

The methyl-CpG-binding protein CIBZ suppresses myogenic differentiation by directly inhibiting *myogenin* expression

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Postnatal growth and regeneration of skeletal muscle are carried out mainly by satellite cells, which, upon stimulation, begin to express myogenin (*Myog*), the critical determinant of myogenic differentiation. DNA methylation status has been associated with the expression of *Myog*, but the causative mechanism remains almost unknown. Here, we report that the level of CIBZ, a methyl-CpG-binding protein, decreases upon myogenic differentiation of satellite-derived C2C12 cells, and during skeletal muscle regeneration in mice. We present data showing that the loss of CIBZ promotes myogenic differentiation, whereas exogenous expression of CIBZ impairs it, in cultured cells. CIBZ binds to a *Myog* promoter-proximal region and inhibits *Myog* transcription in a methylation-dependent manner. These data suggest that the suppression of myogenic differentiation by CIBZ is dependent, at least in part, on the regulation of *Myog*. Our data show that the methylation status of this proximal *Myog* promoter inversely correlates with *Myog* transcription in cells and tissues, and during postnatal growth of skeletal muscle. Notably, induction of *Myog* transcription by CIBZ suppression is independent of the demethylation of CpG sites in the *Myog* promoter. These observations provide the first reported molecular mechanism illustrating how *Myog* transcription is coordinately regulated by a methyl-CpG-binding protein and the methylation status of the proximal *Myog* promoter.

Keywords: CIBZ; MBP; myogenin; muscle differentiation; regeneration

Cell Research (2011) 21:1578-1590. doi:10.1038/cr.2011.90; published online 31 May 2011

Introduction

In rodents, skeletal muscles grow dramatically from birth to adulthood. Adult skeletal muscle is capable of repeated self-repair, throughout life, in response to damage caused by injury or myopathies [1, 2]. Such postnatal development and self-repair of skeletal muscles is mainly attributable to muscle progenitor cells called satellite cells, which are located between the basal lamina and the plasma membrane of muscle fibers [1, 3]. Satellite cells account for about 30% of the muscle nuclei in neonatal mice, but decline to 2-6% in 2-month-old adults [3]. As

adulthood is reached, the residual satellite cells become quiescent, but upon activation they divide to repopulate the satellite cell pool and to generate a large number of daughter myoblasts. These daughter myoblasts subsequently undergo myogenic differentiation, in which they fuse and form multinucleated myotubes [1, 4]. Activation of quiescent satellite cells and subsequent myogenic differentiation are controlled by various transcription factors, chief among which are the myogenic regulatory factors (MRFs) Myf5, MyoD, myogenin (*Myog*) and MRF4 [5]. MyoD and Myf5 are expressed in proliferating and undifferentiated myoblasts, whereas *Myog* is induced during early differentiation. In agreement with these expression patterns, MyoD and Myf5 establish the myogenic lineage, while *Myog* directly controls the differentiation of myoblasts [4, 5]. MRF4, on the other hand, appears to function as a differentiation factor in later fibers. Induction of *Myog* is essential for the differentiation of

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Received 26 July 2010; revised 20 January 2011; accepted 10 February 2011; published online 31 May 2011

myoblasts that contributes to the formation of myotubes and fibers: mice deficient for *Myog* form myoblasts but do not develop mature skeletal muscle [6]. Hence, a deeper understanding of the transcriptional regulation of *Myog* will provide important insights into the molecular mechanism of myogenic differentiation.

