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DEVELOPMENTALLY REGULATED ALTERNATIVE SPLICING IS PERTURBED IN TYPE 1 DIABETIC SKELETAL MUSCLE

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Abstract

Introduction—Type 1 diabetic patients can develop skeletal muscle weakness and atrophy by molecular mechanisms that are not well understood. Alternative splicing (AS) is critical for gene expression in the skeletal muscle, and its dysregulation is implicated in muscle weakness and atrophy. Therefore, we investigated whether AS patterns are affected in type 1 diabetic skeletal muscle contributing to skeletal muscle defects.

Methods—AS patterns were determined by reverse transcription–polymerase chain reaction and levels of RNA binding proteins were assessed by Western blot in type 1 diabetic mouse skeletal muscle and during normal mouse skeletal muscle development.

Results—Five genes with critical functions in the skeletal muscle are misspliced in type 1 diabetic skeletal muscle, resembling their AS patterns at embryonic stages. AS of these genes undergoes dramatic transitions during skeletal muscle development, correlating with changes in specific RNA binding proteins.

Conclusion—Embryonic spliced variants are inappropriately expressed in type 1 diabetic skeletal muscle.

Keywords

alternative splicing; muscle development; RNA binding proteins; skeletal muscle; type 1 diabetes

Type 1 diabetic patients exhibit multisystemic complications, such as nephropathy, retinopathy, neuropathy, myopathy, and cardiovascular disease.[1–9] Diabetic myopathy is characterized by skeletal muscle weakness and atrophy.[5] Under diabetic conditions, skeletal

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muscle function, growth, development, and repair are compromised.[5,10–12] In type 1 diabetic patients, muscle mass and myofiber size are reduced, and muscle metabolism switches to a glycolytic pathway.[11–14] In addition to growth and function, repair capacity of skeletal muscle is blunted in type 1 diabetes due to the adverse effects of hyperglycemia on muscle progenitor (satellite) cells.[11,15–18] However, molecular mechanisms responsible for diabetic myopathy are not well understood.

Alternative splicing (AS) is critical for muscle growth, development, and function via directly regulating gene expression.[17–21] AS is a complex process controlled by RNA binding proteins that bind to specific motifs in the pre-mRNA.[22] The abundance and activity of these regulators are important for AS decisions.[22] **R**NA **b**inding protein **F**ox-1 homolog (RBFOX), **M**uscle**b**li**n**d-**l**ike (MBNL), and **C**UG-binding protein **E**lav-**l**ike **f**amily (CELF) of RNA binding proteins are linked to AS regulation during heart and skeletal muscle development.[23–27] Dysregulation of AS is linked to skeletal muscle and heart pathophysiology of myotonic dystrophy.[28–33]

We have recently shown that global changes in AS occur in type 1 diabetic mouse heart due to changes in RNA binding proteins, negatively affecting gene expression and contributing to diabetic cardiomyopathy.[27,34] Therefore, we hypothesized that type 1 diabetic skeletal muscle may also display abnormal AS signatures mediated by RNA binding proteins.

METHODS

Type 1 Diabetic Mice

A well-established type 1 diabetic mouse model, the non-obese diabetic (NOD) mouse, which can closely mimic human type 1 diabetes,[10,35,36] was used in our experiments. In this mouse model, females develop diabetes around 30 weeks of age due to autoimmune-mediated destruction of β islet cells.[10,35,36] NOD mice (≥5 months of age) were purchased (NOD/ShiLtj, Stock #001976; Jackson Laboratory, Bar Harbor, Maine). Fasting blood glucose levels of both NOD and control mice were tested weekly using tail vein blood by a OneTouch glucometer. Female NOD mice that exhibited fasting glucose levels >400 mg/dl for 3 weeks were used in our experiments because female mice develop diabetes at an earlier time-point and more consistently than males.[27] Age-matched ICR (ICR/HaJ, Stock #009122; Jackson Laboratory) female mice were used as non-diabetic controls. ICR mice have the same major histocompatibility complex (MHC) haplotype as NOD mice, but they do not develop insulitis or diabetes. In addition, NOD mice descend from an ICR strain, making them ideal controls. NOD and ICR mice were euthanized and the gastrocnemius muscle was isolated for RNA and protein extraction.

