GSK3β mediates muscle pathology in myotonic dystrophy

Karlie Jones,1 Christina Wei,1 Polina Iakova,2 Enrico Bugiardini,3 Christiane Schneider-Gold,4 Giovanni Meola,3 James Woodgett,5 James Killian,6 Nikolai A. Timchenko,2 and Lubov T. Timchenko1

1Department of Molecular Physiology and Biophysics and 2Department of Pathology and Immunology and Huffington Center on Aging, Baylor College of Medicine, Houston, Texas, USA. 3Department of Neurology, University of Milan, IRCCS Policlinico San Donato, Milan, Italy. 4Department of Neurology, St. Josep Hospital of the Ruhr-University of Bochum, Bochum, Germany. 5Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada. 6Department of Neurology, Baylor College of Medicine, Houston, Texas, USA.

Myotonic dystrophy type 1 (DM1) is a complex neuromuscular disease characterized by skeletal muscle wasting, weakness, and myotonia. DM1 is caused by the accumulation of CUG repeats, which alter the biological activities of RNA-binding proteins, including CUG-binding protein 1 (CUGBP1). CUGBP1 is an important skeletal muscle translational regulator that is activated by cyclin D3–dependent kinase 4 (CDK4). Here we show that mutant CUG repeats suppress Cdk4 signaling by increasing the stability and activity of glycogen synthase kinase 3β (GSK3β). Using a mouse model of DM1 (HSA18), we found that CUG repeats in the 3′ untranslated region (UTR) of human skeletal actin increase active GSK3β in skeletal muscle of mice, prior to the development of skeletal muscle weakness. Inhibition of GSK3β in both DM1 cell culture and mouse models corrected cyclin D3 levels and reduced muscle weakness and myotonia in DM1 mice. Our data predict that compounds normalizing GSK3β activity might be beneficial for improvement of muscle function in patients with DM1.

Introduction

Myotonic dystrophy type 1 (DM1) is a complex disease affecting primarily skeletal muscle, causing myotonia, skeletal muscle weakness, and wasting (1). DM1 is caused by the expansion of polymorphic, noncoding CTG repeats in the 3′ untranslated region (UTR) of the dystrophia myotonica protein kinase (DMPK) gene (2, 3). The severity of DM1 correlates with the length of CTG expansions. The longest CTG expansions are observed in patients with a congenital form of DM1 that affects newborn children (1). Congenital DM1 is characterized by a delay in skeletal muscle development, leading to extreme muscle weakness and a weak respiratory system, which has been associated with a high mortality rate (4, 5). Expanded CTG repeats cause the disease through RNA CUG repeats that misregulate several CUG RNA-binding proteins, including CUGBP1 (CUGBP Elav-like family member 1, CELF1) and muscleblind 1 (MBNL1) (6–24). The mutant CUG aggregates sequester MBNL1, reducing splicing of MBNL1-regulated mRNAs (11, 12, 17). A portion of the mutant CUG repeats bind to CUGBP1 and elevate CUGBP1 protein levels through an increase in its stability (14). Phosphorylation of CUGBP1 by PKC also contributes to the increase in CUGBP1 stability (24).

CUGBP1 is a highly conserved, multifunctional protein that regulates RNA processing on several levels, including translation, RNA stability, and splicing (9, 14–16, 18, 20–22, 25–32). The increase in CUGBP1 to the levels observed in the congenital DM1 leads to the delay of myogenesis in the CUGBP1 transgenic mouse model (18). Multiple functions of CUGBP1 are tightly regulated by phosphorylation at distinct sites (21, 22, 24). Phosphorylation of CUGBP1 by AKT at S28 controls nucleus-cytoplasm distribution and increases CUGBP1 affinity toward certain mRNA targets (21, 22). Translational activity of CUGBP1 is regulated by cyclin D3/CDK4 phosphorylation at S302 (21, 22, 29, 32).

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: J Clin Invest. 2012;122(12):4461–4472. doi:10.1172/JCI64081.

Significance

p-S302–CUGBP1 binds to the active eukaryotic initiation translation factor 2α (eIF2α) (22, 29, 32). The CUGBP1-eIF2 complex delivers mRNAs to polysomes and promotes their translation (29). Since CUGBP1 regulates translation of proteins crucial for skeletal muscle development (such as a cyclin-dependent kinase inhibitor, p21, cyclin D1, and myocyte enhancer factor 2A), the translational function of CUGBP1 is important for normal myogenesis, especially at the stage of formation of multinucleated myotubes (15, 18, 21). In DM1 myotubes, CUGBP1 does not bind to active eIF2 because CUGBP1 is under-phosphorylated at S302. This reduction of phosphorylation of CUGBP1 is associated with the reduction of cyclin D3 levels in DM1 myotubes (21). As a result, the fusion of DM1 myotubes occurs with low efficiency, delaying myogenesis. Correction of cyclin D3 levels in DM1 myoblasts improves formation of multinucleated myotubes (21). Based on these data, we hypothesized that the normalization of cyclin D3 in DM1 might be beneficial for DM1 patients and might correct DM1 skeletal muscle pathology.

In the current study, we tested this hypothesis by investigating upstream events that cause the reduction of cyclin D3 in DM1. We found that the reduction of cyclin D3 in DM1 muscle biopsy samples is associated with the phosphorylation of cyclin D3 at T283 by active GSK3β kinase. Our data show that correction of the GSK3β/cyclin D3 pathway in the DM1 mouse model (HSA18 mice) improves skeletal muscle strength and reduces myotonia.

Results

Increase in GSK3β in muscle biopsy samples from patients with DM1. Our previous study has shown that cyclin D3 is reduced in the cytoplasm of DM1 cultured myotubes (21). To determine whether cyclin D3 is also reduced in mature muscle in DM1, we initially examined cyclin D3 levels in muscle biopsies from 2 sex- and age-matched normal controls and from 2 patients with DM1. This analysis showed that the protein levels of cyclin D3 are reduced in DM1 skeletal muscle (Figure 1A).
One of the well-characterized markers of DM1 molecular pathology is the increase in CUGBP1 (14, 16, 19, 23). We found that the same muscle extracts from patients with DM1 showed elevation of CUGBP1 relative to normal muscle samples (Figure 1, B and C).

