ≥10% for six of these genes (**Figure 7** and **Supplementary Figure S4**). The effect of metformin was beneficial on *INSR* exon 11, *TNNT2* exon 5, *ATP2A1* exon 22, *DMD* exon 71, *DMD* exon 78, and *KIF13A* exon 32, as the isoform ratio shifted towards control values. The drug did not affect the 16 other splicing events that were tested (including those for which no splicing event is observed in absence of treatment in this cell type) (**Supplementary Figure S5**). The involvement of AMPK activation in the regulation of the six DM1 splicing defects modified by metformin was tested in one of the DM1 myoblast cultures. Experiments confirm a partial implication of the AMPK as AICAR treatment restored the splicing defects of *ATP2A1* exon 22 and *TNNT2* exon 5, while no modulations of *DMD* exon 78 and *INSR* exon 11 were observed (**Figure 7** and **Supplementary Figure S4**). Interestingly, AICAR promoted the inclusion of *DMD* exon 71. The splicing defect of *KIF13A* exon 32, not detected in these myoblasts, could not be analyzed. The efficacy of metformin treatment on DM1-associated splicing defects was next compared to pentamidine, a compound shown to revert some splicing defects associated with DM1.[6] Interestingly, a decreased expression of RBM3 is also observed after pentamidine treatment (**Supplementary Figure S6a,b**). Concordant with this observation, similar efficiency was observed between metformin and pentamidine with reference to their ability to restore the inclusion of *ATP2A1* exon 22, corresponding to the most affected splicing event in muscle biopsies of DM1 patients,[30] as well as on DMD exon 71 (**Figure 7**, **Supplementary Table S4**, and **Supplementary Figures S4** and **S6c**). However, in contrast to metformin treatment, no significant effect (>10%) of pentamidine was detected on *DMD* exon 78, *INSR* exon 11, and *TNNT2* exon 5.

In vivo effects of metformin on RNA alternative splicing

In order to explore the effects of metformin on alternative splicing at therapeutic concentrations in humans, we investigated patients currently being treated with metformin for Type 2 diabetes. To perform a clinical trial in which metformin would be temporarily replaced by another antidiabetic drug, namely sitagliptin, the lack of efficacy of sitagliptin was verified on *INSR* exon 11 alternative splicing in preliminary experiments carried
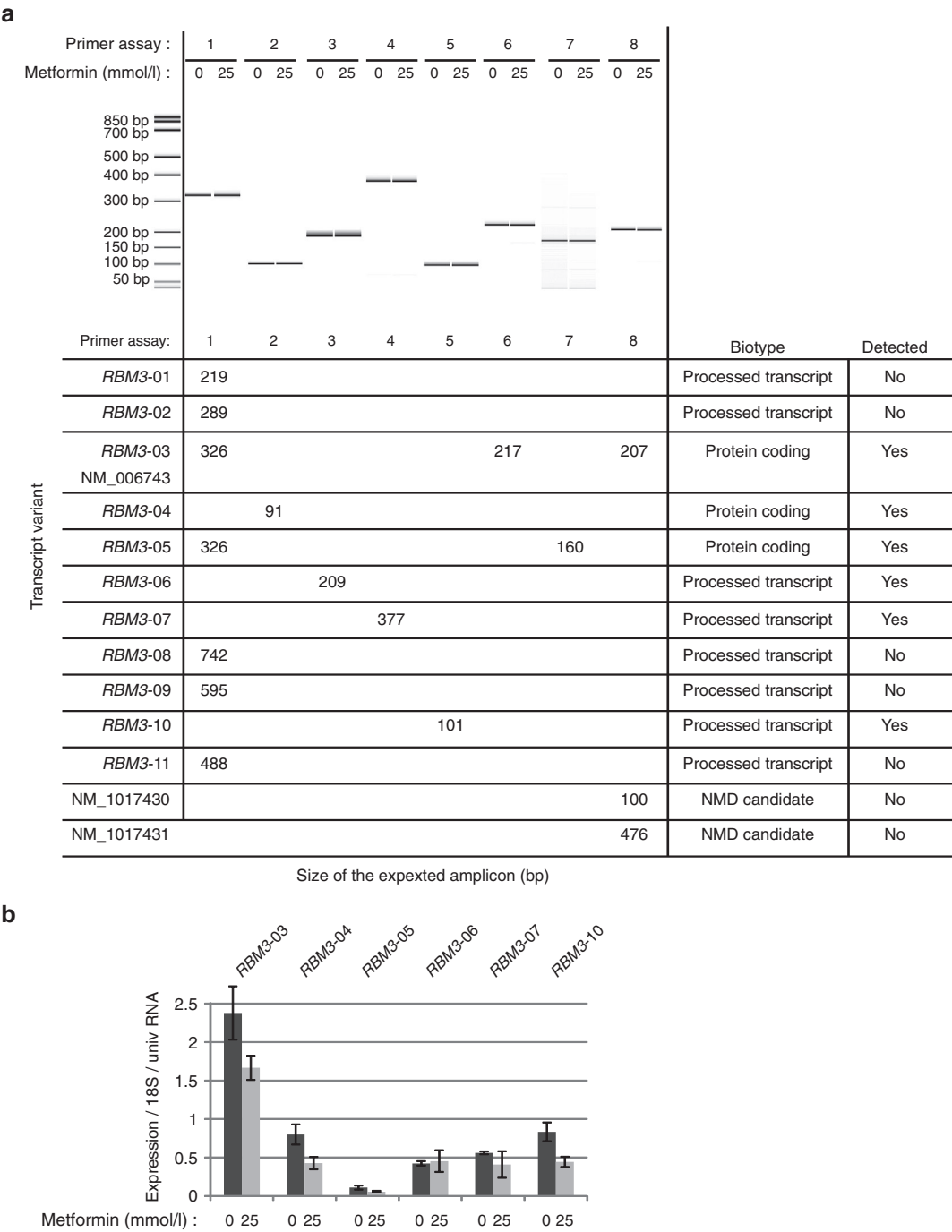
a

Transcript variant	1	2	3	4	5	6	7	8	Biotype	Detected
RBM3-01	219								Processed transcript	No
RBM3-02	289								Processed transcript	No
RBM3-03 NM_006743	326					217		207	Protein coding	Yes
RBM3-04		91							Protein coding	Yes
RBM3-05	326						160		Protein coding	Yes
RBM3-06			209						Processed transcript	Yes
RBM3-07				377					Processed transcript	Yes
RBM3-08	742								Processed transcript	No
RBM3-09	595								Processed transcript	No
RBM3-10					101				Processed transcript	Yes
RBM3-11	488								Processed transcript	No
NM_1017430							100		NMD candidate	No
NM_1017431							476		NMD candidate	No

Size of the expexted amplicon (bp)

b

Figure 5 Analysis of the effect of metformin on *RBM3* alternative splicing. (**a**) Expression profile of 11 *RBM3* transcripts and 2 nonsense-mediated mRNA decay candidates in myotonic dystrophy type I (DM1) mesodermal precursor cells (MPCs) treated or not with 25 mmol/l of metformin for 48 hours. Primer assay corresponds to a specific pair of primers designed to match with distinct splice variants of *RBM3*. (**b**) Expression level of *RBM3* variants were quantified in DM1 MPC after a 48 hours treatment with 25 mmol/l metformin by reverse transcription–quantitative PCR. Primer assays that amplified one variant were used as followed: for *RBM3*-03, primer assay 6; for *RBM3*-04, primer assay 2; for *RBM3*-05, primer assay 7; for *RBM3*-06, primer assay 3; for *RBM3*-07, primer assay 4; for primer *RBM3*-10, primer assay 5. Data are represented as mean + SD. Expression level of each variant was normalized on 18S and on the expression in universal RNA. NMD, nonsense-mediated mRNA decay.