Mouse Development

Friend virus B (FVB/NJ, Stock #001800) wild-type mice were purchased (Jackson Laboratory). Timed matings were conducted. Embryonic day 18 (E18) and newborn (NB) mice were euthanized and hindlimbs were obtained. Quadricep muscles were isolated from 6-month-old wild-type FVB (adult) mice.

All mouse experiments were conducted in accordance with the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals* and approved by the institutional animal care and use committee of the University of Texas Medical Branch (#1101001).

RNA Extraction

RNA was extracted from mouse tissues using TRIzol (15596018; Thermo Fisher Scientific/Invitrogen, Waltham, Massachusetts) following the manufacturer's protocol. RNA concentrations were measured using a microplate spectrometer (Epoch; BioTek, Winooski, Vermont).

Quantitative Reverse Transcription–Polymerase Chain Reaction and Statistical Analysis

Quantitative reverse transcription–polymerase chain reaction (qRT-PCR) and statistical analysis were performed as described elsewhere.[27,34] Briefly, a cDNA library was generated by annealing 125 ng of oligo(dT) (S1316S; New England BioLabs, Ipswich, Massachusetts) to the poly(A) tail of total RNA (1 μg) at 65° C for 10 min and then incubated with 15 units/μg Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT) (AMV 007-1; Life Sciences Advance Technologies, St. Petersburg, Florida) and 10 μmol/L deoxynucleotide triphosphates (Thermo Fisher Scientific/Invitrogen) at 42° C for 1 hour. For the PCR reaction, primer sequences were designed on exons upstream and downstream of alternative exons. Genes and primer sequences used for this study are included in the Supplementary Material (available online).

PCR amplification was carried out in a 20-μl reaction using 5 μl of cDNA, 15 units/μg Biolase Taq polymerase (21066; Bioline, London, UK) and 200 ng of each primer under the following conditions: 95° C, 45 s; 59° C, 45 s; and 75° C, 1 min, for 25 cycles. PCR products were resolved with 5% non-denaturing polyacrylamide gels and stained with ethidium bromide. DNA bands were imaged using Gel Doc XR + (Bio-Rad, Hercules, California) and quantified by normalizing the ethidium bromide signal to the size of the DNA bands using Bio-Rad Image Lab software.[27] Percent inclusion of alternative exons was calculated using the formula as we have described elsewhere.[27] The Student's *t*-test was used to determine statistical significance between 2 groups and one-way analysis of variance (ANOVA) for more than 2 groups using GraphPad Prism (GraphPad, Inc., La Jolla, California).

Western Blotting

Thirty to fifty μg of protein was separated on 10% sodium dodecylsulfate–polyacrylamide gel electrophoresis gels, and transferred to Immobilon-P polyvinylidene fluoride membranes (EMD IPVH00010; EMD Millipore, Temecula, California). Membranes were stained with Ponceau S (P7170-1L; Sigma Co. St. Louis, Missouri) to assess transfer quality and equal protein loading. Membranes were blocked using 5% dry fat-free milk solution in phosphate-buffered saline with 0.1% Tween 20 (PBST) for 1 hour, followed by overnight incubation with the specified primary antibody at 4° C. Membranes were subsequently washed 3 times for 15 minutes each with PBST followed by incubation with the relevant horseradish peroxidase (HRP)–labeled secondary antibody for 3 hours at room temperature. HRP activity was determined using Immobilon Western Chemiluminescent (WBKLS0500; EMD

Millipore) or SuperSignal West Femto Chemiluminescent (34096; Thermo Fisher Scientific/Pierce) followed by exposure to GeneMate X-ray film (F-9024-8x10; Bioexpress, Kaysville, Utah). Protein levels were quantified by measuring protein band intensity and normalizing it to loading controls using the Bio-Rad Image Lab software as described elsewhere.[34,37] Primary antibodies used in WB analyses were as follows: rabbit anti-RBFOX1 (HPA040809; Sigma); mouse anti-CELF1 (ab9549; Abcam, Cambridge, Massachusetts); mouse anti-MBNL1 (M3320; Sigma), rabbit anti-MYF5 (ab125301; Abcam); mouse anti-MYOD1 (ab16148; Abcam); and mouse anti-histone H3 (ab10799; Abcam). Secondary antibodies used in WB analyses were as follows: goat anti-mouse light-chain IgG-HRP (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania) and goat anti-rabbit IgG-HRP (EMD Millipore).