To further examine the mechanism of the reduction in cyclin D3 in DM1, we asked whether the increase in GSK3β in DM1 skeletal muscle might lead to the increased phosphorylation of cyclin D3 at T283 (p-T283–cyclin D3). In these experiments, we also examined the second mechanism of cyclin D3 regulation, which is mediated by the interaction of cyclin D3 with p-Rb. Cyclin D3 was precipitated from normal and from DM1 muscle samples using antibodies to total cyclin D3. The cyclin D3 IPs were divided into two portions; one portion was examined by Western blotting with antibodies to Rb, p-T286 (recognizing p-T286 in cyclin D3), and cyclin D3. The signal of IgGs is the control for antibodies used for immunoprecipitation. Since cyclin D3 is reduced in DM1, the amount of protein from DM1 muscle tissue used for IP was 6-fold higher than that isolated from normal muscle. Ratios of p-T283–cyclin D3 (E) and Rb (F) to total cyclin D3 were determined by quantitating protein expression from DM1 and normal muscle samples (Figure 1A and D).

To confirm the reduction of cyclin D3 in DM1, we performed immunohistochemical analysis of 6 additional muscle biopsy samples from the patients with DM1 and from 2 additional samples from patients with normal muscle histopathology. Cyclin D3 levels were reduced in all 8 examined patients with DM1 (Figure 1, A–C). Western blot analysis also showed that levels of total GSK3β were increased in all studied DM1 muscle biopsy samples (Figure 1, A–C).

GSK3β is a constitutively active protein kinase, the activity of which is inhibited by phosphorylation of S9 by other upstream kinases (35). We measured the levels of p-S9–GSK3β in the muscle biopsy samples from normal patients and patients with DM1 and found that the inactive form (phosphorylated at S9) of GSK3β was almost undetectable in DM1 muscle (Figure 1, B and C). Such reduction in the inactive p-S9–GSK3β and increase in total GSK3β in DM1 muscle shows that active GSK3β is elevated in DM1.

It is known that cyclin D3 is mainly regulated at the level of protein stability by two mechanisms (33, 34). The first mechanism of cyclin D3 regulation involves GSK3β-mediated phosphorylation of cyclin D3 at T283, which triggers degradation of cyclin D3 through the ubiquitin/proteasome pathway (33). The second mechanism is mediated by the interaction of cyclin D3 with phospho–retinoblastoma protein (p-Rb). This interaction protects cyclin D3 from degradation (34). To examine whether GSK3β is involved in the downregulation of cyclin D3 in DM1, we measured the total levels of GSK3β in the muscle biopsy samples from DM1 patients. Western blot analysis showed that the levels of total GSK3β increased in the DM1 muscle samples (Figure 1A).

To examine whether the increase in GSK3β in DM1 skeletal muscle might lead to the increased phosphorylation of cyclin D3 at T283, we performed Western blotting with antibodies to cyclin D3 (Cyc D3), GSK3β, p-S9–GSK3β, CUGBP1, and actin. (C) Average levels of cyclin D3, GSK3β, p-S9–GSK3β, and CUGBP1 presented as ratios to actin. The standard deviations represent quantitation of protein expression based on 3 experiments. (D) Phosphorylation of cyclin D3 at T283 is increased in DM1 skeletal muscle biopsies. IP–Western blot assay. Cyclin D3 was precipitated with antibodies to total cyclin D3. Cyclin D3 IPs were divided into two portions and examined by Western blot assay with antibodies to Rb, p-T286 (recognizing p-T286 in cyclin D3), and cyclin D3. The signal of IgGs is the control for antibodies used for immunoprecipitation. Since cyclin D3 is reduced in DM1, the amount of protein from DM1 muscle tissue used for IP was 6-fold higher than that isolated from normal muscle. Ratios of p-T283–cyclin D3 (E) and Rb (F) to total cyclin D3 were determined by quantitating protein expression from D. The standard deviations show values based on 3 experiments.

Figure 1
Elevation of active GSK3β and reduction of cyclin D3 in DM1 skeletal muscle biopsies. (A) Western blot analyses of the total protein extracts from patients with normal muscle histopathology (N1, N2) and from patients affected with DM1 (D1, D2) were performed, with antibodies shown on the right. (B) Examination of GSK3β/cyclin D3/CUGBP1 pathway in skeletal muscles of 6 additional patients with DM1. Western blotting was performed with antibodies to cyclin D3 (Cyc D3), GSK3β, p-S9–GSK3β, CUGBP1, and actin. (C) Average levels of cyclin D3, GSK3β, p-S9–GSK3β, and CUGBP1 presented as ratios to actin. The standard deviations represent quantitation of protein expression based on 3 experiments. (D) Phosphorylation of cyclin D3 at T283 is increased in DM1 skeletal muscle biopsies. IP–Western blot assay. Cyclin D3 was precipitated with antibodies to total cyclin D3. Cyclin D3 IPs were divided into two portions and examined by Western blot assay with antibodies to Rb, p-T286 (recognizing p-T286 in cyclin D3), and cyclin D3. The signal of IgGs is the control for antibodies used for immunoprecipitation. Since cyclin D3 is reduced in DM1, the amount of protein from DM1 muscle tissue used for IP was 6-fold higher than that isolated from normal muscle. Ratios of p-T283–cyclin D3 (E) and Rb (F) to total cyclin D3 were determined by quantitating protein expression from D. The standard deviations show values based on 3 experiments.
Mutant CUG repeats increase expression of GSK3β in a mouse model of DM1. To determine whether the mutant CUG repeats may be responsible for elevation of GSK3β and downregulation of cyclin D3, we examined levels of GSK3β and cyclin D3 in muscle of a mouse model of DM1, HSA LR mice (11). These mice express an array of the untranslated CUG repeats in the 3’ UTR of human skeletal actin (11). We found that levels of total GSK3β were elevated in skeletal muscle (soleus) of HSA LR mice (at 6 months of age) and extracts from matching WT mice. CRM, cross-reactive material. (B) Ratios of signals of GSK3β and cyclin D3, as presented in A, to actin. The standard deviations are shown for 3 experiments.

