GSK3β mediates muscle pathology in myotonic dystrophy

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Myotonic dystrophy type 1 (DM1) is a complex neuromuscular disease characterized by skeletal muscle wasting, weakness, and myotonia. DM1 is caused by the expansion 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).

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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).
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 confirm the reduction of cyclin D3 in DM1, we performed immunoblot 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.

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 p-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 shown are based on 3 repeats. (C) GSK3β is increased in skeletal muscle of 1-month-old HSA LR mice. Protein extracts from skeletal muscle (gastrocnemius) 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.

GSK3β is increased in DM1 muscle cell precursors due to protein stabilization. Data in Figure 1, B and C, show that the levels of inactive p-S9–GSK3β were reduced in DM1 muscle biopsies. Since the active form of GSK3β is phosphorylated at Y216 (37), we examined the levels of p-Y216–GSK3β in DM1 muscle samples. As shown in Figure 4A, p-Y216–GSK3β is increased in DM1 muscle biopsies. Consistent with this finding, inactive p-S9–GSK3β was reduced in DM1, whereas total GSK3β was increased.

It has been shown that autophosphorylation of GSK3β at Y216 increases stability of GSK3β (37). To determine whether the elevation of GSK3β in DM1 occurs due to increased stability of GSK3β, we treated normal and DM1 myoblasts with cycloheximide (CHX) to block new protein synthesis and measured levels of total GSK3β at different time points after CHX addition. We found that the half-life of GSK3β was very short in normal myoblasts. The levels of GSK3β were sharply reduced in 1 hour after CHX addition and remained at low levels during the whole course of the treatment with CHX (Figure 4, B and C). However, in DM1 myoblasts, levels of GSK3β were essentially unchanged 1 hour after treatment with CHX (Figure 4, B and C). Although GSK3β levels were reduced after 2 and 4 hours of CHX addition to DM1 myoblasts, levels remained higher than those in normal myoblasts. Thus, stability of GSK3β protein is increased in DM1.

To determine whether the inhibition of the CUG-elevated GSK3β normalizes cyclin D3 levels, we utilized recently generated monoclonal CHO cell lines with Tet-regulated transcription of the
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 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
GSK3β protein levels suggests that the inhibition of autophosphorylation of GSK3β might reduce stability of GSK3β and thus normalize its levels. To test this possibility, we measured the levels of p-Y216–GSK3β in CUG-expressing cells treated with lithium and found that the activated p-Y216 form of GSK3β was not detectable in the treated cells (Figure 5E). Thus, inhibition of GSK3β activity by lithium inhibits the autophosphorylation of GSK3β at Y216 and reduces stability of GSK3β. Normalization of GSK3β levels and activity in the CUG-expressing cells treated with lithium corrected levels of cyclin D3 with respect to controls (Figure 5, E and F).

The expanded CUG repeats cause skeletal muscle weakness in the HSA12 mouse model. The above results suggest that the mutant CUG repeats misregulate the GSK3β/cyclin D3 pathway. As previously shown, the reduction in cyclin D3 leads to a reduction in CUGBP1 phosphorylation at S302, and this event inhibits CUGBP1 translational activity 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 strength are severely affected in these mice (C. Wei and L. Timchenko, unpublished observations). Since the GSK3β/cyclin D3 pathway is altered in muscle of HSA12 mice, we tested whether HSA12 mice develop muscle weakness. Grip strength has been shown to be a sensitive measure of muscle strength caused by CUG expansions. The number of these nuclei increased by 44% in the TA of 1-month-old HSA12 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 HSA12 mice show variability in muscle weakness even within the same line. We found that in the line 20L/Rb, the majority of HSA12 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 CTG repeat expansions, with an increase in length of CTG repeats from generation to generation (1). DM1 is also characterized by somatic instability of CTG repeat expansions, with changes in length of CTG repeats in different tissues of patients with DM1 and with an increase in length of CTG repeats with age (1). Recent studies predict that the length of CTG expansions might change in patients with DM1 at different rates (39). Thus, it is predicted that the length of CTG repeats might increase in HSA12 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 littersmates.