RESULTS

Alternative Splicing Is Dysregulated in Type 1 Diabetic Mice

To determine whether AS changes occur in diabetic skeletal muscle and contribute to diabetic myopathy, we tested AS patterns of 5 genes involved in muscle contraction, growth, and metabolism. Using non-obese diabetic (NOD) mice, we assessed AS regulation of calcineurin (*Ppp3ca*), plasma membrane calcium-transporting ATPase 1 (*Atp2b1*), fragile X mental retardation syndrome–related protein 1 (*Fxr1*), myotubularin-related phosphatase 3 (*Mtmr3*), and glutathione peroxidase 8 (*Gpx8*).

We checked in NOD mouse skeletal muscle for splicing of *Ppp3ca* exon 13 that encodes for the auto-inhibitory domain of the protein and found that exon 13 was more excluded in type 1 diabetic mice (Fig. 1A and B). Next, we examined AS of *Atp2b1* exon 21 that is within the calmodulin-binding domain. We found that exon 21 (corresponds to exon 22 in human) of *Atbp2b1* was less included in the NOD mouse gastrocnemius muscle in comparison to control mice (Fig. 1C). AS of *Fxr1* exons 15 and 16 was also altered in type 1 diabetic mouse skeletal muscle (Fig. 1D). AS of *Mtmr3* exon 16 was affected modestly in NOD mice (Fig. 1E). Finally, we checked splicing of *Gpx8* exon 2 that encodes for the thioredoxin superfamily domain essential for antioxidant properties of the enzyme. We found that exon 2 of *Gpx8* was more excluded in the skeletal muscle of NOD mice (Fig. 1F). Overall, all 5 genes exhibited altered AS patterns in type 1 diabetic mouse skeletal muscle when compared with controls (Fig. 1).

Alternative Splicing Events Altered in Diabetic Skeletal Muscle Are Developmentally Regulated

To determine whether AS events affected in diabetic skeletal muscle are developmentally regulated, we tested AS of these genes *in vivo* during normal mouse skeletal muscle development. We examined AS events in wild-type mouse skeletal muscle at embryonic day 18 (E18), newborn, and adult (6-month-old) stages. AS of *Ppp3ca* (Fig. 2A), *Atp2b1* (Fig. 2B), *Fxr1* (Fig. 2C), *and Mtmr3* (Fig. 2D) transitioned in developing skeletal muscle such that alternative exons were included in adult, but excluded in newborn and embryonic skeletal muscle. *Gpx8* exon 2 was included similarly in embryonic and adult skeletal muscle, but it was included less in newborn skeletal muscle (Fig. 2E). AS events altered in

type 1 diabetic skeletal muscle underwent embryonic-to-postnatal transitions during normal skeletal muscle development in mice.

RNA Binding Proteins that Regulate Alternative Splicing Undergo Dynamic Changes during Mouse Skeletal Muscle Development

To determine the protein levels of RNA binding proteins that regulate AS during skeletal muscle development, we examined steady-state levels of CELF1, MBNL1, and RBFOX1 in E18 and newborn limb and adult quadriceps by Western blot (WB). Histone H3 WB and Ponceau S stainings of membranes were used as loading controls. CELF1 protein levels were high at E18 and newborn stages, but were dramatically decreased in adult skeletal muscle relative to embryonic skeletal muscle (Fig. 3). RBFOX1 protein levels increased as the muscle developed. Although 2 isoforms (~48 kDa and ~37–40 kDa) of MBNL1 expressed in skeletal muscle were abundant at embryonic and newborn stages, they were severely downregulated in adult mice relative to embryonic and newborn mice (Fig. 3). In summary, CELF1, RBFOX1, and MBNL1 protein levels were dynamically controlled at embryonic, newborn, and adult stages in skeletal muscle.