out *in vitro* on peripheral blood lymphocytes (PBLs) (**Supplementary Figure S7a,b**). Fifteen diabetic patients, who had been treated with a stable dose of metformin between 2.1 and 3 g/day for more than a year, were recruited in a study where metformin was replaced for 1 month by sitagliptin, and RNA alternative splicing was explored in PBLs (NCT 01349387). There was no change in blood glucose levels during the course of the study. Of the splicing events identified in response to metformin, we chose alternative splicing of *INSR* exon 11, which is expressed in most tissues. Because of its low level of
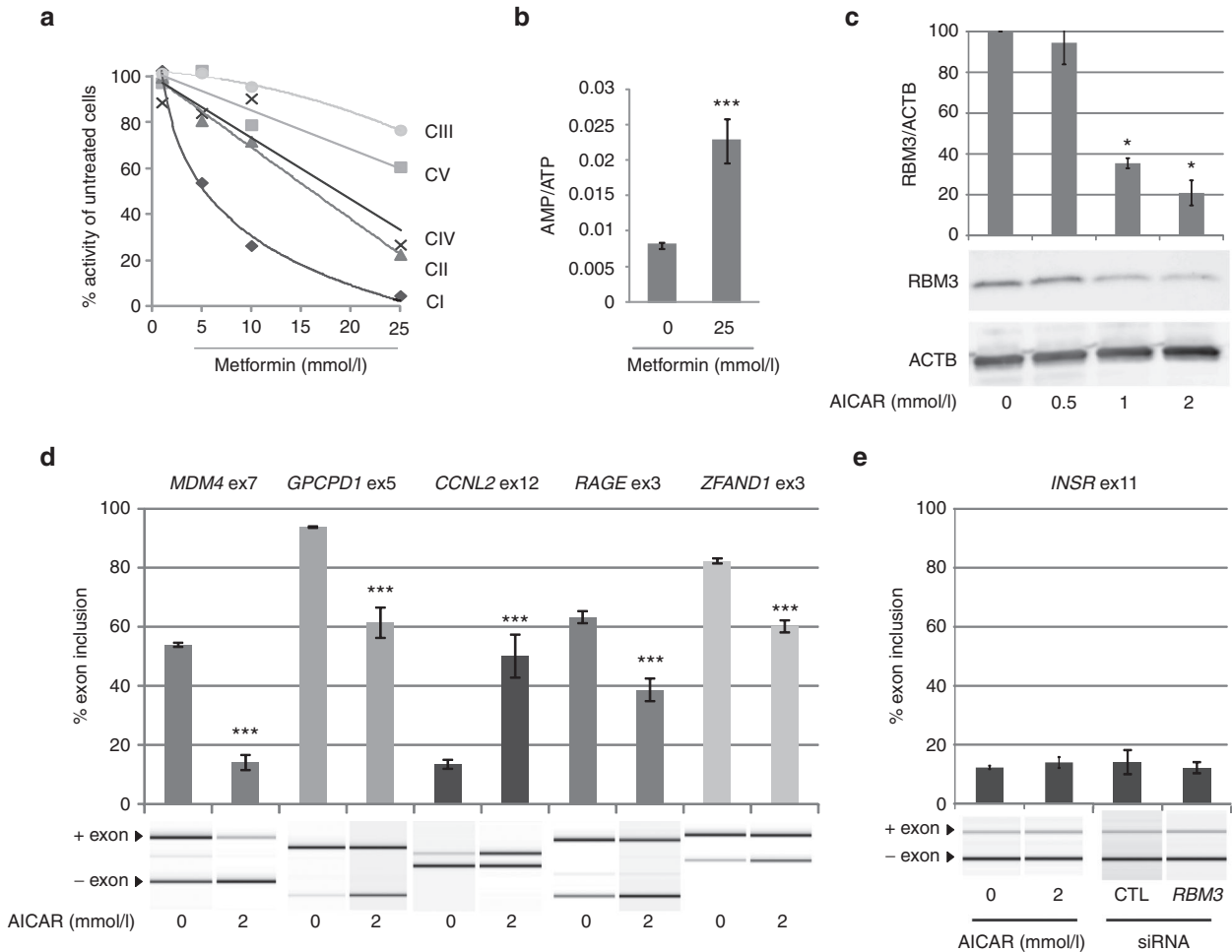
Figure 6 Intracellular modifications induced by metformin and involvement of AMPK in splicing control. (**a**) Activities of the different complexes of the respiratory chain were evaluated by spectrometry in myotonic dystrophy type I (DM1) mesodermal precursor cells (MPCs) treated for 48 hours with 5, 10, and 25 mmol/l metformin. Representative graph of the results obtained with three different samples. CI to CV represent the different complexes of the respiratory chain. (**b**) The effect of metformin on intracellular AMP/ATP content was quantified with high-performance liquid chromatography. Data (mean + SD) were analyzed with analysis of variance (ANOVA) and the Kruskal–Wallis *post hoc* test. (**c**) AICAR inhibits the expression of RBM3, analyzed by western blot. Data (mean + SD) were analyzed with ANOVA and the Kruskal–Wallis *post hoc*. (**d**) AICAR promotes changes in *MDM4* exon 7, *GPCPD1* exon 5, *CCNL2* exon 7, *RAGE* exon 3, and *ZFAND1* exon 3. (**e**) The alternative splicing of *INSR* exon 11, analyzed in DM1 MPCs by reverse transcription–PCR, is not modified by a 24-hour AICAR treatment or the downregulation of *RBM3* induced by the transfection of siRNA for 48 hours. Data (mean + SD) were analyzed with ANOVA and a Tukey–Kramer *post hoc* test. *$P < 0.05$, ***$P < 0.001$. AICAR, 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside, Acadesine, N[1]-(β-D-ribofuranosyl)-5-aminoimidazole-4-carboxamide).

expression in these cells, *INSR* +/− exon 11 transcripts were analyzed with quantitative PCR. Analysis confirmed that metformin triggered *INSR* exon 11 inclusion in this clinical setting (**Figure 8a**). Alternative splicing of *FAS (CD95)* exon 6 was analyzed in order to identify an additional splicing event that could be measured in PBLs from treated patients. *FAS* exon 6 exclusion was also affected by therapeutic doses of metformin in diabetic patients, giving rise to a shift from the anti- to the proapoptotic isoform of the protein (**Figure 8b,c**).

Discussion

The main result of this study is the demonstration that metformin, the antidiabetic biguanide, affects the alternative RNA splicing machinery. Our results point to a molecular mechanism that involves, at least partially, the activation of AMPK and modulation of the RBM3 RNA-binding protein. The demonstration that metformin modulates several splicing events *in vitro* and *in vivo*, including some altered in DM1, suggests that it would be worthwhile to evaluate the efficacy of metformin treatment in alleviating other symptoms than those related to insulin resistance.