Myog transcription is controlled by a 1.5-kb 5'-regulatory region (nucleotides -1 565 to +18), which is sufficient to recapitulate the major features of *Myog* expression during embryonic and fetal development [7]. Cumulative evidence indicates that the promoter region (-184 to +18) is indispensable for *Myog* expression [8, 9]. Transcription of *Myog* is stimulated mainly by MRFs or by members of the myocyte enhancer factor 2 (MEF2) family, through binding to the E-box elements or to the MEF2-binding site of the *Myog* promoter, respectively [5, 10]. By contrast, *Myog* expression is negatively regulated by the inhibitors of DNA-binding (Id) family, which block the stimulatory effect of MRFs by forming inactive heterodimers with them [11]. *Myog* is also negatively regulated by other transcriptional repressors that bind to the E-box elements, such as MyoR [12]. Whereas considerable progress has been made in elucidating how *Myog* is regulated through its E-box elements and MEF2-binding site, much less is known about the patterns of DNA methylation of this muscle-specific gene.

Cytosine-5 DNA methylation in mammals is essential for important functions such as cell differentiation, imprinting and X-inactivation [13]. Treatment of 10T1/2 fibroblasts with the DNA demethylating reagent 5-aza-dC, or expression of antisense *Dnmt1*, results in myotube formation, suggesting that global demethylation plays a role in myogenic differentiation [14, 15]. Similarly, 5-aza-dC treatment induces *Myog* expression in myoblast cells, implying that DNA methylation is also involved in the suppression of *Myog* transcription [16, 17]. Since the 51-kb region between *Myog* and its upstream gene lacks CpG islands, DNA methylation in the vicinity of the promoter is probably responsible for *Myog* silencing. Consistent with this, Lucarelli *et al.* reported that the methylation status of a single CCGG site at 340 bp upstream from the transcriptional start site (TSS) affects *Myog* transcription in mouse tissues and in C2C12, a skeletal muscle satellite-derived myoblast cell line [17]. This *HpaII* recognition site (CCGG), which is in the region proximal to the *Myog* promoter, was identified using the methylation-sensitive endonuclease *HpaII*-PCR assay. However, we currently lack information about which methylated CpG-binding proteins (MBPs) bind to this site, or a detailed CpG methylation profile of the proximal *Myog* promoter around the site.

The C2C12 cell line is a well-established model to

investigate the cellular and molecular mechanisms of muscle differentiation [18]. This *in vitro* system faithfully recapitulates the *in vivo* differentiation program. When cultured in differentiation medium (DM), C2C12 cells undergo terminal myogenic differentiation. We reported previously that a novel MBP member, CIBZ (ZBTB38 in human) [19], represses the Gal4-driven SV40 promoter [20]; it can bind to methylated CpG through its zinc fingers (unpublished data). We found that CIBZ is localized in both the nucleus and the cytoplasm of NIH3T3 cells [20] and C2C12 cells (unpublished data); its level is high in C2C12 cells but decreases upon DM induction [21].

We now show that CIBZ is down-regulated during skeletal muscle regeneration, and that it suppresses C2C12 myoblast differentiation. Our data reveal that CIBZ's role in myogenic differentiation is dependent, at least in part, on the negative regulation of its direct target, *Myog*. CIBZ binds to a 451-bp proximal region of the *Myog* promoter (Myog451). Using a combination of approaches, we show that CIBZ binds directly to and represses the activity of Myog451 in a methylation-dependent manner. We provide evidence that the methylation status of Myog451 correlates with *Myog* transcriptional activation in cells and tissues, and during postnatal growth of skeletal muscle. These findings suggest a viable therapeutic approach for the treatment of skeletal muscle disorders.

Results

CIBZ expression is down-regulated during myoblast differentiation and skeletal muscle regeneration

We sought to determine the role of CIBZ in myogenic differentiation. Expression of Myog and myosin heavy chain (MHC), markers of early and late stages of myogenic differentiation, respectively, was monitored to assess the cells' differentiation state [22]. As seen in Figure 1A, RT-PCR and immunoblot analysis showed that induction of C2C12 cells in DM resulted in early differentiation within 8-24 h, and in late differentiation after 48 h. CIBZ protein declined quickly within 8 h and was barely detectable at 24-72 h during differentiation, whereas *CIBZ* mRNA levels remained relatively stable.