RNA Binding Protein Levels Are Altered in Type 1 Diabetic Skeletal Muscle

To determine whether expression of embryonic spliced variants in type 1 diabetic skeletal muscle correlates with changes in RBFOX1, CELF1, and MBNL1, protein levels of these RNA binding proteins were determined by WB analysis using gastrocnemius muscle from control ICR or NOD mice. RBFOX1 protein was increased by 2.65-fold (Fig. 4A). There was no statistically significant change in MBNL1 protein levels in NOD mice skeletal muscle (Fig. 4A). CELF1 protein levels were upregulated by 2.78-fold in the skeletal muscle of NOD mice in comparison to control mice (Fig. 4B). These results show that RBFOX1 and CELF1 proteins are modulated in skeletal muscle under type 1 diabetic conditions. Notably, upregulation of CELF1 protein levels in diabetic skeletal muscle mimicked its protein levels at embryonic and newborn stages in muscle, consistent with more embryonic/newborn-like AS patterns of *Atp2b1, Fxr1*, and *Mtmr3* in diabetic skeletal muscle (Fig. 1C–E vs. Fig. 2B–D).

Next, we tested whether CELF1 increase in diabetic skeletal muscle is a primary event or is secondary due to muscle regeneration. Therefore, we assessed protein levels of MYF5 and MYOD1 transcription factors that are upregulated in regenerating muscle. Figure 4B shows that CELF1 protein levels were elevated in NOD mice where MYOD1 and MYF5 levels were significantly downregulated – 2.17-fold and –1.79-fold, respectively (Fig. 4B). Overall, we found that increases in CELF1 protein levels correlated with expression of embryonic spliced variants in type 1 skeletal muscle and this increase was not due to muscle regeneration.

DISCUSSION

We have previously identified genome-wide AS changes in diabetic hearts.[27,34] In this study we examined whether AS defects occur in type 1 diabetic skeletal muscle. We found that 5 genes relevant to skeletal muscle physiology were misspliced in type 1 diabetic skeletal

muscle. Because all the alternative exons of these genes are located within a functional domain of the protein, and these exons are excluded in diabetic skeletal muscle, AS changes likely affect their functions/activities under diabetic conditions. We found that AS patterns of these genes are regulated dramatically during mouse skeletal muscle development. Expression of “fetal-like” spliced variants is implicated in skeletal muscle pathogenesis of myotonic dystrophy, in which there is severe muscle wasting and atrophy.[28–33] Although diabetic myopathy displays less severe muscle atrophy and weakness, expression of embryonic spliced variants may contribute to myopathy in diabetic patients. In this study we tested only 5 genes, but it is possible that there is a global switch to embryonic AS programs in diabetic skeletal muscle. Future studies are needed to test this possibility. Several other studies, including ours, have shown that embryonic splicing patterns are also activated in failing and diabetic hearts.[27,38,39] Therefore, a defect in developmental regulation of AS patterns may be a common phenomenon in striated muscle under pathological conditions.

We found that CELF1 and RBFOX1 protein levels are elevated in type 1 diabetic skeletal muscle (Fig. 4). Importantly, expression of embryonic spliced variants was in agreement with upregulation of CELF1 protein levels. Increase in CELF1 protein levels and expression of embryonic-like spliced variants in diabetic skeletal muscle supports our previous results showing that high CELF1 protein level correlates with subsequent reactivation of embryonic splicing programs in diabetic hearts.[27] In support of our findings, it has been shown that AS of *Fxr1, Mtmr3*, and *Atp2b1* revert back to an embryonic pattern in CELF1 heart-specific transgenic mice, suggesting that CELF1 controls AS of these genes.[23] In addition, our results show that CELF1 upregulation is not a secondary effect from muscle regeneration and supports previous findings that muscle growth, development, and regeneration are diminished under diabetic conditions.[5,10–12,40]

RBFOX1 protein levels are also increased in type 1 diabetic skeletal muscle (Fig. 4A). Interestingly, we did not see a switch to an embryonic expression pattern of RBFOX1 in diabetic skeletal muscle. RBFOX1 protein levels were low at embryonic stages but high at adult stages in skeletal muscle (Fig. 3). In diabetic skeletal muscle protein levels increased further. The mechanism for this increase and its consequences on alternative splicing patterns are unknown.