We next asked whether the reduction in grip strength in HSA12 mice was due to a myofiber loss. Total myofibers were counted on maximal transverse sections of gastrocnemius (gastroc) and tibialis anterior (TA) muscles from 6-month-old HSA12 and WT mice. We found that the number of myofibers was reduced by 27% in TA (P < 0.05) and by 44% in gastroc (P < 0.0005) in the 6-month-old HSA12 mice (Figure 6B). The total number of myofibers was also reduced in other muscle groups in HSA12 mice of this age (data not shown). The reduction in myofiber number in skeletal muscle of the 6-month-old HSA12 mice was accompanied by an increase in the average myofiber area of approximately 27% in TA (P < 0.0005) and by 46% in gastroc (P < 0.0005) relative to WT mice of the same age (Figure 6C), probably due to compensatory response to the loss of myofiber number.

In contrast to the 6-month-old HSA12 mice, the total number of myofibers in the 1-month-old HSA12 mice increased by 32% in gastroc and by 28% in TA (Figure 6B). However, myofibers in the 1-month-old HSA12 mice were smaller in size relative to those in the 1-month-old WT mice (Figure 6C). The average myofiber area was reduced in TA of 1-month-old HSA12 mice by 13% and in gastroc by 20% in comparison to age-matched WT controls (Figure 6C). We suggest that the increase in myofiber number in 1-month-old HSA12 mice is mediated by the activation of muscle regeneration in response to the accumulation of CUG repeats that 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 HSA12 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 HSA12 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

Figure 4
GSK3β is increased in DM1 due to stabilization of the protein. (A) p-Y216–GSK3β is increased in DM1 muscle biopsies. Western blot analysis of total protein extracts from 2 normal and 2 DM1 patients with antibodies to active p-Y216–GSK3β, inactive p-S9–GSK3β, total GSK3β, and actin is shown. Antibodies to p-Y216–GSK3β cross-react with other proteins (CRM). (B) GSK3β stability is increased in DM1 muscle cell precursors. Protein synthesis was inhibited by CHX; and GSK3β levels were measured by Western blot analysis in normal and in DM1 myoblasts at different time points (1, 2, and 4 hours) after CHX addition. β-Actin shows the loading of proteins. (C) Quantification of GSK3β stability in normal and DM1 myoblasts. The y axis shows GSK3β signals (as ratios to β-actin) in cells treated with CHX as percentages of GSK3β signal in the untreated cells. The signal of GSK3β in the untreated cells was counted as 100%. The x axis shows the time of treatment with CHX (in hours). The standard deviations represent values of 3 experiments.
was reduced by 13% in TA of the 6-month-old HSA\textsuperscript{LR} 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\textsuperscript{LR} 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 satellite 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\textsuperscript{LR} 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\textsuperscript{LR} mice than in matching WT mice (P < 0.005) (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\textsuperscript{LR} mice. As shown in Figure 6F, the levels of Pax-7 were increased in young HSA\textsuperscript{LR} muscle, but they were reduced in the muscle from adult HSA\textsuperscript{LR} mice. These results suggest that the activation of satellite cells in the 6-month-old HSA\textsuperscript{LR} 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\textsuperscript{LR} 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\textsuperscript{LR} 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\textsuperscript{LR} 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\textsuperscript{LR} mice develop muscle weakness accompanied by a reduction in activated satellite cells, an increase in internal nuclei, suggesting repetitive cycles of degeneration and regeneration, and a reduction in myofiber number.

Treatments of HSA\textsuperscript{LR} 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\textsuperscript{LR} mice with lithium and found that levels of GSK3β were reduced in skeletal muscle (gastroc) of HSA\textsuperscript{LR} mice after treatment (Figure 7A). The levels of cyclin D3 were also normalized. Since lithium also acts as a Mg\textsuperscript{2+} 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\textsuperscript{LR} 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 significant increase in grip strength was observed in the treated groups compared to the control groups (Figure 6A). In addition, we examined the number of myofibers, myofiber area, and the number of activated Pax-7–positive cells in the skeletal muscles of HSA LR mice treated with lithium and TDZD-8 (Figures 6B, 6C, and 6D). We observed a significant increase in the total number of myofibers and a decrease in myofiber area in the treated groups compared to the control groups. The number of activated Pax-7–positive cells was also increased in the treated groups (Figure 6E).