In response to muscle injury in which myofibers are damaged, satellite cells begin to expand and differentiate to replace the lost myofibers. To investigate the expression of CIBZ protein during muscle regeneration in mice, cardiotoxin (CTX) was used. Treatment of the tibialis anterior muscle with CTX is a common and reproducible method to induce myonecrosis and subsequent muscle regeneration while leaving the innervating nerve intact [2, 23]. As shown in Figure 1B, treatment with CTX in skel-

etal muscle induced regeneration on day 3, evidenced by the up-regulation of *Myog* at both the mRNA and protein levels. These observations confirm previous reports [24, 25]. Conversely, CIBZ protein was down-regulated on days 1 and 3 post-injection, whereas *CIBZ* mRNA levels did not change appreciably.

As the down-regulation of CIBZ occurred before the up-regulation of *Myog* (Figure 1A and 1B) in these experiments, abrogation of CIBZ may be required for myogenic differentiation in C2C12 myoblasts, and for skeletal muscle regeneration.

CIBZ regulates myogenic differentiation in C2C12 cells

To clarify the role of CIBZ in myogenic differentiation, we first examined the effect of its depletion by siRNA. Inhibition of CIBZ 24–48 h after single siRNA transfection in C2C12 myoblasts induced *Myog*, but not MHC (data not shown). Since this period may be insufficient to yield an observable phenotype, we used repeated transfection of siRNA, to sustain a long-term knockdown effect. siRNA-mediated knockdown of CIBZ on day 5, three days after a second transfection, resulted in induction of the MHC protein (Figure 2A), although

the efficiency was much lower than that attained by DM treatment. These data indicate that CIBZ abrogation is capable, at least to some extent, of inducing myogenic differentiation.

To examine whether CIBZ silencing accelerates DM-induced multinucleated myotube formation, we knocked down the expression of CIBZ by siRNA in C2C12 cells for 1 day, and then cultured the cells in DM. Immunoblot analysis showed that *CIBZ* siRNA, but not the control siRNA, enhanced the up-regulation of MHC induced by culturing in DM, in a time-dependent manner (Supplementary information, Figure S1A). CIBZ inhibition resulted in a remarkable enhancement of myotube formation and up-regulation of MHC on days 2 (Supplementary information, Figure S1B–S1D) and 3 (Figure 2B and 2C) after switching to DM.

To explore whether ectopically expressed CIBZ has a reciprocal effect on myogenic differentiation, we transfected Myc-tagged *CIBZ* into C2C12 cells for 1 day (Figure 2D, left panel), and then treated the cells with DM for 2 days. As anticipated, Myc-CIBZ significantly reduced the DM-induced up-regulation of *Myog* and MHC protein levels (Figure 2D, right panel), indicating that ectopic CIBZ expression impedes myogenic differentiation.

CIBZ's role in myogenic differentiation is at least partially dependent on Myog gene regulation

To investigate the molecular mechanism underlying CIBZ-regulated myogenic differentiation, we examined whether CIBZ abrogation affects the transcription of MRFs. RT-PCR analysis showed that CIBZ depletion activates the expression of only *Myog* among the MRF genes; *MyoD*, *Myf5* and *MRF4* transcription was unaffected (Figure 3A). Immunoblot analysis revealed that the inhibition of CIBZ expression by siRNA-1 and -2 resulted in a significant up-regulation of *Myog* protein, but not of *MyoD*. These data suggest that CIBZ acts as a negative regulator of the *Myog* gene.