In our study, *Fxr1* exons 15 + 16 were mostly excluded in type 1 diabetic mouse skeletal muscle. FXR1 regulates mRNA translation of muscle structural genes such as Talin2 and Desmoplakin.[41] *Fxr1* ablation in mice leads to defects in formation of limb musculature and reduced *Fxr1* expression in zebrafish induces muscle dystrophy.[42,43] *Fxr1* exon 15 and 16 splicing is regulated by both CELF and RBFOX proteins.[23,34] Exons 15 and 16 are critical for binding to the muscle-specific target mRNAs of FXR1. A naturally occurring constitutively active cal-cineurin is generated via exclusion of *Ppp3ca* exons 13 and 14 that encode for the autoinhibitory domain, and this calcineurin isoform is thought to affect muscle regeneration.[44–46] Therefore, the change in AS pattern of *Ppp3ca* (calcineurin) in diabetic mice generates a constitutively active isoform that may play a role in the muscle fiber changes and regeneration defects observed in type 1 diabetic skeletal muscle.[13] *Atp2b1* exon 21 is mostly excluded in type 1 diabetic mouse skeletal muscle, similar to its AS pattern in myotonic dystrophy patient myoblasts.[47] Exclusion of the same exon, which is

important for calmodulin interactions, may contribute to defects in calcium homeostasis in type 1 diabetic skeletal muscle. *Atp2b1* exon 21 inclusion was shown to be controlled in an RBFOX and CELF-dependent manner.[23,34] *Gpx8* has 3 exons, exon 2 is skipped more in type 1 diabetic mouse skeletal muscle. This exon encodes for the thioredoxin-like superfamily domain found in antioxidant proteins.[48,49] Exclusion of this exon may impact the antioxidant activity of GPX8 in type 1 diabetic skeletal muscle, but it is still unclear what regulates inclusion/exclusion of this exon under diabetic conditions.

Identifying biologically relevant AS changes in type 1 diabetic skeletal muscle may provide new targets for potential treatment options in patients. In the future, the use of modified oligonucleotides that are currently being tested for treatment of other muscle diseases[50–52] may provide novel tools to correct AS changes in diabetic patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ANOVA	analysis of variance
AS	alternative splicing
CELF1	CUG-binding protein Elav-like family member 1
E18	embryonic day 18
FVB	Friend virus B
HRP	horseradish peroxidase
MHC	histocompatibility complex
MBNL1	muscleblind-like splicing regulator 1
NB	newborn
NOD	non-obese diabetic
qRT-PCR	quantitative reverse transcript–polymerase chain reaction
RBFOX1	RNA binding protein Fox-1 homolog 1
T1D	type 1 diabetes

WB Western blot

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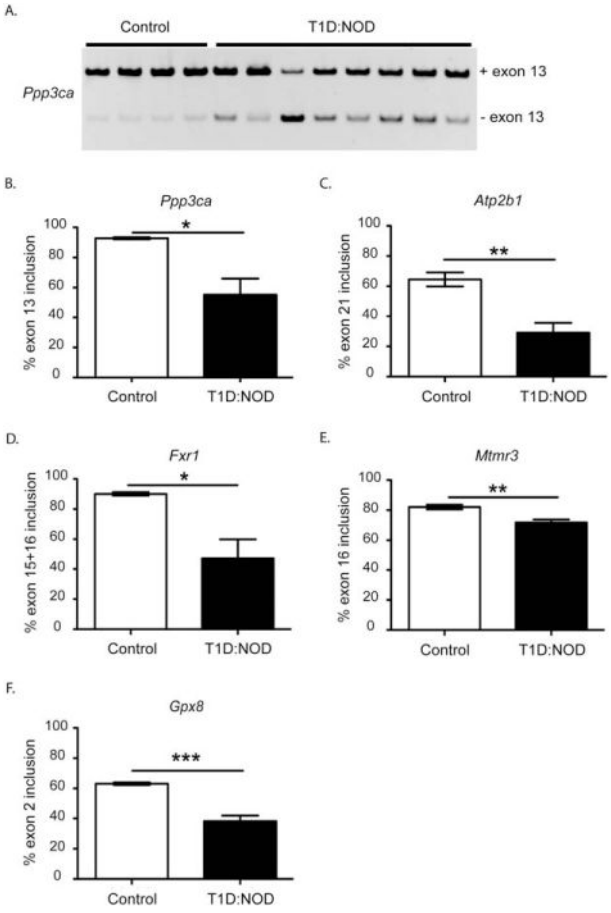