In summary, the correction of the GSK3β/cyclin D3/CUGBP1 pathway by lithium and TDZD-8 treatments led to improvements in muscle strength, increased myofiber number, reduced myofiber area, and increased the number of activated Pax-7–positive cells in skeletal muscle of HSA LR mice.
2-week lithium-containing diet improved skeletal muscle strength in HSA\textsuperscript{LR} mice from 73% to 93% (P < 0.0001) of the WT mouse strength measurements (Figure 8A). Lithium improved muscle strength in HSA\textsuperscript{LR} mice of both 3 and 6 months of age.

We next examined whether lithium improves muscle histopathology in HSA\textsuperscript{LR} mice. H&E staining showed that lithium treatment reduced the number of internal nuclei in TA of treated HSA\textsuperscript{LR} mice from 7.6 nuclei to 4.4 nuclei per field (P < 0.0001) (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{LR} 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{LR} 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{LR} 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{LR} mice; however, the beneficial effect of the 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{LR} mice.

To confirm that the beneficial effect of lithium on muscle pathology in HSA\textsuperscript{LR} mice is mediated by inhibition of GSK3\textbeta, we examined the effect of TDZD-8 on the muscle strength of HSA\textsuperscript{LR} mice. We found that the treatment of HSA\textsuperscript{LR} mice with TDZD-8 improved the grip strength by 20.1% (P < 0.0009880) (Figure 9A).

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

Correction of Pax-7 levels in the HSA\textsuperscript{LR} 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
Inhibition of GSK3\textbeta corrects levels of cyclin D3 and translational activity of CUGBP1 in skeletal muscle of HSA\textsuperscript{LR} mice. Western blot analysis of GSK3\textbeta and cyclin D3 in skeletal muscle (gastroc) of 6-month-old WT and HSA\textsuperscript{LR} mice before and after treatment with lithium (A) and TDZD-8 (B). β-Actin shows protein loading. (C) Lithium reduces amounts of the translation repressor complexes CUGBP1–p-S51–eIF2α in skeletal muscle of HSA\textsuperscript{LR} mice. Top panel: CUGBP1 was precipitated with anti-CUGBP1 from skeletal muscle of 6-month-old WT and HSA\textsuperscript{LR} mice, treated and untreated with lithium, and the IPs were probed with antibodies to p-S51–eIF2α. Heavy chain IgGs signals are also shown. Bottom panels (input): Western blotting with antibodies to the total eIF2α. The membrane was reprobed with β-actin.

Figure 8
Lithium reduces skeletal muscle weakness in HSA\textsuperscript{LR} mice. (A) Improvement of grip strength in the HSA\textsuperscript{LR} mice treated with lithium. Grip strength in 3-month-old HSA\textsuperscript{LR} mice before and after treatment with lithium is shown. ***\textit{P} < 0.0001 (treated HSA\textsuperscript{LR} mice vs. untreated HSA\textsuperscript{LR} 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{LR} mice before and after treatment with lithium. Arrowheads indicate internal nuclei in TA of HSA\textsuperscript{LR} mice before treatment with lithium. Scale bars: 75 μm. (C) Lithium reduces the number of internal nuclei in TA muscle from HSA\textsuperscript{LR} 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{LR} 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. *\textit{P} < 0.01172 (treated HSA\textsuperscript{LR} mice vs. untreated HSA\textsuperscript{LR} mice). SEM is shown.


Table 1

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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.

Discussion

Despite numerous investigations into DM1 pathology, the detailed mechanism underlying this multisystem disease is not well understood, and therapeutic approaches have remained underdeveloped. In this study, we found that the active GSK3<sub>β</sub> is elevated in skeletal muscle biopsy samples from DM1 patients (Figure 1). The increase in GSK3<sub>β</sub> in DM1 occurs due to accumulation of mutant CUG repeats. This conclusion was made based on studies of two DM1 models: HSA<sup>LR</sup> mice, expressing CUG repeats in the 3' UTR of skeletal muscle actin; and CHO double-stable clones expressing Tet-regulated pure CUG repeats.

In agreement with our findings, a recent study has shown disruption of AKT/GSK3<sub>β</sub> signaling in neuronal cells in response to the expression of CUG repeats (40).