Mutant CUG repeats increase expression of GSK3β in a mouse model of DM1. To determine whether the mutant CUG repeats may be responsible for elevation of GSK3β and downregulation of cyclin D3, we examined levels of GSK3β and cyclin D3 in muscle of a mouse model of DM1, HSA LR mice (11). These mice express an array of the untranslated CUG repeats in the 3’ UTR of human skeletal actin (11). We found that levels of total GSK3β were elevated in skeletal muscle (soleus) of HSA LR mice; whereas cyclin D3 was reduced in the same protein extracts (Figure 2, A and B). The elevation of GSK3β in the muscle of HSA LR mice was detected in adult (6-month-old) (Figure 2, A and B) and in young (1-month-old) mice (Figure 2C). In skeletal muscle of young HSA LR mice, GSK3β migrated as a double band that may represent differentially phosphorylated GSK3β (Figure 2C). Since many functions of GSK3α and -β are similar, we analyzed the levels of GSK3ε in skeletal muscle of these mice and found no significant changes for this enzyme (Figure 2A). Analysis of CUGBP1 levels showed that, similar to that in DM1 muscle, CUGBP1 levels were increased in skeletal muscle of HSA LR mice (Figure 2D, E). Taken together, these data show that the mutant CUG repeats increase levels of GSK3β and reduce cyclin D3.

Misregulation of GSK3β in DM1 myogenesis. Our previous studies with the DM1 human muscle cell model showed that cyclin D3 is increased in normal myogenesis at the stage of formation of multinucleated myotubes (21). We have also shown that cyclin D3 activates phosphorylation of CUGBP1 at S302 and that the amounts of active CUGBP1-eIF2 complexes are increased during normal myotube differentiation (21). In contrast to normal myogenesis, DM1 myoblasts have reduced levels of cytoplasmic cyclin D3 (21). Since GSK3β is elevated in DM1 myofibers and because GSK3β regulates cyclin D3, we thought that GSK3β might be misregulated in DM1 myogenesis. To examine this suggestion, we have compared the levels of total GSK3β in the course of normal and DM1 myogeneses using human muscle cell models. As seen in Figure 3, A, C, and D, protein levels of GSK3β are reduced during normal differentiation in both cytoplasm and nuclei of myotubes. However, in cytoplasm of DM1 myoblasts, the expression of GSK3β is increased compared with that in normal myogenesis (Figure 3, A–C). In normal nuclei, GSK3β levels are reduced during differentiation; however, in DM1 nuclei the levels of GSK3β remain high in myoblasts and in myotubes (Figure 3, A, B, and D). In agreement with data showing that active GSK3β reduces cyclin D3, the cells with increased levels of GSK3β had reduced levels of cyclin D3 (Figure 3, A, B, E, and F). These data show that DM1 muscle cell precursors have lost proper regulation of nucleus-cytoplasm distribution of GSK3β during myogenesis and that this aberrant expression of GSK3β is involved in a failure of DM1 myoblasts to increase cyclin D3.

**Figure 2**
The expansion of CUG repeats increases GSK3β protein in skeletal muscle from HSA LR mice. (A) Levels of total GSK3α, GSK3β, cyclin D3, and actin were determined by Western blot analyses of total protein extracts from skeletal muscle (soleus) of HSA LR mice (at 6 months of age) and extracts from matching WT mice. CRM, cross-reactive material. (B) Ratios of signals of GSK3β and cyclin D3, as presented in A, to actin. The standard deviations for 3 experiments are shown. (C) GSK3β is increased in skeletal muscle of 1-month-old HSA LR mice. Protein extracts from skeletal muscle (gastroc) of 1-month-old WT and HSA LR mice were analyzed by Western blot with antibodies to total GSK3β and re-probed with antibodies to actin. Shown are ratios of GSK3β signals, as presented in C, to actin. The standard deviations shown are based on 3 repeats. (D) CUGBP1 is increased in muscle of HSA LR mice. Protein extracts from skeletal muscle (soleus) of age-matched HSA LR and WT mice were analyzed by Western blot assay. The membrane was re-probed with antibodies to actin and stained with Coomassie blue to verify protein loading and integrity. (E) Ratios of CUGBP1 signals presented in D (as an average of 4 mutant and 2 WT mice) to actin signals. The standard deviations are shown for 3 experiments.
mutant CUG repeats (CUG914) (22, 38). In this DM1 cell model, the transcription of the mutant CUG repeats by Northern blot and FISH assays at 7 hours after Dox addition (refs. 22, 38, and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI64081DS1). It has been previously shown that the mutant CUG repeats are almost undetectable at 3 and 6 hours after Dox addition, because only a small percentage of cells (2%–4%) showed CUG repeat aggregates at these time points (38). However, at 7 hours after Dox addition, the mutant CUG repeats are detectable by FISH hybridization (Supplemental Figure 1 and ref. 38). Immunoanalysis of GSK3β in this cell model showed that the mutant CUG repeats increase total levels of GSK3β at 7 hours after Dox addition (Figure 5, A and B). GSK3β levels continued to increase at 17 hours after Dox addition and then were reduced at 24 and 48 hours. We examined whether GSK3β is elevated during the period 1–7 hours after Dox addition and detected the increase of GSK3β as early as 2 hours after Dox addition (Figure 5, C and D). These results show that elevation of GSK3β occurs at an early stage of the expression of CUG repeats before the buildup of CUG aggregates. The kinetics of GSK3β elevation mimicked early accumulation and partial degradation of the mutant CUG repeats (Figure 5A, Supplementary Figure 1, and refs. 22, 38). Examination of the levels of the p-Y216 form of GSK3β after induction of transcription of the mutant CUG repeats showed that the increase in the levels of p-Y216–GSK3β mimicked the kinetics of the elevation of total GSK3β (Figure 5A). Thus, the mutant CUG repeats increase GSK3β stability through the increase in autophosphorylation of GSK3β. In agreement with the elevation of GSK3β, cyclin D3 levels were reduced after CUG914 transcription was initiated. The reduction in cyclin D3 at 7 hours after Dox addition was significantly weaker (1.2-fold) than the increase in GSK3β (5-fold), suggesting that the reduction in cyclin D3 follows the increase of GSK3β. In agreement with this suggestion, an 8-fold increase in GSK3β at 17 hours after Dox addition led to a 2.5-fold reduction in cyclin D3 (Figure 5, A and B).