FIGURE 1.

Alternative splicing is dysregulated in skeletal muscle of non-obese diabetic mice. Alternative splicing analysis of **(A)** and **(B)**, *Ppp3ca* exon 13, **(C)** *Atp2b1* exon 21, **(D)** *Fxr1* exon 15 + 16, **(E)** *Mtmr3* exon 16, and **(F)** *Gpx8* exon 2 in ICR/HaJ (control) (white bars) or type 1 non-obese diabetic (T1D:NOD) (black bars) mouse gastrocnemius muscle, as determined by qRT-PCR ($n \geq 4$; *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$).

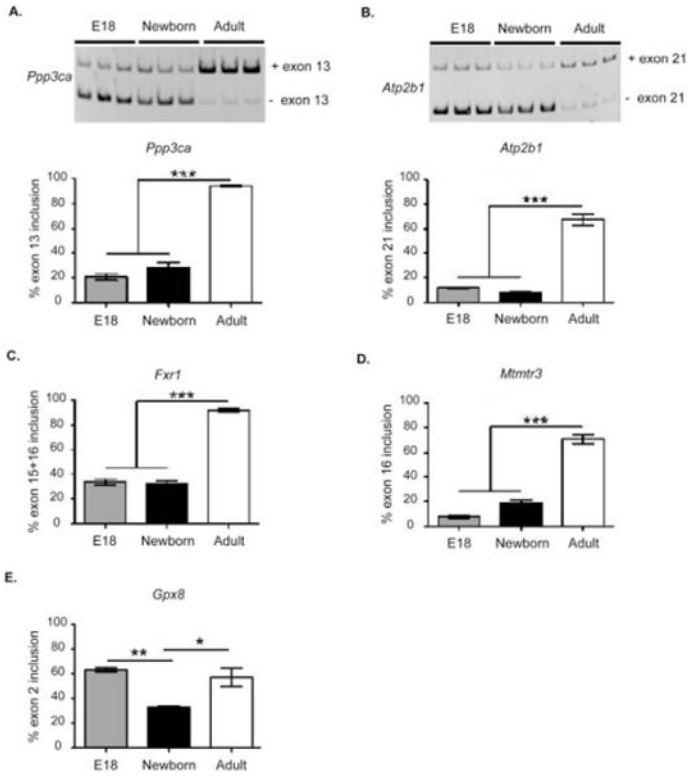

FIGURE 2.

Alternative splicing events affected in type 1 diabetic skeletal muscle undergo changes during mouse skeletal muscle development. Representative gel images of **(A)** *Ppp3ca* and **(B)** *Atp2b1* AS in normal FVB mouse skeletal muscle during development. Quantification of alternative exon inclusions of **(A)** *Ppp3ca*, **(B)** *Atp2b1*, **(C)** *Fxr1*, **(D)** *Mtmr3*, and **(E)** *Gpx8* in embryonic day 18 (E18) limb, newborn limb, and adult mouse quadriceps ($n = 3$; $*P < 0.05$, $**P < 0.01$, $***P < 0.005$).