The importance of gene regulation at the level of RNA transcripts by alternative splicing has been reported increasingly in fields such as development,[31] cancer,[32] metabolism,[33] and monogenic diseases.[1] Alternative RNA splicing thus opens new opportunities for therapeutic approaches.[34] However, it seems unlikely that selective modulation of a single specific splicing event could be carried out without generating concomitant adverse effects. However, this goal might be achieved with the use of marketed drugs whose biodistribution
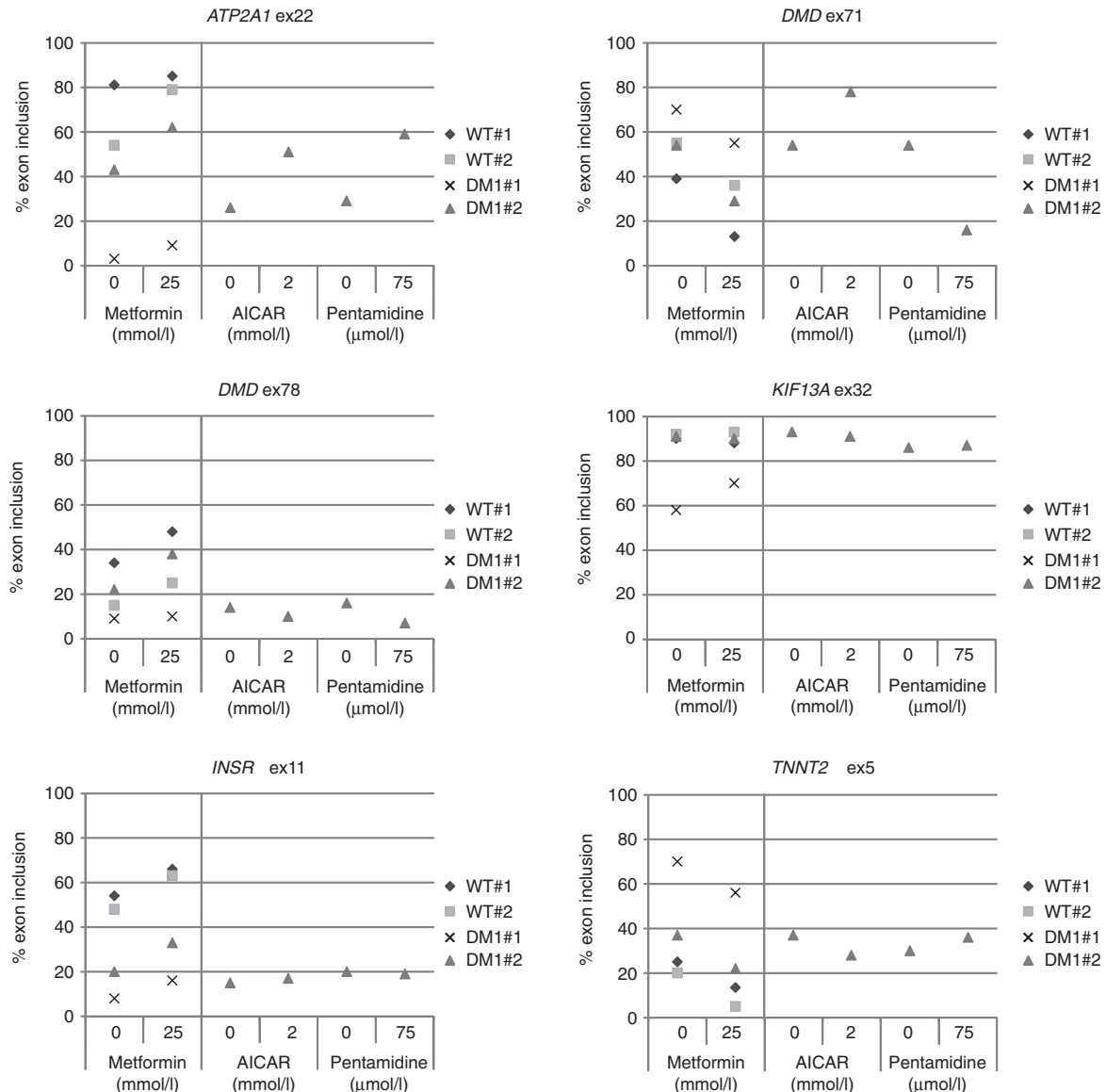
Figure 7 Metformin impacts several splicing defects associated with DM1 in mutated human myoblasts. Reverse transcription–PCR analysis of DM1-associated splicing defects in myoblasts from two DM1 patients and two healthy individuals treated for 48 hours with 25 mmol/l metformin or for 24 hours with 75 μmol/l pentamidine or AICAR 2 mmol/l. Representative data from two independent experiments are shown. AICAR, 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside, Acadesine, N[1]-(β-D-ribofuranosyl)-5-aminoimidazole-4-carboxamide; DM1, myotonic dystrophy type I; WT, wild type.

is known and which regulate alternative splicing.[4,5,35] These compounds could be reconsidered as modulators of alternative RNA splicing in addition to the effects on cellular targets for which they have been screened. It is worth mentioning that an analysis by exon array demonstrated that compounds such as clotrimazole, flunarizine, and chlorhexidine targeted different signal transduction pathways and caused distinct changes in alternative splicing of a number of genes.[4] This suggests that discrete targeting of the alternative splicing of specific genes associated with a particular disease may be possible with selected pharmacological agents.

In this context, we focused on metformin, which is indicated as a first-line oral therapy for treatment of hyperglycemia in individuals with Type 2 diabetes. Even though this drug has

been in use for several decades, most of its cellular effects are still under investigation and new emerging effects, such as inhibition of cell proliferation, suggest its potential repurposing to treat cancer. Metformin was recently reported to selectively inhibit the translation of several RNA-binding proteins concomitantly with its blockade of cell proliferation.[7] Our results confirm that metformin treatment downregulates RBM3 in DM1 MPCs and induces splicing of a restricted set of primary transcripts. The cellular model we used allowed us to verify the involvement in this process of AMPK, the classical cellular target of metformin.[28,29] The characterization of metformin treatment in DM1 MPCs pointed to a signal associated with energy depletion and blockade of cell proliferation induced by inhibition of complex 1 of the mitochondria, ATP decrease, and activation

a

INSR ex11

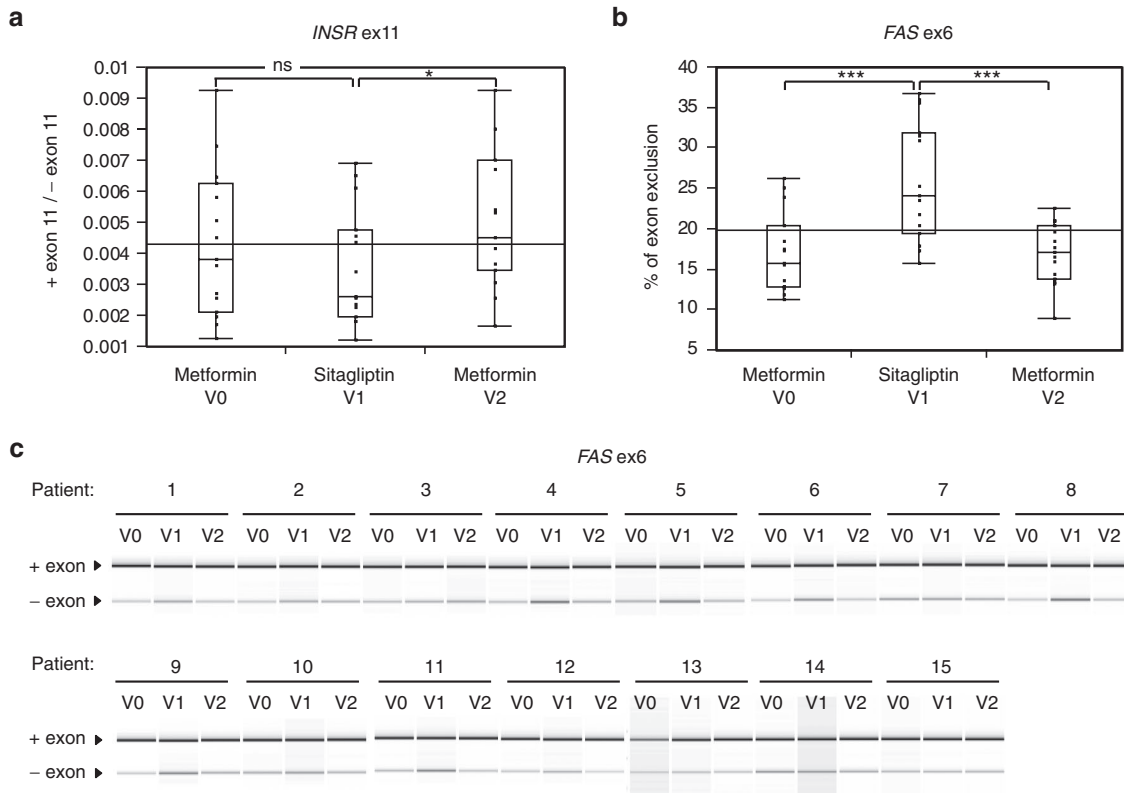**b**

FAS ex6

c

FAS ex6

Figure 8 Regulation of alternative splicing of *INSR* exon 11 and *FAS* exon 6 by therapeutic doses of metformin can be followed *in vivo* in patients. (**a**) The *INSR* + exon 11 / – exon 11 ratio was monitored with reverse transcription–quantitative PCR (RT–qPCR) in peripheral blood lymphocytes from diabetic patients treated with metformin (V0), with sitagliptin instead of metformin for 1 month (V1) and 1 month after restarting metformin treatment (V2). (**b,c**) The same samples were also analyzed with RT–PCR to quantify the alternative splicing of *FAS* exon 6, which is also regulated by metformin. Data represent the individual responses in each group (*n* = 15 patients) and were analyzed with the Wilcoxon paired test. *$P < 0.05$, ***$P < 0.001$. NS, nonsignificant.