To examine whether inhibition of GSK3β could correct cyclin D3 levels, monoclonal CHO cells expressing the mutant CUG repeats after Dox addition were treated with lithium, a known inhibitor of GSK3 (35). This treatment partially corrected the total levels of GSK3β (Figure 5, E and F). Partial reduction of
GSTβ protein levels suggests that the inhibition of autophosphorylation of GSTβ might reduce stability of GSTβ and thus normalize its levels. To test this possibility, we measured the levels of p-Y216–GSTβ in CUG-expressing cells treated with lithium and found that the activated p-Y216 form of GSTβ was not detectable in the treated cells (Figure 5E). Thus, inhibition of GSTβ activity by lithium inhibits the autophosphorylation of GSTβ at Y216 and reduces stability of GSTβ. Normalization of GSTβ levels and activity in the CUG-expressing cells treated with lithium corrected levels of cyclin D3 with respect to controls (Figure 5, F and G).

The expanded CUG repeats cause skeletal muscle weakness in the HSALR mouse model. The above results suggest that the mutant CUG repeats misregulate the GSTβ/cyclin D3 pathway. As previously shown, the reduction in cyclin D3 leads to a reduction in CUGBP1 phosphorylation at S302, and this event inhibits translation in DM1 muscle cells (21, 22). Such inhibition of CUGBP1 could reduce translation of some mRNAs essential for muscle function, leading to the skeletal muscle wasting and weakness. In agreement with this prediction, our recent analysis of muscle structure and function in a mouse model with disrupted CUGBP1 and in mice in which S302 is replaced with alanine shows that myogenesis and muscle regeneration in response to the accumulation of CUG repeats initiate muscle damage. This idea is consistent with a significant increase in nuclei located beneath the basal lamina surrounding myofibers in the 1-month-old HSALR mice (Figure 6D), which likely represent satellite cells activated in response to myofiber damage caused by CUG expansions. The number of these nuclei increased by 44% in the TA of 1-month-old HSALR mice and by 31% in gastroc relative to that in the 1-month-old WT mice. In contrast, the number of nuclei beneath the basal lamina compared with that in WT mice of the same age (Figure 6A). It is important to note that HSALR mice show variability in muscle weakness even within the same line. We found that in the line 20LRb, the majority of HSALR mice developed muscle weakness, whereas approximately 20% of mice showed the same grip strength as age- and sex-matched WT mice. Such variability of phenotype in the mouse model of DM1 correlates with a strong variability of DM1 severity, ranging from asymptomatic to lethal (1). The phenotype variability in DM1 is associated with meiotic instability of CUG repeat expansions, with an increase in the length of CUG repeats from generation to generation (1). DM1 is also characterized by somatic instability of CUG repeat expansions, with changes in the length of CUG repeats in different tissues of patients with DM1 and with an increase in the length of CUG repeats with age (1). Recent studies predict that the length of CUG expansions might change in patients with DM1 at different rates (39). Thus, it is predicted that the length of CUG repeats might increase in HSALR mice in the succeeding generations due to meiotic instability. It is also possible that other factors (genetic modifiers) might have different effect on the instability of the mutant CUG repeats in different littermates.
was reduced by 13% in TA of the 6-month-old HSA<sup>LR</sup> mice and by 32% in gastroc (P < 0.05) (Figure 6D).

The alterations in the number of nuclei beneath the basal lamina prompted us to compare the number of activated satellite cells in young and adult HSA<sup>LR</sup> muscle to that in age- and sex-matched WT mice. We examined the paired box protein Pax-7 as a marker of newly activated satellite cells. To compare the number of activated proliferating cells, we extracted myogenic cells from the whole gastroc of matching mice, plated them on cell culture slides, and subjected them to immunofluorescence analysis with antibodies to Pax-7. We found that the number of activated Pax-7–positive cells extracted from gastroc of young (2 months old) HSA<sup>LR</sup> mice was 2.3-fold greater than that in gastroc of the matching WT mice (P < 0.0004) (Figure 6E and Supplemental Figure 2). In contrast to young mice, the number of newly activated proliferating satellite cells was around 1.7 times lower in gastroc of 7-month-old HSA<sup>LR</sup> mice than in matching WT mice (P < 0.036) (Figure 6E and Supplemental Figure 3).

We compared Pax-7 expression in muscle extracts from young (1-month-old) and adult (6-month-old) WT and HSA<sup>LR</sup> mice. As shown in Figure 6F, the levels of Pax-7 were increased in young HSA<sup>LR</sup> muscle, but they were reduced in the muscle from adult HSA<sup>LR</sup> mice. These results suggest that the activation of satellite cells in the 6-month-old HSA<sup>LR</sup> mice is probably diminished, leading to reduced fiber regeneration, a reduction in total fiber number, and skeletal muscle weakness. This is in contrast to the young HSA<sup>LR</sup> mice, in which Pax-7 was elevated and muscle weakness was not detected.

One of the outcomes of CUG toxicity in muscle is the accumulation of internal myonuclei. H&E staining showed small numbers of internal nuclei in 1-month-old muscle of HSA<sup>LR</sup> mice: an average of 1.4 nuclei per field in TA and 0.8 nuclei per field in gastroc (Figure 6G). However, at 6 months of age, there was a significant increase in internal nuclei in muscle of HSA<sup>LR</sup> mice: 6.6 nuclei per field in TA (P < 0.05) and 11 nuclei per field in gastroc (P < 0.005). Taken together, these results show that adult HSA<sup>LR</sup> mice develop muscle weakness accompanied by a reduction in internal myonuclei, suggesting repetitive cycles of degeneration and regeneration, and a reduction in myofiber number.