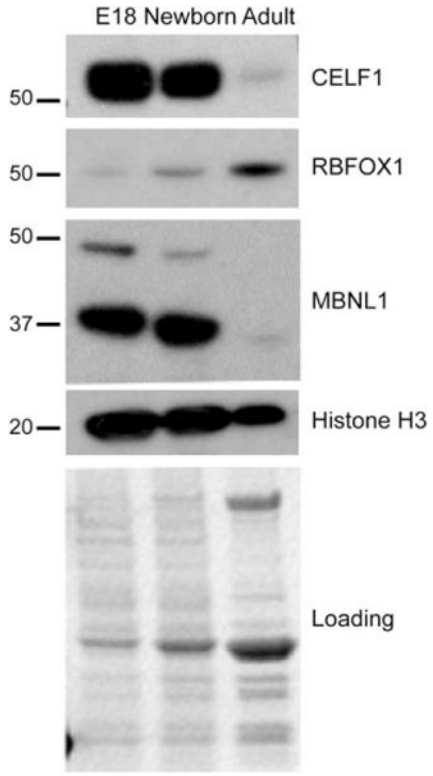

FIGURE 3.

Expression of splicing regulators is dynamically controlled during skeletal muscle development. Western blot (WB) analysis of CELF1, RBFOX1, MBNL1, and Histone H3 in wild-type E18 embryonic mouse limb, newborn mouse limb, and adult mouse quadriceps. Ponceau S staining and Histone H3 WB were used to monitor protein loading.

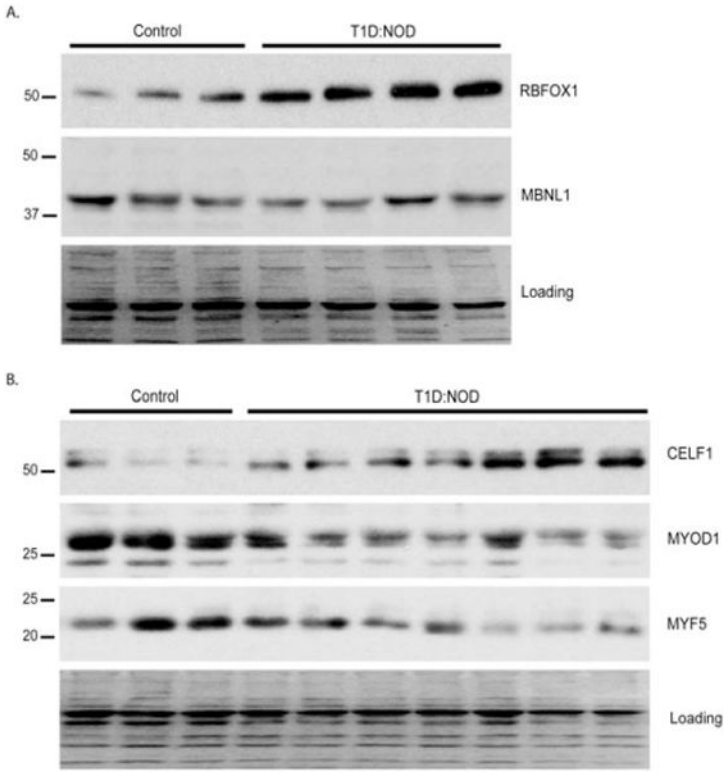

FIGURE 4.

Protein levels of splicing regulators are modulated in non-obese diabetic skeletal muscle. Western blot analysis of **(A)** RBFOX1 and MBNL1 in ICR (control) or non-obese diabetic (T1D:NOD) gastrocnemius muscle of mice. **(B)** CELF1, MYOD1, and MYF5 in control ($n = 3$) and NOD ($n = 7$) mice gastrocnemius muscle. Ponceau S staining was used to monitor protein loading. Fold-change in each protein in NOD mice skeletal muscle was quantified after normalizing to the loading control and compared with control ICR mice (*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$, NS = not significant). Fold changes in protein levels were as follows: RBFOX1 protein 2.65 ± 0.15 ($P < 0.01$); MBNL1 protein 1.1 ± 0.1 (not significant); CELF1 protein 2.78 ± 0.36 ($P < 0.05$); MYOD1 protein −2.17 ± 0.06 ($P < 0.005$); and MYF5 protein −1.79 ± 0.09 ($P < 0.05$).