of the AMPK metabolic sensor. This reveals a molecular signature at the level of RNA transcripts that is associated with metabolic stress and similar to that identified for other genotoxic or oxidative stress inducers.[36,37] In parallel to these events, the absence of modulation of several DM1-associated splicing defects by the AMPK activator, AICAR, reveals the existence of additional molecular mechanisms by which metformin modulates the alternative splicing of certain primary transcripts. Metformin has been described as diminishing tyrosine kinase receptor signaling *in vitro* and *in vivo*.[38,39] These tyrosine kinase receptors include epidermal growth factor receptor, the signaling pathway of which controls *INSR* exon 11 inclusion through inhibition of *hnRNPA1* and *hnRNPA2B1* expression.[40] Whether such a mechanism is involved in other alternative splicing events regulated by metformin remains to be explored.

Among the regulated splicing events, we explored the impact of metformin treatment on those affected in DM1, because this drug is used to treat Type 2 diabetes in patients with DM1.[8] This well-tolerated drug could be an efficient way to alleviate DM1 missplicing in several organs affected by this multisystemic disease. It is commonly thought that most clinical manifestations of DM1 are linked with defects in alternative splicing due to the loss of MBNL1 function.[41] Accordingly, most attempts at finding treatments for this as yet incurable disease have focused on the release of MBNL1 from ribonucleoprotein intranuclear inclusions, and reversion of splicing

defects has been observed with ribozymes,[42] antisense oligonucleotides,[43] and chemical compounds such as pentamidine.[6,44] Metformin is shown here to alter splicing through a different mechanism that targets the splicing machinery. The downregulation of RBM3 by metformin in DM1 but also wild-type MPCs reveals a mode of splicing regulation that is not specific to DM1. The impact of metformin on DM1-associated splicing defects could be in part defined by the overlap between the targets of RBM3 and those of MBNL1. Our results indicated that metformin is capable of alleviating several splicing defects in cells differentiated from pluripotent stem cells derived from a DM1-mutant embryo, as well as in myoblasts sampled from DM1 patients. To focus on DM1 splicing defects that would be therapeutically significant, we analyzed the impact of metformin treatment in DM1 myoblasts on 20 splicing defects identified in DM1 skeletal muscle tissue that were correlated with muscle weakness using a genome-wide approach.[30] Experiments confirmed the partial modulation of these splicings by metformin, including *ATP2A1* exon 22, identified as the most affected in correlation with muscle weakness in DM1 patients, and *INSR* exon 11 or *TTN* exon 5 that belong to the early transition splicing group that are strongly affected by DM1 (>30% shift of exon inclusion), yet not associated with muscle weakness ($r \leq 0.5$).[30] Within the *DMD* transcript, metformin enhanced the inclusion of exon 78 but also increased the DM1-associated skipping of exon 71.

A transcript lacking the *DMD* exon 71 is normally expressed in normal skeletal muscle but is overexpressed in DM1 patients. Immunoblot analysis shows no change in dystrophin protein expression in skeletal muscle between DM1 and non-DM individuals,[45] indicating that the functional impact of *DMD* exon 71 exclusion remains to be functionally tested. Notably, the skipping of *DMD* exon 71 is also observed in DM1 myoblasts in response to pentamidine treatment, indicating that treatments that restore MBNL1 expression also provide partial correction of the DM1-associated splicing defects. In parallel to the modulation of splicing, metformin could be of interest for DM1 as an activator of AMPK. AICAR treatment tested on muscle function in mdx mice[46] has been reported to promote significant improvements in disease phenotype (a gain in body and muscle weight, a decrease in muscle inflammation and in the number of fibers with central nuclei and an increase in fibers with peripheral nuclei), including an increase in overall behavioral activity and significant gains in forelimb and hind limb strength. Since metformin is commonly used to treat insulin resistance in DM1 patients at doses that were shown in the present study to induce shifts in transcript isoform ratios, we decided to investigate modulation of splicings by metformin as well as drug efficacy on several functional parameters in a clinical trial with DM1 patients (EudraCT number: 2013-001732-21). This study will determine the therapeutic potential of metformin to treat DM1 patients for aspects of their disorder other than insulin resistance.

Considering that metformin has been used for decades in millions of patients without major toxicity, one may consider targeting alternative splicing in order to obtain a therapeutic effect. Accordingly, a systematic search for the effects on alternative splicing of drugs that are already in current use may eventually allow clinicians to extend their indications to diseases in which a change in isoform ratios of specific genes may be therapeutically beneficial. For example, the two isoforms of *FAS* (CD95) have opposite effects, being either pro- or antiapoptotic,[47,48] and, in PBLs sampled from diabetic patients, treatment with metformin induced a shift from the antiapoptotic variant to the proapoptotic variant of CD95. This effect could influence FAS-mediated apoptosis, which could be relevant for Ewing and other sarcomas[49] or autoimmune lymphoproliferative syndromes resulting from the failure of *FAS* exon 6 inclusion.[50]

Materials and Methods

Reagents. Primers, probes, and siRNA sequences are listed in **Supplementary Table S5**. The *RBM3* siRNA came from Qiagen (Courtaboeuf, France). Sitagliptin was obtained from Januvia 100-mg tablets (MSD Merck Sharp & Dohme Ltd, Hoddesdon, UK). Metformin, AICAR (5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside, Acadesine, N¹-(β-D-Ribofuranosyl)-5-aminoimidazole-4-carboxamide), pentamidine isethionate salt, cycloheximide, staurosporine, and ionomycin were obtained from Sigma. Primary antibodies used in this study were raised against SRSF1 (Cliniscienes, Nanterre, France; LSB2340, 1/500), RBM3 (Abcam, Cambridge, UK; ab134946, 1/1,000), SFPQ (Abcam; ab117617, 1/500), RBM45 (Abcam; ab105770, 1/200), SRSF6

(Cliniscienes; LS-B5712, 1/2,000), CELF1 (Millipore, Darmstadt, Germany; 05621, 1/2,000), Ki-67 (Millipore; MAB4190), and ACTB-peroxidase (Sigma-Aldrich, Saint-Louis, MO; A3854). Horseradish peroxidase-conjugated secondary antibodies used for western blot were goat anti-mouse IgG-horseradish peroxidase or goat anti-rabbit IgG-horseradish peroxidase (1:10,000; Amersham Bioscience, GE Healthcare, Saclay, France). MBNL1 was detected by the use of the MANDYS1 antibody, kindly provided by Prof. Glenn Morris (Center for Inherited Neuromuscular Disease, Oswestry, UK) and obtained from the MDA Monoclonal Antibody Resource.

Pluripotent stem cells culture. The two hESC lines used in this study came from the Department of Embryology and Genetics of the Vrije Universiteit, AZ-VUB Laboratory, Brussels, Belgium: the VUB03_DM1 (XX, passages 66–67) carrying the DM1 mutation (1,330 CTG repeats) and the VUB01_CTL (XY, passage 83) used as a control.[51] Human pluripotent stem cells were maintained on a layer of mitotically inactivated murine embryonic STO fibroblasts in Knockout Dulbecco's Modified Eagle's Medium supplemented with 20% knockout serum replacement, 1 mmol/l Glutamax, 1 mmol/l nonessential amino acids, 1% penicillin/streptomycin, 0.1% β-mercaptoethanol, and 5 ng/ml recombinant human FGF2 (all from Invitrogen, Carlsbad, CA). Medium was changed daily and cells were passaged every 5–7 days. Manual dissection was routinely used to passage the cells.