Treatments of HSA<sup>LR</sup> mice with lithium and TDZD-8 correct the GSK3β/cyclin D3 pathway in skeletal muscle. To examine whether the inhibition of GSK3β could correct cyclin D3, we treated HSA<sup>LR</sup> mice with lithium and found that levels of GSK3β were reduced in skeletal muscle (gastroc) of HSA<sup>LR</sup> mice after treatment (Figure 7A). The levels of cyclin D3 were also normalized. Since lithium also acts as a Mg<sup>2+</sup> competitive inhibitor and may have other targets, we used an additional inhibitor of GSK3β, 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8). Similar to the treatment with lithium, the treatment of HSA<sup>LR</sup> mice with TDZD-8...
normalized the levels of GSK3β in HSA LR muscle and corrected the expression of cyclin D3 (Figure 7B).

Correction of the GSK3β/cyclin D3 pathway in muscles of HSA LR mice treated with lithium also restored CUGBP1 translational function. CUGBP1 forms a complex with inactive p-S51–eIF2 in skeletal muscle of HSA LR mice (Figure 7C). It has been previously shown that this complex is a repressor of translation (22).

After the animals were exposed to a lithium-containing diet, the amounts of complexes containing CUGBP1 and inactive p-S51–eIF2 were significantly reduced. This result shows that the improvement of GSK3β/cyclin D3 in skeletal muscle of HSA LR mice reversed inhibition of CUGBP1 translational activity caused by the mutant CUG repeats.

Lithium and TDZD-8 treatments improve skeletal muscle strength and reduce myotonia in skeletal muscle of HSA LR mice. To examine whether the correction of the GSK3β/cyclin D3/CUGBP1 pathway improved muscle strength, we measured grip strength before and after the treatment of HSA LR mice. We found that a...
2-week lithium-containing diet improved skeletal muscle strength in HSA\textsuperscript{Li} mice from 73% to 93% (\(P < 0.0001\)) of the WT mouse strength measurements (Figure 8A). Lithium improved muscle strength in HSA\textsuperscript{Li} mice of both 3 and 6 months of age.

We next examined whether lithium improves muscle histopathology in HSA\textsuperscript{Li} mice. H&E staining showed that lithium treatment reduced the number of internal nuclei in TA of treated HSA\textsuperscript{Li} mice from 7.6 nuclei to 4.4 nuclei per field (\(P < 0.01172\)) (Figure 8, B and C). However, in gastroc, the number of internal nuclei was almost unchanged (data not shown).

Since accumulation of CUG repeats in HSA\textsuperscript{Li} mice also causes myotonia, we examined whether lithium treatment has any effect on myotonia. Like that of muscle weakness, the severity of myotonia is variable in the 20LRb line of HSA\textsuperscript{Li} mice. We found that lithium treatment reduced myotonia in the treated animals (Table 1). A positive effect of lithium on myotonia was observed in HSA\textsuperscript{Li} mice of different ages, including 3-month- and 6-month-old mice. Switching to the regular diet after 2 weeks of lithium-containing chow reversed muscle weakness in HSA\textsuperscript{Li} mice; however, the beneficial effect of lithium on myotonia remained during 4 weeks after lithium withdrawal (data not shown). This finding suggests that even small treatment terms of lithium are beneficial for reduction of myotonia in HSA\textsuperscript{Li} mice.

To confirm that the beneficial effect of lithium on muscle pathology in HSA\textsuperscript{Li} mice is mediated by inhibition of GSK3\(\beta\), we examined the effect of TDZD-8 on the muscle strength of HSA\textsuperscript{Li} mice. We found that the treatment of HSA\textsuperscript{Li} mice with TDZD-8 improved the grip strength by 20.1% (\(P < 0.009880\)) (Figure 9A).

Improvement of muscle strength in HSA\textsuperscript{Li} mice treated with lithium and TDZD-8 suggests that the correction of GSK3\(\beta\) might have a positive effect on myofiber regeneration. Since levels of the marker of satellite cells Pax-7 are reduced in adult HSA\textsuperscript{Li} mice (Figure 6F), we examined whether TDZD-8 treatment improves Pax-7 expression in HSA\textsuperscript{Li} muscle. Western blot analysis showed that TDZD-8 normalized the levels of Pax-7 in gastroc from HSA\textsuperscript{Li} mice (Figure 9B).

Correction of Pax-7 levels in the HSA\textsuperscript{Li} mice treated with TDZD-8 suggested that activation of satellite cells in the treated mice might be improved. We compared the number of activated Pax-7 satel-

![Figure 7](https://example.com/figure7.png)

**Figure 7**

Inhibition of GSK3\(\beta\) corrects levels of cyclin D3 and translational activity of CUGBP1 in skeletal muscle of HSA\textsuperscript{Li} mice. Western blot analysis of GSK3\(\beta\) and cyclin D3 in skeletal muscle (gastroc) of 6-month-old WT and HSA\textsuperscript{Li} mice before and after treatment with lithium (A) and TDZD-8 (B). \(\beta\)-Actin shows protein loading. (C) Lithium reduces amounts of the translation repressor complexes CUGBP1–p-S51–eIF2\(\alpha\) in skeletal muscle of HSA\textsuperscript{Li} mice. Top panel: CUGBP1 was precipitated with anti-CUGBP1 from skeletal muscle of 6-month-old WT and HSA\textsuperscript{Li} mice, treated and untreated with lithium, and the IPs were probed with antibodies to p-S51–eIF2\(\alpha\). Heavy chain IgGs signals are also shown. Bottom panels (input): Western blotting with antibodies to the total eIF2\(\alpha\). The membrane was reprobed with \(\beta\)-actin.