Next, we examined whether overexpression of CIBZ (Supplementary information, Figure S2) inhibits *Myog*. As shown in Figure 3B, overexpression of CIBZ in C2C12 cells suppressed the up-regulation of *Myog* at both the mRNA and protein levels under DM induction. Considering that CIBZ is an MBP [19], we next asked whether the CIBZ zinc finger 1-5 (ZF1-5) region, which can bind independently to methylated CpG (Figure 5B), was required for the suppression of *Myog* transcription. As seen in Figure 3B, the CIBZ Δ ZF1-5 mutant (lacking the ZF1-5 domain) failed to repress *Myog* expression. This result reveals that the suppression of *Myog* gene by CIBZ may be DNA methylation-dependent, and, in

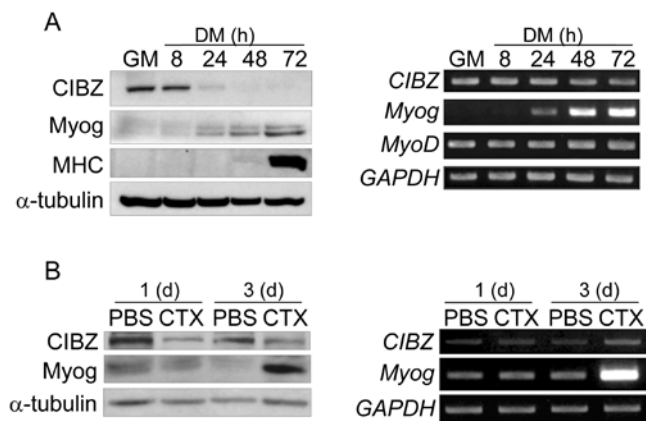


Figure 1 CIBZ is down-regulated during myogenic differentiation in C2C12 cells, and in skeletal muscle regeneration. **(A)** DM was used to induce myogenic differentiation. Total cell lysates were collected at the indicated time points, and analyzed by western blotting (left panels) and semiquantitative RT-PCR (right panels). GM: growth medium. The indicated proteins were detected by their corresponding antibodies. α -tubulin and the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene were used as controls for western blots and RT-PCR, respectively. **(B)** Tibialis anterior muscles from 6-week-old C57BL/6 mice, 1 and 3 days after CTX-induced injury or PBS control injection, were dissected, and whole-cell lysates were prepared and analyzed by western blotting (left panels) and semiquantitative RT-PCR (right panels). Results shown are representative of three independent experiments.