Differentiation of hESC lines in MPCs. MPCs were generated by differentiation from hESCs according to the protocol described previously by Marteyn *et al.*[19] MPCs derived from the VUB03_DM1 and VUB01_CTL hES cell lines were cultured on 0.1% gelatin-coated flasks and plates (Sigma-Aldrich) using Knockout Dulbecco's Modified Eagle's Medium (Invitrogen) supplemented with 20% fetal bovine serum (Eurobio, Les Ulis, France), 1 mmol/l Glutamax (Invitrogen), 1 mol/l nonessential amino acids (Invitrogen), and 0.1% β-mercaptoethanol (Invitrogen).

Culture of human myoblasts. Control and DM1 myoblasts were obtained from the Myobank in accordance with the French legislation on ethical rules (kindly provided by Dr. D. Furling). Two control myoblasts were originally isolated from the quadriceps of a 5-day-old infant (CHQ) and from a week 14 fetus (Me16). Two DM1 myoblasts were originally isolated from the quadriceps of a 11-day-old infant carrying more than 2,500 CTG (DM11) and from a week 14 fetus carrying 800 CTG (DM16). WT#1 and WT#2 correspond to Me16 and CHQ myoblasts while DM1#1 and DM1#2 match with DM11 and DM16 myoblasts. Cells were cultured on 0.1% gelatin-coated flasks and plates using Dulbecco's Modified Eagle's Medium-F12 + glutamax medium (Invitrogen) supplemented with 20% fetal bovine serum (Eurobio).

Culture of human PBLs. Freshly isolated wild-type and mutated PBLs, provided by Dr. Guillaume Bassez (CHU Henri Mondor, Creteil, France), were obtained from the Genethon DNA and Cells Bank (Evry, France). Cells were thawed and cultured in RPMI medium supplemented with 20% of fetal bovine serum (Eurobio) and 1% penicillin–streptomycin (Invitrogen)

according to cell bank instructions. Chemical treatments were performed once a day for 2 days. Freshly isolated PBLs from diabetic patients treated by metformin or sitagliptin were obtained from the CERITD (Evry, France) (NCT 01349387).

Measurement of sitagliptin activity and cell viability. Sitagliptin activity was measured *in vitro* using the luminescent DPPIV-Glo Protease Assay (Promega, Madison, WI) according the manufacturer's instructions. Viability of lymphocytes treated with a range of sitagliptin doses was monitored with the CellTiter-Glo assay (Promega) according the manufacturer's instructions.

Transfection of DNA constructs and siRNAs. MPCs were seeded in 24-well plates and transfected with 600 ng of plasmid, 0.6 µl of PLUS (Invitrogen) and 1.5 µl Lipofectamine LTX (Invitrogen). The RTB300 minigene used to analyze the splicing of exogenous human *cTNT* transcripts was kindly provided by Prof. TA Cooper (Baylor College of Medicine, Houston, TX). We constructed the minigene used to study *Clcn1* exon 7a splicing as described by Kino et al.[26] The genomic fragment covering exons 6 to 7 from mouse genomic DNA was PCR amplified using the *Clcn1* cloning primers described in **Supplementary Table S5**, cloned in the pCR-BluntII-TOPO vector (Invitrogen) using the BamHI/SalI restriction enzymes and then subcloned into the BglII-SalI site of pEGFP-C1 (Clontech, Mountain View, CA). For siRNA transfection, MPCs were seeded in 24-well plates and transfected with 10 nmol/l siRNA RBM3 (Qiagen) listed in **Supplementary Table S5** using 2.5 µl LipoRNAiMax (Invitrogen).

RNA sequencing library preparation and sequencing. Sequencing libraries were prepared according to the Illumina TruSeq Stranded mRNA Sample Prep Kit (according to manufacturer's protocol) (Illumina, San Diego, CA). A 2×101 bp paired-end sequencing was performed on the HiSeq2000 instrument, using half a lane per sample, to produce on average 80 million read pairs per sample (160 million sequences) with an average insert length of 130 bp. Trimmomatic,[52] Tophat2,[53] Picard suite (http://www.broadinstittute.github.io/picard), RNA-SeQC,[54] and in-house metrics were used to evaluate data quality.

RNA sequencing data analysis and identification of differential genes and splicing events. Reads were aligned using TopHat2 (v2.0.8[53]). TopHat2 was run with the assistance of gene annotations (Illumina's iGenomes based on EnsEMBL r70), which means that the alignment was performed in three steps: transcriptome mapping, genome mapping, and spliced mapping. The minimum and maximum intron lengths were also re-evaluated, respectively, to 30 and 1,200,000 to maximize the number of introns detected. The mate inner distances were set to their corresponding values. Alignment files in bam format were then filtered to removed poor mapping quality score (<10) and not primary alignments and read pairs with one single read mapped were filtered using samtools (v0.1.8[55]).

For the differential gene expression analysis, reads mapping to genes were first quantified using the HTSeq-count script provided by the HTSeq python package (v0.5.4[56]). The R/Bioconductor package DESeq2 (v1.4.5[57]) was then used to identify genes regulated by the drug treatment. A filter was then applied to DESeq2 results to select genes with an adjusted *P* value ≤0.05 and the mean of normalized counts ≥10.

For the alternative splicing analysis, reads crossing the exon–exon junction ("junction reads") were extracted from the read alignment files to detect the exon skipping events. In order to avoid spurious read alignments, we applied additional filters for the junction reads considered: no indels at the junction site, no hard clipping, and a minimal overlap of 4 bp over the junction site. FasterDB[58] gene and exon annotations were used as a guide to detect known and new exon skipping events. For each exon skipping event detected across all samples, junction reads corresponding to the inclusion of the exon and junction reads corresponding to the exclusion of the exon were quantified. The differential analysis was performed using KissDE, an R package developed as part of the KisSplice post-processing workflow.[59] KissDE works on pairs of variants for which read counts are available in each replicate of each condition and tests if a variant is enriched in one condition. Counts are modeled using a negative binomial distribution. KissDE fits a generalized linear model and tests for the effect of an interaction between the variant and the condition using a likelihood ratio test with a 5% false discovery rate to control for multiple testing. A percent splicing index (PSI or Ψ) value was then estimated for each sample as the ratio of inclusion junction reads to the sum of inclusion and exclusion junction reads. As the datasets are paired, the difference of Ψ values for each event (deltaPSI or $\Delta\Psi$) was calculated as the median of $\Delta\Psi$ values for each replicate. A filter was then applied on exon skipping events detected to select significant variants with an adjusted *P* value ≤0.05 and $\Delta\Psi$ value ≥10%.

Gene expression and splicing analysis by RT–PCR. Total RNA was extracted using the RNeasy Micro/Mini kit (Qiagen) and reverse transcribed using random hexamers and Superscript III Reverse Transcriptase kit (Invitrogen). For splicing analysis, PCR amplification was carried out with recombinant Taq DNA polymerase (Invitrogen) and the primers listed in **Supplementary Table S5**. The amplification was performed using a first step at 94 °C for 3 minutes followed by 30 cycles of 45 seconds at 94 °C, 30 seconds at 55 °C, 30 seconds at 72 °C, and finished with a final 10 minutes extension at 72 °C. The PCR products were quantified using the Bioanalyzer 2100 and DNA 1000 LabChip kit (Agilent, Santa Clara, CA). Primers used for splicing analysis in human myoblasts are described in Nakamori et al.[30] except those used to analyze *ATP2A1* exon 22 and *TNNT2* exon 5 splicings.

RBM3 gene expression and splicing analyses by quantitative PCR. Quantitative PCR reactions were carried out in 384-well plates using a QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems, ThermoFisher Scientific, Illkirch Graffenstaden, France) with Power SYBR Green 2× Master Mix (Life Technologies, ThermoFisher Scientific, Illkirch Graffenstaden, France), 0.5 µl of cDNA, and 100 nmol/l of primers (Invitrogen) in a final volume of 10 µl. Detailed information on the primers sequences is provided in **Supplementary Table S5**. The relative expression level of each gene was calculated with the method described by Pfaffl.[60] A precise description of samples preparation and

experiment procedure are compiled in **Supplementary Table S6**. Data were expressed as mean ± SD.