![Figure 8](https://example.com/figure8.png)

**Figure 8**

Lithium reduces skeletal muscle weakness in HSA\textsuperscript{Li} mice. (A) Improvement of grip strength in the HSA\textsuperscript{Li} mice treated with lithium. Grip strength in 3-month-old HSA\textsuperscript{Li} mice before and after treatment with lithium is shown. **\(P < 0.0001\)** (treated HSA\textsuperscript{Li} mice vs. untreated HSA\textsuperscript{Li} mice). SEM is shown. (B) H&E staining. Representative images of transverse cross-sections stained with H&E from TA of 6-month-old WT and HSA\textsuperscript{Li} mice before and after treatment with lithium. Arrowheads indicate internal nuclei in TA of HSA\textsuperscript{Li} mice before treatment with lithium. Scale bars: 75 \(\mu\)m. (C) Lithium reduces the number of internal nuclei in TA muscle from HSA\textsuperscript{Li} mice. The y axis shows the number of internal nuclei, determined by H&E staining, based on analysis of 200–300 fibers in a maximal region of the transverse sections of TA of 6-month-old HSA\textsuperscript{Li} mice before and after treatment with lithium. As a normal control, internal nuclei were counted in 200–300 fibers in matching muscle of 6-month-old WT mice. *\(P < 0.01172\)** (treated HSA\textsuperscript{Li} mice vs. untreated HSA\textsuperscript{Li} mice). SEM is shown.
Table 1  
Lithium reduces myotonia in 3-month-old HSA<sup>LR</sup> mice

<table>
<thead>
<tr>
<th>Mouse no.</th>
<th>Myotonia before Li+</th>
<th>Myotonia after Li+</th>
<th>Change in myotonia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+++ (5)</td>
<td>+ (5)</td>
<td>↓</td>
</tr>
<tr>
<td>2</td>
<td>+++ (5)</td>
<td>+ (3) and ++ (2)</td>
<td>↓</td>
</tr>
<tr>
<td>3</td>
<td>+++ (5)</td>
<td>+ (3) and ++ (2)</td>
<td>↓</td>
</tr>
<tr>
<td>4</td>
<td>++ (2) and +++ (3)</td>
<td>+ (3) and ++ (2)</td>
<td>↓</td>
</tr>
<tr>
<td>5</td>
<td>+++ (5)</td>
<td>+ (1) and 0 (4)</td>
<td>↓</td>
</tr>
<tr>
<td>6</td>
<td>+++ (5)</td>
<td>0 (1) and + (4)</td>
<td>↓</td>
</tr>
</tbody>
</table>

Myotonic runs were measured 5 times in each HSA<sup>LR</sup> mouse, and the severity of myotonia is expressed from + to ++++, where + corresponds to weak myotonia and +++ corresponds to severe myotonia. The values in parentheses show the number of measurements of myotonia with the same severity.

Figure 9  
TDZD-8 treatment improves skeletal muscle strength in HSA<sup>LR</sup> mice.  
(A) Improvement of grip strength in HSA<sup>LR</sup> mice treated with TDZD-8. Grip strength in the 3-month-old HSA<sup>LR</sup> mice before and after treatment with TDZD-8. SEM is shown. **P < 0.009880, ***P < 0.000006.  
(B) Treatment of HSA<sup>LR</sup> mice with TDZD-8 normalizes levels of Pax-7. Western blot analysis of protein extracts from matching muscles (gastroc) from 3-month-old WT mice, untreated HSA<sup>LR</sup> mice, and HSA<sup>LR</sup> mice treated with TDZD-8. The y-axis shows total number of activated Pax-7-positive cells isolated from gastroc of 3-month-old WT and HSA<sup>LR</sup> mice, untreated (*P < 0.010838, untreated HSA<sup>LR</sup> mice vs. matching WT mice) and treated with TDZD-8 (*P < 0.02952, treated HSA<sup>LR</sup> mice vs. untreated HSA<sup>LR</sup> mice) (see Methods).
show that muscle in the 1-month-old HSA LR mice was characterized by an increased number of nuclei located beneath the basal lamina and by an increased number of small-size myofibers (Figure 6). In agreement with this, the levels of marker of satellite cells Pax-7 were significantly increased in skeletal muscle of 1-month-old HSA LR mice. The number of newly activated proliferating satellite cells also increased in young HSA LR mice. These data suggest that in young HSA LR mice, muscle is actively regenerating due to activation and proliferation of satellite cells. As a result, muscle regeneration prevents the development of muscle weakness. However, muscles in adult (6-month-old) HSA LR mice had a reduced number of nuclei located beneath the basal lamina, reduced numbers of Pax-7–positive satellite cells, and reduced numbers of myofibers. These data suggest that muscle in adult HSA LR mice cannot efficiently regenerate and, as a result, myofibers are degenerating.

Since lithium has other targets in addition to GSK3β, we used a highly selective inhibitor of GSK3β, TDZD-8. Similar to lithium, TDZD-8 reduced muscle weakness in HSA LR mice. This improvement in muscle strength was accompanied by a correction of basal lamina and by an increased number of small-size myofibers. These data suggest that inhibition of GSK3β in HSA LR mice at a young age, when they show an insignificant reduction in grip strength, delays development of muscle weakness at 3 months of age. Use of inducible mouse models with temporary expression of CUG repeats at a young age, with simultaneous treatment with lithium or other potent inhibitors of GSK3β such as TDZD-8, would be a good model for determining the best timing of treatment of these mice.

In conclusion, this study shows that the mutant CUG repeats elevate active GSK3β in DM1 muscle and that inhibition of GSK3β with lithium or TDZD-8 improves muscle strength and reduces myotonia in the DM1 mouse model. Results in this study and the data described in the literature suggest that CUG repeats cause the disease through several pathways: (a) elevation of active CUGBP1 due to an increase of its stability, causing mis-regulation of translation, splicing, and stability of mRNAs controlled by CUGBP1; (b) reduction of MBNL1 due to sequestration by foci, causing reduction of splicing of MBNL1-regulated mRNAs; and (c) mis-regulation of signaling in DM1 cells, in particular GSK3β signaling, which leads to elevation of the inactive form of CUGBP1 in DM1 muscle. The positive effect of lithium and TDZD-8 on skeletal muscle function in HSA LR mice suggests that lithium or other GSK3β antagonists that correct the GSK3β/cyclin D3/CUGBP1 pathway might be candidates for DM1 therapy.

### Methods

**Chemicals.** TDZD-8 was obtained from Sigma-Aldrich. TDZD-8 was dissolved in DMSO at 10 mg/ml and kept at −80°C until use.