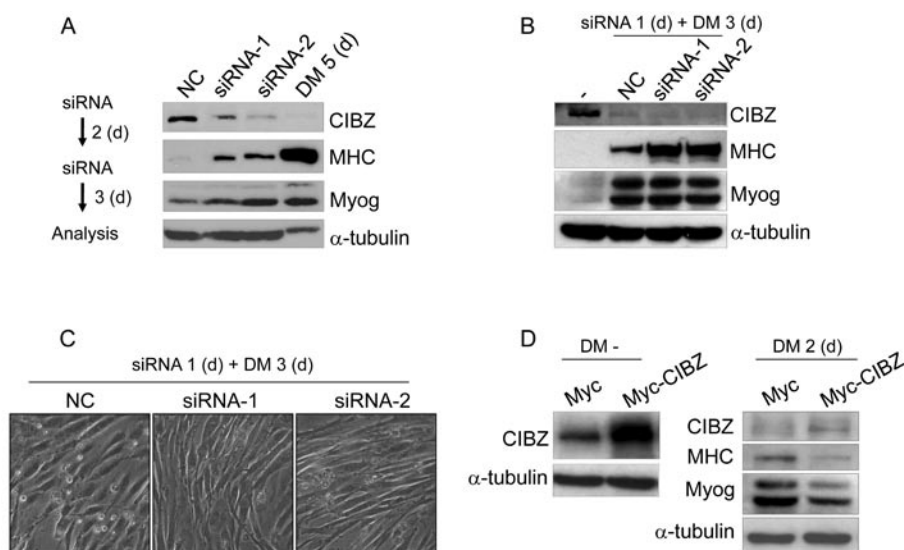


Figure 2 CIBZ negatively regulates terminal differentiation of C2C12 myoblasts. **(A)** Repeated knockdown of *CIBZ* up-regulates the expression of MHC in C2C12 myoblasts. C2C12 cells were transfected with two *CIBZ* siRNAs or a scrambled siRNA negative control (NC). After the first transfection, the cells were transfected again with siRNA on day 2. Total cell lysates were isolated on day 5, and subjected to immunoblot analysis with the indicated antibodies. α -Tubulin was used as a loading control. **(B, C)** C2C12 cells were transfected with *CIBZ* siRNA or NC for 1 day, and then cultured in DM for an additional 3 days. **(B)** Total cell lysates were subjected to immunoblot analysis with the indicated antibodies. Untransfected C2C12 cells were also used as a control (lane 1, “-”). **(C)** *CIBZ* siRNA-treated C2C12 cells were photographed under a phase-contrast microscope. **(D)** Exogenous *CIBZ* expression in C2C12 cells impedes myogenic differentiation induced by DM. C2C12 cells were transiently transfected with Myc or Myc-*CIBZ* for 1 day, and then treated with DM for 2 days. Total cell lysates were subjected to immunoblot analysis. Results shown are representative of three independent experiments.

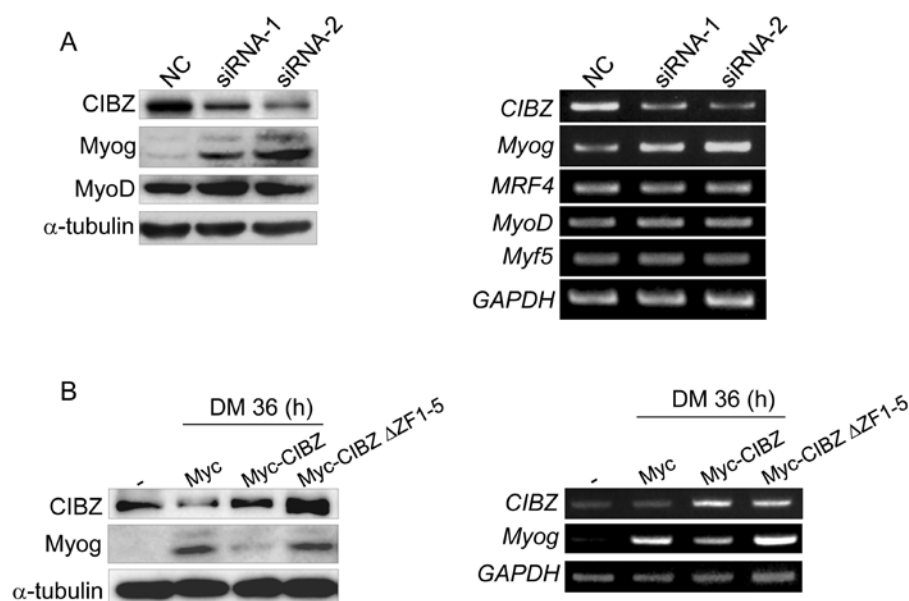


Figure 3 CIBZ protein level regulates the expression of Myog in C2C12 myoblasts. **(A)** At 36 h after transfection with two *CIBZ* siRNAs or NC, expression of the indicated proteins and genes was detected by western blotting (left panels) and semi-quantitative RT-PCR (right panels), respectively. α -tubulin and *GAPDH* served as loading controls for immunoblotting and RT-PCR, respectively. **(B)** C2C12 cells were transfected with Myc, Myc-*CIBZ* and Myc-*CIBZ* Δ ZF1-5 for 24 h, treated with DM for 36 h, and then cell lysates were analyzed by western blotting (left panels) or by semi-quantitative RT-PCR (right panels).

conjunction with the CIBZ loss-of-function data (Figure 3A), suggests that CIBZ's role in differentiation is dependent, at least in part, on the negative regulation of *Myog* transcription.