Protein extraction and western blot analysis. Cells were homogenized in radioimmunoprecipitation assay buffer (Sigma-Aldrich) containing 1% protease inhibitors (Sigma-Aldrich) and 10% phosphatase inhibitors (Roche, Paris, France). After electrophoresis on 4–12% Nu-PAGE Bis-Tris gels (Invitrogen) under reducing conditions, proteins were transferred to nitrocellulose membranes (Invitrogen), blocked with phosphate-buffered saline (PBS) containing 0.1% Tween-20 and 5% bovine serum albumin (BSA) or 5% non-fat dry milk, depending on the primary antibody used, and incubated overnight with the primary antibody diluted in PBS containing 0.1% Tween-20 and 5% BSA or 5% nonfat dry milk. Membranes were then incubated for 1 hour with the corresponding secondary antibody and immunoreactive protein bands were detected by ECL Plus detection reagents (Amersham Bioscience) according to the manufacturer's protocol using an ImageQuant CDD camera (GE Healthcare).

Enzymatic activities. Respiratory chain enzyme activities were spectrophotometrically measured using a Cary 50 UV–visible spectrophotometer (Varian, Les Ulis, France) as described by Bénit *et al.*[61] Mitochondrial substrate oxidation was polarographically estimated using a Clark oxygen electrode (Hansatech Instruments, Norfolk, England) in a magnetically stirred 250-μl chamber maintained at 37 °C in 250 μl of a respiratory medium consisting of 0.3 mol/l mannitol, 5 mmol/l KCl, 5 mmol/l $MgCl_2$, 10 mmol/l phosphate buffer (pH 7.2), and 1 mg/ml BSA, plus substrates or inhibitors as described by Rustin *et al.*[62] Protein concentration was measured according to the Bradford assay.

Viability, cytotoxicity, and caspase assay. MPCs DM1 were seeded at 5,000 cells/well in 96-well plate and were treated with dose range of metformin for 48 hours or dose range of ionomycin and staurosporine for 24 hours. Viability, cytotoxicity, and apoptosis events were assessed using the ApoTox-Glo Triplex Assay (Promega). After incubation of cells with the "Viability/Cytotoxicity reagent" for 50 minutes at 37 °C, the resulting cell viability and cytotoxicity fluorescences were measured respectively at 400Ex/505Em and 485Ex/520Em using the CLARIOstar microplate reader (BMG LABTECH, Champigny-sur-Marne, France). Cells were then incubated with the "Caspase-Glo 3/7 reagent" for 30 minutes at room temperature in dark, and caspase activation (a hallmark of apoptosis) was determined with luminescence measurement using the CLARIOstar microplate reader (BMG LABTECH).

Ki-67 proliferation assay. DM1 MPCs were treated with metformin dose range for 48 hours, daily repeated. After treatment, cells were fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature and incubated overnight at 4 °C with Ki-67 antibody diluted in PBS solution with 0.1% BSA and 0.3% Triton. After three washings in PBS, cells were incubated for 1 hour at room temperature with Alexa Fluor 647 goat anti-mouse IgG (ref. A21235; 1:1,000; Invitrogen) and Hoechst (ref. H3570; 1:3,000; Invitrogen) diluted in the same blocking solution as previously. After three washings in PBS,

percentage of cells in proliferation was assessed by counting Ki-67–positive nuclei number using a Cellomics Arrayscan automated microscope (Thermo Scientific, Hudson, NH).

Metforgene clinical trial for the INSR exon 11 splicing monitoring in diabetic patients. An interventional clinical trial was promoted by CERITD in the Centre Hospitalier Sud Francilien (Corbeil-Essonnes, France) (NCT 01349387) to investigate whether a treatment with metformin in patients with Type 2 diabetes had an effect on *INSR* exon 11 alternative splicing of the insulin receptor. During their visit of consultation on the follow-up to the Type 2 diabetes, 15 patients were selected on the basis of active metformin treatment at a dose greater than or equal to 1,400 mg/day. After inclusion in the study to day 0, metformin treatment will be interrupted between day 1 and day 30, replaced by Januvia 100 mg/day dose, and then resumed at day 31. Patients had to achieve a 10 ml blood sample at day 0, day 30, and 1 month after metformin retreatment. Blood samples were processed by Ficoll gradient centrifugation to isolate the circulating leukocytes. Total RNA was extracted using the RNeasy Micro/Mini kit (Qiagen) and reverse transcribed using random hexamers and Superscript III Reverse Transcriptase kit (Invitrogen). Expressions of *INSR* +/– exon 11 transcripts and 18S were monitored with TaqMan gene expression assays using the primers and MGB probes described in **Supplementary Table S5** and TaqMan Gene Expression Master Mix (Applied Biosystems) using the 7900HT Fast Real-Time PCR System (Applied Biosystems). The method described by Pfaffl[60] was used to determine the relative expression level of each gene. *FAS* exon 6 alternative splicing was additionally tested by RT–PCR. PCR amplification was carried out with recombinant Taq DNA polymerase (Invitrogen) and the primers listed in **Supplementary Table S5**. The amplification was performed using a first step at 94 °C for 3 minutes followed by 30 cycles of 45 seconds at 94 °C, 30 seconds at 55 °C, 30 seconds at 72 °C, and finished with a final 10 minutes extension at 72 °C. The PCR products were quantified using the Bioanalyzer 2100 and DNA 1000 LabChip kit (Agilent). Statistics were computed using JMP9 software (SAS, Cary, NC). Statistical differences were determined with a Wilcoxon paired test. Differences between groups were considered significant when P <0.05 (*$P < 0.05$; **$P < 0.01$; *** $P < 0.001$).

Statistical analysis. Statistics were computed in JMP using P values. Values are reported as mean and SD. Differences between groups were considered significant when P <0.05 (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). According the size of the experiment, samples parametric (ANOVA and *post hoc* tests) or nonparametric tests were chosen.

Supplementary Material

Figure S1. Western blot analysis of RBM3, SRSF1, SRSF6, RBM45 and SFPQ expressions in wild type or DM1 MPCs in response to metformin treatment.
Figure S2. Heatmap representation of the splicing events modulated by metformin in DM1 MPCs and analysis of RBM3 RNA-binding protein involvement in this regulation.
Figure S3. Analysis of RBM3 transcript variants that are candidate to the non sense mediated decay in response to metformin and cycloheximide treatments in DM1 MPCs.

Figure S4. RT-PCR detection of 6 DM1 associated splicing defects in myoblasts from 2 non affected individuals or 2 DM1 patients, treated with metformin, pentamidine or AICAR.

Figure S5. Metformin does not impact the alternative splicing of 16 splicing defects in DM1 mutated myoblasts (DM16) treated for 48 hours with a range of dose of metformin.

Figure S6. Impact of pentamidine on RBM3 expression and DM1 associated splicing defects in DM1 human myoblasts.

Figure S7. In vitro evaluation of metformin and sitagliptin treatments on *INSR* exon 11 splicing in peripheral blood lymphocytes.

Table S1. List of genes modulated by 10mM metformin treatment for 48 hours in DM1 MPCS.

Table S2. List of genes modulated by 25mM metformin treatment for 48 hours in DM1 MPCS.

Table S3. List of splicing events modulated by 10 mM and 25 mM metformin treatments for 48 hours in DM1 MPCS.

Table S4. Effect of 25 mM metformin, 75 µM pentamidine and 2 mM AICAR on the regulation of alternative splicings altered in the DM1 mutated human myoblasts DM16.

Table S5. Human primers, probes and siRNA sequences.

Table S6. Detailed procedures of reverse transcription-quantitative PCR experiments according to the MIQE Guideli.