**Muscle biopsy samples.** Muscle biopsy samples from biceps brachii of 8 patients with DM1 of both sexes, aged 42–56 years, were used. Four control samples from patients with normal muscle histology were derived from 2 males and 2 females of 46, 52, 53, and 54 years of age. Muscle biopsies were kept frozen at −80°C until use.

**Animals.** Homozygous HSA LR mice were a gift from Charles A. Thornton of the University of Rochester Medical Center, Rochester, New York, USA. Skeletal muscle histology of several muscle groups of HSA LR mice of different age (1, 3, 6, and 9 months) and age- and sex-matched WT mice was examined using H&E staining. To quantify the number of fibers, the myofibers (200–300 fibers) in the maximal cross-sectional area were counted using MetaMorph (Molecular Devices) software. Nuclei were counted in 200–300 fibers of each muscle group in maximal cross-sectional areas in the sex- and age-matched mouse cohorts using MetaMorph software, and the results were averaged. The average area of myofibers was calculated using MetaMorph software based on the analysis of 200–300 fibers in maximal cross-sectional areas of each muscle group. The grip strength in HSA LR and matching WT mice was examined using a grip strength meter (Columbus Instruments). Measurement of grip strength in mice of different ages was performed in comparable environments. Prior the grip strength test, mice were acclimated by grasping the wire from the grip strength meter several times. During this test, the mouse was allowed to establish a firm grip on the wire, and then the mouse was slowly pulled away until the grip was released. The tension of the wire was recorded, and the average grip strength was determined based on 5 grasps for each mouse. The measurements were repeated 5 times with 5 grasps each time, and average grip strength was calculated. In the experiments with lithium

#### Table 2

<table>
<thead>
<tr>
<th>Mouse no.</th>
<th>Myotonia before TDZD-8</th>
<th>Myotonia after TDZD-8</th>
<th>Change in myotonia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+ (3)</td>
<td>+ (3) and ++ (2)</td>
<td>↓</td>
</tr>
<tr>
<td>2</td>
<td>+ (3)</td>
<td>+ (4) and ++ (1)</td>
<td>↓</td>
</tr>
<tr>
<td>3</td>
<td>+ (4) and 0 (1)</td>
<td>++ (5)</td>
<td>↓</td>
</tr>
<tr>
<td>4</td>
<td>+ (3) and +++ (2)</td>
<td>++ (4) and ++ (1)</td>
<td>↓</td>
</tr>
<tr>
<td>5</td>
<td>+ (4) and 0 (1)</td>
<td>(4) and ++ (1)</td>
<td>↓</td>
</tr>
</tbody>
</table>

Myotonic runs were measured 5 times in each HSA LR mouse before and after treatment with TDZD-8, and the severity of myotonia is expressed from + to ++++, where + corresponds to weak myotonia and ++++ corresponds to severe myotonia.
treatment, mice of 3 months of age showing muscle weakness were main-
tained for 2 weeks on the basic 2016 rodent diet supplemented with 0.24%
lithium carbonate (Teklad, Harlan). In the experiments with TDZD-8
treatment, TDZD-8 was administered in 3-month-old HSAΔβ mice via i.p.
injections at a dose of 10 mg/kg for 2 days. Skeletal muscle strength in
HSAΔβ mice was determined before and after treatment with lithium or
TDZD-8. Since TDZD-8 was dissolved in DMSO, matching HSAΔβ mice
were injected with the same amounts of DMSO as control. For biochemi-
cal analyses where indicated, soleus, gastroc, and TA from HSAΔβ mice
were collected, cut in pieces, and subjected to immediate freezing in liquid
nitrogen. As a control, corresponding muscles were collected from the sex-
and age-matched WT mice. Frozen muscle samples were kept at ~80°C
until use. In experiments with myotonia, HSAΔβ mice showing muscle
weakness were treated with lithium or TDZD-8, and myotonia was mea-
ured in the proximal rear leg muscle with monopolar electrodes before
and after treatment in the EMG laboratory (Baylor College of Medicine).
Each measurement was repeated 5 times at the same temperature (32°C),
and myotonic runs were recorded. No sedation was applied. The severity
of myotonia was estimated from + to ++++, with + corresponding to weak
myotonia and +++ corresponding to severe myotonia.

Analysis of activated satellite cells. To isolate activated satellite cells, whole
muscle (gastroc) from sex- and age-matched WT (n = 5), HSAΔβ (n = 4), and
HSAΔβ mice treated with TDZD-8 (n = 2) were minced and digested in a solu-
tion of 0.5% collagenase II (Gibco, Invitrogen) in 1× PBS for 45 minutes
at 37°C with gentle agitation. The digested muscle was filtered through
Netwell inserts with mesh size polyester membrane, and supernatants were
plated in 2-chamber slides in HAM’s F-10 medium, containing 15% FBS, 5%
defined supplemented calf serum, 2 mmol/l glucose, 100 μg/ml penicillin,
 streptomycin, and 0.5 μg/ml human basic fibroblast growth factor. Cells
maintained for 17 hours in 5% CO2 at 37°C were washed in 1× PBS and fixed
in 4% formaldehyde. Satellite cells were identified by immunofluorescence
analysis with polyclonal antibodies to Pax-7 (no. ab34360) from Abcam.
Fixed cells were sequentially incubated in a solution of 1× PBS, containing
normal goat serum (1:25) and 0.5% BSA, then with primary antibodies to
Pax-7 (diluted 1:150 in 1× PBS, supplemented with normal goat serum, 1:200,
and 0.2% BSA), and with secondary goat anti-rabbit antibodies labeled with
FITC (1:200, Santa Cruz Biotechnology Inc.). Slides were mounted in Vecta-
shield medium (Vector Laboratories) containing DAPI. Pax-7+–positive cells
were visualized by fluorescence microscopy as shown in Supplemental Fig-
ures 2 and 3. As a control for the immunofluorescence study, the primary
antibodies were omitted (Supplemental Figure 2A). For comparison of the
number of satellite cells in the matching WT, HSAΔβ, and HSAΔβ mice treat-
ed with TDZD-8, each slide chamber was divided into 40 sections, and all
Pax-7+–positive cells were counted under the same conditions (magnification,
time of exposure, contrast, and brightness) using MetaMorph software.
The average values of 3 experiments were obtained and normalized to the weight
of the whole gastroc in the analyzed mice.