Our data from the monoclonal CHO cell lines indicate that the elevation of GSK3<sub>β</sub> is an early event caused by the accumulation of small amounts of mutant CUG repeats. The mutant CUG repeats in this cell model are detectable by FISH and Northern blot assays mainly at 7 hours after Dox addition (Supplemental Figure 1 and ref. 38). We found that GSK3<sub>β</sub> increased within 2 hours after induction of CUG<sub>914</sub> expression, suggesting that even small amounts of CUG repeats, which are difficult to detect by FISH assay, are sufficient to elevate GSK3<sub>β</sub>. The mechanism of CUG repeat–dependent elevation of active GSK3<sub>β</sub> involves the stabilization of GSK3<sub>β</sub> through an increase in its activity. This conclusion is supported by results indicating that inhibition of GSK3<sub>β</sub> activity by lithium corrected GSK3<sub>β</sub> protein levels (Figure 5E). Since the stabilization of GSK3<sub>β</sub> correlates with an increase in its autophosphorylation, the most likely mechanism is that CUG repeats first increase activity of GSK3<sub>β</sub>, and this in turn activates GSK3<sub>β</sub> phosphorylation, stabilizing GSK3<sub>β</sub>. How might CUG repeats activate GSK3<sub>β</sub>? Since RNA-binding activity of GSK3<sub>β</sub> has not been reported, it is clear that there is a pathway mediator that is activated by CUG repeats. Protein factors or toxic peptides, synthesized from expanded CUG RNA through an AUG-independent mechanism, might trigger the elevation of GSK3<sub>β</sub> activity (41). These factors involved in activating a CUG-mediated increase in GSK3<sub>β</sub> remain to be identified.

One of the most important results of this study is the identification of GSK3<sub>β</sub> as a crucial signaling molecule associated with the reduction of cyclin D3 and muscle weakness and myotonia in HSA<sup>LR</sup> mice. Whereas initial reports had not described progressive muscle weakness and wasting in these mice, we found reproducible and statistically significant reduction of muscle strength in 3-month- and 6-month-old HSA<sup>LR</sup> mice. Our data suggest that GSK3<sub>β</sub> may be a novel therapeutic target for the treatment of DM1.

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 was performed with antibodies to Pax-7. β-Actin shows protein loading. (C) TDZD-8 treatment increases the number of activated Pax-7–positive cells. The y-axis shows total number of Pax-7–positive cells isolated from gastroc of 4-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 the inactive form of CUGBP1 in DM1 muscle. The positive elevation occurs prior to buildup of CUG foci that sequester MBNL1 (Figure 5, C and D, Supplemental Figure 1, and refs. 22, 38).

One of the important questions to be answered is, What is the most appropriate timing for treatment of DM1 with GSK3β inhibitors? We found that TDZD-8 treatment of young (6 weeks old) HSA LR mice for 1 week improved their grip strength by 10.9% (Supplemental Figure 4). We tested grip strength in the same mice at 3 months of age and found that their grip strength was 9.2% higher than in the age- and sex-matched untreated HSA LR mice. These data suggest that the 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 GSK3 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 the age- and sex-matched WT mice was examined by 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 to 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>
<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>+++ (5)</td>
<td>+ (3) and ++ (2)</td>
<td>↓</td>
</tr>
<tr>
<td>2</td>
<td>+++ (5)</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>++ (5)</td>
<td>++ (3) and +++ (2)</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 HSAiL mice via i.p.
injectons at a dose of 10 mg/kg for 2 days. Skeletal muscle strength in
HSAiL mice was determined before and after treatment with lithium or
TDZD-8. Since TDZD-8 was dissolved in DMSO, matching HSAiL mice
were injected with the same amounts of DMSO as control. For biochemi-
cal analyses where indicated, soleus, gastroc, and TA from HSAiL 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, HSAiL 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 as + 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), HSAiL (n = 4), and
HSAiL 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-glutamine, 100 μg/ml penicil-
lin/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% paraformaldehyde. 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 1:500 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, HSAiL, and HSAiL 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-
um lacking 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 peptatin (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
total cyclin D3. The second portion of the 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 μM) 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 CUG914 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%
Tc-free FBS and antibiotics, Geneticin (250 μg/ml) and Hygromycin B
(400 μg/ml). In parallel plates, LiCl (20 μM) was added. Transcription
of CUG914 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 CUG914 RNA in this cell cul-
ture model, examined by Northern blot hybridization with 32P-labeled
CAGβ probe, was previously reported (22, 38). Expression of GFP after
Dox addition was examined by monitoring of ﬂuorescent signal. Pro-
tein extracts were isolated 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 CUG914 RNA was
examined by FISH hybridization with CAG13 probe, labeled with AlexFluor
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-
dues (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