1. Cooper, TA, Wan, L and Dreyfuss, G (2009). RNA and disease. *Cell* **136**: 777–793.
2. Keren, H, Lev-Maor, G and Ast, G (2010). Alternative splicing and evolution: diversification, exon definition and function. *Nat Rev Genet* **11**: 345–355.
3. Oana, K, Oma, Y, Suo, S, Takahashi, MP, Nishino, I, Takeda, S *et al.* (2013). Manumycin A corrects aberrant splicing of Clcn1 in myotonic dystrophy type 1 (DM1) mice. *Sci Rep* **3**: 2142.
4. Younis, I, Berg, M, Kaida, D, Dittmar, K, Wang, C and Dreyfuss, G (2010). Rapid-response splicing reporter screens identify differential regulators of constitutive and alternative splicing. *Mol Cell Biol* **30**: 1718–1728.
5. Stoilov, P, Lin, CH, Damoiseaux, R, Nikolic, J and Black, DL (2008). A high-throughput screening strategy identifies cardiotonic steroids as alternative splicing modulators. *Proc Natl Acad Sci USA* **105**: 11218–11223.
6. Warf, MB, Nakamori, M, Matthys, CM, Thornton, CA and Berglund, JA (2009). Pentamidine reverses the splicing defects associated with myotonic dystrophy. *Proc Natl Acad Sci USA* **106**: 18551–18556.
7. Larsson, O, Morita, M, Topisirovic, I, Alain, T, Blouin, MJ, Pollak, M *et al.* (2012). Distinct perturbation of the translatome by the antidiabetic drug metformin. *Proc Natl Acad Sci USA* **109**: 8977–8982.
8. Kouki, T, Takasu, N, Nakachi, A, Tamanaha, T, Komiya, I and Tawata, M (2005). Low-dose metformin improves hyperglycaemia related to myotonic dystrophy. *Diabet Med* **22**: 346–347.
9. Ho, TH, Charlet-B, N, Poulos, MG, Singh, G, Swanson, MS and Cooper, TA (2004). Muscleblind proteins regulate alternative splicing. *EMBO J* **23**: 3103–3112.
10. Kanadia, RN, Johnstone, KA, Mankodi, A, Lungu, C, Thornton, CA, Esson, D *et al.* (2003). A muscleblind knockout model for myotonic dystrophy. *Science* **302**: 1978–1980.
11. Miller, JW, Urbinati, CR, Teng-Umnuay, P, Stenberg, MG, Byrne, BJ, Thornton, CA *et al.* (2000). Recruitment of human muscleblind proteins to (CUG)(n) expansions associated with myotonic dystrophy. *EMBO J* **19**: 4439–4448.
12. Brook, JD, McCurrach, ME, Harley, HG, Buckler, AJ, Church, D, Aburatani, H *et al.* (1992). Molecular basis of myotonic dystrophy: expansion of a trinucleotide (CTG) repeat at the 3' end of a transcript encoding a protein kinase family member. *Cell* **69**: 385.
13. Fu, YH, Pizzuti, A, Fenwick, RG Jr, King, J, Rajnarayan, S, Dunne, PW *et al.* (1992). An unstable triplet repeat in a gene related to myotonic muscular dystrophy. *Science* **255**: 1256–1258.
14. Mahadevan, M, Tsilfidis, C, Sabourin, L, Shutler, G, Amemiya, C, Jansen, G *et al.* (1992). Myotonic dystrophy mutation: an unstable CTG repeat in the 3' untranslated region of the gene. *Science* **255**: 1253–1255.
15. Kuyumcu-Martinez, NM, Wang, GS and Cooper, TA (2007). Increased steady-state levels of CUGBP1 in myotonic dystrophy 1 are due to PKC-mediated hyperphosphorylation. *Mol Cell* **28**: 68–78.
16. Fugier, C, Klein, AF, Hammer, C, Vassilopoulos, S, Ivarsson, Y, Toussaint, A *et al.* (2011). Misregulated alternative splicing of BIN1 is associated with T tubule alterations and muscle weakness in myotonic dystrophy. *Nat Med* **17**: 720–725.
17. Charlet-B, N, Savkur, RS, Singh, G, Philips, AV, Grice, EA and Cooper, TA (2002). Loss of the muscle-specific chloride channel in type 1 myotonic dystrophy due to misregulated alternative splicing. *Mol Cell* **10**: 45–53.
18. Mankodi, A, Takahashi, MP, Jiang, H, Beck, CL, Bowers, WJ, Moxley, RT *et al.* (2002). Expanded CUG repeats trigger aberrant splicing of ClC-1 chloride channel pre-mRNA and hyperexcitability of skeletal muscle in myotonic dystrophy. *Mol Cell* **10**: 35–44.
19. Marteyn, A, Maury, Y, Gauthier, MM, Lecuyer, C, Vernet, R, Denis, JA *et al.* (2011). Mutant human embryonic stem cells reveal neurite and synapse formation defects in type 1 myotonic dystrophy. *Cell Stem Cell* **8**: 434–444.
20. Denis, JA, Gauthier, M, Rachdi, L, Aubert, S, Giraud-Triboult, K, Poydenot, P *et al.* (2013). mTOR-dependent proliferation defect in human ES-derived neural stem cells affected by myotonic dystrophy type 1. *J Cell Sci* **126**(Pt 8): 1763–1772.
21. Gauthier, M, Marteyn, A, Denis, JA, Cailleret, M, Giraud-Triboult, K, Aubert, S *et al.* (2013). A defective Krab-domain zinc-finger transcription factor contributes to altered myogenesis in myotonic dystrophy type 1. *Hum Mol Genet* **22**: 5188–5198.
22. Holt, I, Mittal, S, Furling, D, Butler-Browne, GS, Brook, JD and Morris, GE (2007). Defective mRNA in myotonic dystrophy accumulates at the periphery of nuclear splicing speckles. *Genes Cells* **12**: 1035–1048.
23. Whelan, RS, Konstantinidis, K, Wei, AC, Chen, Y, Reyna, DE, Jha, S *et al.* (2012). Bax regulates primary necrosis through mitochondrial dynamics. *Proc Natl Acad Sci USA* **109**: 6566–6571.
24. Chinnaiyan, AM, Tepper, CG, Seldin, MF, O'Rourke, K, Kischkel, FC, Hellbardt, S *et al.* (1996). FADD/MORT1 is a common mediator of CD95 (Fas/APO-1) and tumor necrosis factor receptor-induced apoptosis. *J Biol Chem* **271**: 4961–4965.
25. Philips, AV, Timchenko, LT and Cooper, TA (1998). Disruption of splicing regulated by a CUG-binding protein in myotonic dystrophy. *Science* **280**: 737–741.
26. Kino, Y, Washizu, C, Oma, Y, Onishi, H, Nezu, Y, Sasagawa, N *et al.* (2009). MBNL and CELF proteins regulate alternative splicing of the skeletal muscle chloride channel CLCN1. *Nucleic Acids Res* **37**: 6477–6490.
27. Durand, S, Cougot, N, Mahuteau-Betzer, F, Nguyen, CH, Grierson, DS, Bertrand, E *et al.* (2007). Inhibition of nonsense-mediated mRNA decay (NMD) by a new chemical molecule reveals the dynamic of NMD factors in P-bodies. *J Cell Biol* **178**: 1145–1160.
28. El-Mir, MY, Nogueira, V, Fontaine, E, Avéret, N, Rigoulet, M and Leverve, X (2000). Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. *J Biol Chem* **275**: 223–228.
29. Zhou, G, Myers, R, Li, Y, Chen, Y, Shen, X, Fenyk-Melody, J *et al.* (2001). Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* **108**: 1167–1174.
30. Nakamori, M, Sobczak, K, Puwanant, A, Welle, S, Eichinger, K, Pandya, S *et al.* (2013). Splicing biomarkers of disease severity in myotonic dystrophy. *Ann Neurol* **74**: 862–872.
31. Gabut, M, Samavarchi-Tehrani, P, Wang, X, Slobodeniuc, V, O'Hanlon, D, Sung, HK *et al.* (2011). An alternative splicing switch regulates embryonic stem cell pluripotency and reprogramming. *Cell* **147**: 132–146.
32. Anczuków, O, Rosenberg, AZ, Akerman, M, Das, S, Zhan, L, Karni, R *et al.* (2012). The splicing factor SRSF1 regulates apoptosis and proliferation to promote mammary epithelial cell transformation. *Nat Struct Mol Biol* **19**: 220–228.
33. Pihlajamäki, J, Lerin, C, Itkonen, P, Boes, T, Floss, T, Schroeder, J *et al.* (2011). Expression of the splicing factor gene SFRS10 is reduced in human obesity and contributes to enhanced lipogenesis. *Cell Metab* **14**: 208–218.
34. Naryshkin, NA, Weetall, M, Dakka, A, Narasimhan, J, Zhao, X, Feng, Z *et al.* (2014). Motor neuron disease. SMN2 splicing modifiers improve motor function and longevity in mice with spinal muscular atrophy. *Science* **345**: 688–693.
35. Anderson, ES, Lin, CH, Xiao, X, Stoilov, P, Burge, CB and Black, DL (2012). The cardiotonic steroid digitoxin regulates alternative splicing through depletion of the splicing factors SRSF3 and TRA2B. *RNA* **18**: 1041–1049.
36. Dutertre, M, Sanchez, G, De Cian, MC, Barbier, J, Dardenne, E, Gratadou, L *et al.* (2010). Cotranscriptional exon skipping in the genotoxic stress response. *Nat Struct Mol Biol* **17**: 1358–1366.