Western blot assay. Normal and DM1 myoblasts were grown as previ-
ously described (15, 21). Myotube differentiation was initiated by the
switching of the growth medium containing FBS to the fusion medi-
unlacking FBS (15, 21). The efficiency of myotube differentiation
was monitored by light microscopy and by Western blot analysis with
antibodies against protein markers of differentiation. Cytoplasmic and
nuclear protein extracts were isolated from cultured cells as described
previously (7, 8). The efficiency of the separation of cytoplasm and
nucleus was examined by Western blot assay with antibodies to Rb
(nuclear protein) and to HSP70 (preferentially cytoplasmic protein).
Frozen human and mouse muscle samples were homogenized in RIPA
buffer containing pepstatin (0.7 μg/ml), leupeptin (0.5 μg/ml), and a
cocktail of inhibitors of phosphatases (Sigma-Aldrich) (1 μg/ml using
an electric homogenizer. Protein extracts were centrifuged at 2.4 g for
10 minutes at 4°C. Supernatants were collected and frozen in portions
at ~80°C. Proteins (50 μg) were separated by SDS–gel electrophoresis,
transferred onto nitrocellulose, and probed with monoclonal antibod-
ies to CUGBP1 (no. sc-56649), cyclin D3 (no. sc-182), total GSK3β (no.
sc-71186), p-S9–GSK3β (no. sc-11757), p-Y216–GSK3β (no. sc-135653),
total GSK3α (no. sc-5264), Pax-7 (no. sc-81975) (all from Santa Cruz
Biotechnology Inc.), p-T283–cyclin D1 (no. ab55322) from Abcam, and
β-actin (no. A5441) from Sigma-Aldrich.

Immunoprecipitation–Western blot assay. Total cyclin D3 was precipitated
with antibodies to cyclin D3 (sc-18, Santa Cruz Biotechnology Inc.) from
protein extracts isolated with RIPA buffer. Since cyclin D3 is reduced in
DM1 muscle, the amount of DM1 muscle tissue used for the IP was
600 mg, whereas the amount of normal muscle tissue used was 100 mg.
The cyclin D3 IPs were divided into two portions. One portion was used for
Western blot analyses with antibodies to p-T283–cyclin D1 and to
whole cyclin D3 IP was examined by Western blot antibodies to Rb. The experiments were repeated 3 times, and
average values were presented.

Stability of GSK3β. Normal and DM1 myoblasts (grown no more than
12 passages) were maintained at 50% density as described previously (15).
CHX (final concentration, 10 mM) was added to the growth medium, and
cytoplasmic and nuclear proteins were collected at 0, 1, 2, and 4 hours after
CHX addition. Proteins were analyzed by Western blot assay with antibod-
ies to total GSK3β and β-actin as control for loading.

Treatment of GSK3β with lithium in Tet-regulated CUG15 CHO monoclonal
cell lines. The generation, conditions of growth, and characterization of the
Tet-on CHO cell line expressing noncoding RNA containing 914 pure
CUG repeats and GFP from the two independent CMV promoters
have been described previously (22, 38). Briefly, the CHO monoclonal
cell line (clone 2) was grown to 70% density in DMEM containing 10%
FCS and antibiotics, Geneticin (250 μg/ml) and Hygromycin B
(400 μg/ml). In parallel plates, LiCl (20 mM) was added. Transcription of
CUG15 was induced by addition of Dox (350 ng/ml); RNA and pro-
tein extracts were collected at different time points after Dox addition
(0, 7, 17, 24, and 48 hours). Expression of CUG15 RNA in this cell cul-
ture model, examined by Northern blot hybridization with 32P-labeled
CAGi probe, was previously reported (22, 38). Expression of GFP after
Dox addition was examined by monitoring of fluorescent signal. Pro-
tein extracts were extracted from nuclei and cytoplasm as described previously
(7, 8) and used for Western blot analysis (50 μg) with antibodies to total
GSK3β, cyclin D3, and β-actin as a control for loading. The accumula-
tion of CUG foci in CHO monoclonal cells expressing CUG15 RNA was
examined by FISH hybridization with CAG15 probe, labeled with Alexa
Fluor 555, as described previously (38).

Statistical analyses and densitometric analysis. Western blot and IP–Western
blot images were quantified by scanning densitometry using 3 measure-
ments. The values were normalized to actin expression levels. Mean val-
ues (based on 3 independent experiments) were presented as fold change
relative to controls. For statistical analysis of the grip strength in mice of
different ages, 2-way ANOVA and 2-tailed Student’s t test were used. Sta-
tistical analysis of total fiber number, myofiber area, and number of inter-
nal and external nuclei in 2 muscle groups of mice of 2 genotypes and of
different ages was performed using 3-way ANOVA and 2-tailed Student’s
t test. For statistical analysis of grip strength in mice treated with lithium
and TDZD-8, 2-tailed Student’s t test was used. A P value less than 0.05 was
considered statistically significant.

Study approval. A protocol dealing with animal use was approved by the
Institutional Animal Care and Use Committee at Baylor College of
Medicine. All animal work was performed in accordance with the NIH
The authors are thankful to rotation student John Leach for the initial experiments with mouse tissue. This work was supported by NIH grants 2R01-AR044387-13, 2R01-AR052791-07, R21-NS078659 (to L.T. Timchenko), 2T32HL007676-21A1 (to K. Jones), GM551888, CA100070, AG039885, AG028865, and CA159942 (to N.A. Timchenko) and a grant from Association Française Contre Les Myopathies (to G. Meola).

Received for publication April 5, 2012, and accepted in revised form September 21, 2012.

Address correspondence to: Lubov Timchenko, Department of Molecular Physiology and Biophysics, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030, USA. Phone: 713.798.3142; E-mail: lubovt@bcm.edu.