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37. Vivarelli, S, Lenzken, SC, Ruepp, MD, Ranzini, F, Maffioletti, A, Alvarez, R *et al.* (2013). Paraquat modulates alternative pre-mRNA splicing by modifying the intracellular distribution of SRPK2. *PLoS One* **8**: e61980.

38. Quinn, BJ, Dallos, M, Kitagawa, H, Kunnumakkara, AB, Memmott, RM, Hollander, MC *et al.* (2013). Inhibition of lung tumorigenesis by metformin is associated with decreased plasma IGF-I and diminished receptor tyrosine kinase signaling. *Cancer Prev Res (Phila)* **6**: 801–810.

39. Iglesias, DA, Yates, MS, van der Hoeven, D, Rodkey, TL, Zhang, Q, Co, NN *et al.* (2013). Another surprise from Metformin: novel mechanism of action via K-Ras influences endometrial cancer response to therapy. *Mol Cancer Ther* **12**: 2847–2856.

40. Chettouh, H, Fartoux, L, Aoudjehane, L, Wendum, D, Clapéron, A, Chrétien, Y *et al.* (2013). Mitogenic insulin receptor-A is overexpressed in human hepatocellular carcinoma due to EGFR-mediated dysregulation of RNA splicing factors. *Cancer Res* **73**: 3974–3986.

41. Du, H, Cline, MS, Osborne, RJ, Tuttle, DL, Clark, TA, Donohue, JP *et al.* (2010). Aberrant alternative splicing and extracellular matrix gene expression in mouse models of myotonic dystrophy. *Nat Struct Mol Biol* **17**: 187–193.

42. Wheeler, TM, Leger, AJ, Pandey, SK, MacLeod, AR, Nakamori, M, Cheng, SH *et al.* (2012). Targeting nuclear RNA for *in vivo* correction of myotonic dystrophy. *Nature* **488**: 111–115.

43. Leger, AJ, Mosquea, LM, Clayton, NP, Wu, IH, Weeden, T, Nelson, CA *et al.* (2013). Systemic delivery of a Peptide-linked morpholino oligonucleotide neutralizes mutant RNA toxicity in a mouse model of myotonic dystrophy. *Nucleic Acid Ther* **23**: 109–117.

44. Coonrod, LA, Nakamori, M, Wang, W, Carrell, S, Hilton, CL, Bodner, MJ *et al.* (2013). Reducing levels of toxic RNA with small molecules. *ACS Chem Biol* **8**: 2528–2537.

45. Nakamori, M, Kimura, T, Fujimura, H, Takahashi, MP and Sakoda, S (2007). Altered mRNA splicing of dystrophin in type 1 myotonic dystrophy. *Muscle Nerve* **36**: 251–257.

46. Jahnke, VE, Van Der Meulen, JH, Johnston, HK, Ghimbovschi, S, Partridge, T, Hoffman, EP *et al.* (2012). Metabolic remodeling agents show beneficial effects in the dystrophin-deficient mdx mouse model. *Skelet Muscle* **2**: 16.

47. Cheng, J, Zhou, T, Liu, C, Shapiro, JP, Brauer, MJ, Kiefer, MC *et al.* (1994). Protection from Fas-mediated apoptosis by a soluble form of the Fas molecule. *Science* **263**: 1759–1762.

48. Cascino, I, Fiucci, G, Papoff, G and Ruberti, G (1995). Three functional soluble forms of the human apoptosis-inducing Fas molecule are produced by alternative splicing. *J Immunol* **154**: 2706–2713.

49. Paronetto, MP, Bernardis, I, Volpe, E, Bechara, E, Sebestyén, E, Eyras, E *et al.* (2014). Regulation of FAS exon definition and apoptosis by the Ewing sarcoma protein. *Cell Rep* **7**: 1211–1226.

50. Liu, C, Cheng, J and Mountz, JD (1995). Differential expression of human Fas mRNA species upon peripheral blood mononuclear cell activation. *Biochem J* **310 (Pt 3)**: 957–963.

51. Mateizel, I, De Temmerman, N, Ullmann, U, Cauffman, G, Sermon, K, Van de Velde, H *et al.* (2006). Derivation of human embryonic stem cell lines from embryos obtained after IVF and after PGD for monogenic disorders. *Hum Reprod* **21**: 503–511.

52. Bolger, AM, Lohse, M and Usadel, B (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**: 2114–2120.

53. Kim, D, Pertea, G, Trapnell, C, Pimentel, H, Kelley, R and Salzberg, SL (2013). TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol* **14**: R36.

54. DeLuca, DS, Levin, JZ, Sivachenko, A, Fennell, T, Nazaire, MD, Williams, C *et al.* (2012). RNA-SeQC: RNA-seq metrics for quality control and process optimization. *Bioinformatics* **28**: 1530–1532.

55. Li, H, Handsaker, B, Wysoker, A, Fennell, T, Ruan, J, Homer, N *et al.*; 1000 Genome Project Data Processing Subgroup. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25**: 2078–2079.

56. Anders, S, Pyl, PT and Huber, W (2014). HTSeq–a Python framework to work with high-throughput sequencing data. *Bioinformatics* **31**: 166–169.

57. Anders, S and Huber, W (2010). Differential expression analysis for sequence count data. *Genome Biol* **11**: R106.

58. Mallinjoud, P, Villemin, JP, Mortada, H, Polay Espinoza, M, Desmet, FO, Samaan, S *et al.* (2014). Endothelial, epithelial, and fibroblast cells exhibit specific splicing programs independently of their tissue of origin. *Genome Res* **24**: 511–521.

59. Sacomoto, GA, Kielbassa, J, Chikhi, R, Uricaru, R, Antoniou, P, Sagot, MF *et al.* (2012). KISSPLICE: de-novo calling alternative splicing events from RNA-seq data. *BMC Bioinformatics* **13** (suppl. 6): S5.

60. Pfaffl, M (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**: e45.

61. Bénit, P, Goncalves, S, Philippe Dassa, E, Brière, JJ, Martin, G and Rustin, P (2006). Three spectrophotometric assays for the measurement of the five respiratory chain complexes in minuscule biological samples. *Clin Chim Acta* **374**: 81–86.

62. Rustin, P, Chretien, D, Bourgeron, T, Gérard, B, Rötig, A, Saudubray, JM *et al.* (1994). Biochemical and molecular investigations in respiratory chain deficiencies. *Clin Chim Acta* **228**: 35–51.

Supplementary Information accompanies this paper on the Molecular Therapy–Nucleic Acids website (http://www.nature.com/mtna)