CIBZ binds to the Myog promoter in a DNA methylation-dependent manner in C2C12 cells

We next used a chromatin immunoprecipitation (ChIP) assay to investigate whether CIBZ binds to the *Myog* promoter in proliferating C2C12 cells. Chromatin from C2C12 cells was prepared and immunoprecipitated with anti-MyoD antibody, as a positive control, normal rabbit IgG, as a negative control, and a new anti-CIBZ antibody (see Methods) which is able to immunoprecipitate endogenous CIBZ in C2C12 cells (data not shown). PCR primers for the ChIP assay were designed to amplify the promoter region (-185 to -1) or a more distally located sequence (-1565 to -1447) in the 5'-regulatory region. As expected, MyoD bound to the promoter region containing its binding sites [9, 26], but not to the distal sequence, which lacks a binding site (Figure 4A and 4B). Similarly, strong binding of CIBZ was observed at the promoter region but not at the distal region.

To determine whether this binding of CIBZ is dependent on the methylation status of *Myog*, we treated proliferating C2C12 cells with 1 μM 5-aza-dC for 3 days, conditions that are sufficient for almost complete demethylation of the proximal *Myog* promoter (see Figure 7A, Supplementary information, Figure S5A). Immunoblot data showed that the expression of either CIBZ or MyoD was not greatly changed after the 5-aza-dC treatment (Supplementary information, Figure S3). ChIP analysis revealed that 5-aza-dC treatment almost abolished the binding of CIBZ to the *Myog* promoter region, but did not weaken the binding of MyoD, which binds to CpG-free E-box motifs at the *Myog* promoter [9], to this region (Figure 4C). These results strongly suggest that binding of CIBZ to the *Myog* promoter is methylation-dependent.

CIBZ binds directly to the proximal Myog promoter in a methylation-dependent manner

To obtain direct evidence that CIBZ binds specifically to the *Myog* promoter *in vitro*, we conducted gel mobility shift assays. The methyl-CpG-binding motif of CIBZ, ZF1-5, was fused to glutathione S-transferase

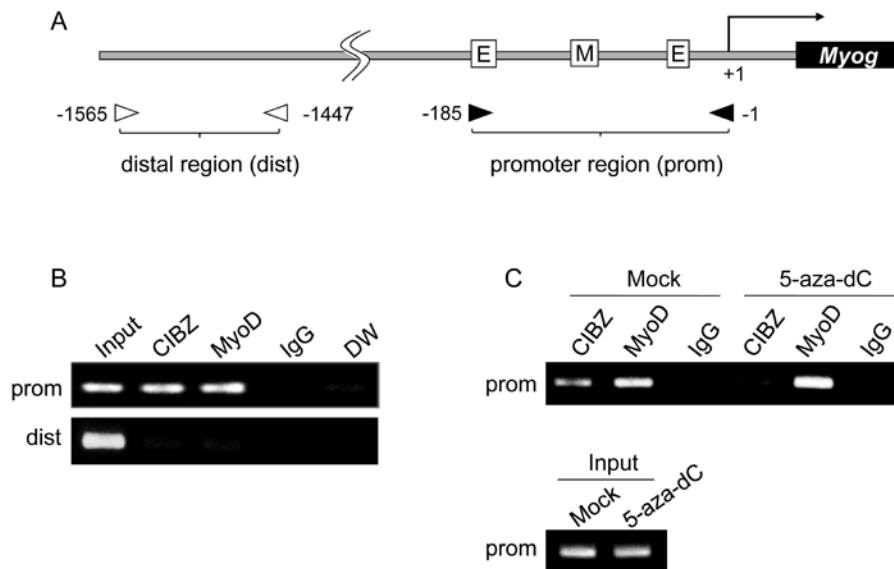


Figure 4 Methylation-sensitive binding of CIBZ to the 5'-regulatory region of *Myog*. **(A)** Schematic structure of the 5'-regulatory region of the mouse *Myog* gene, which contains the amplified promoter fragment (-185 to -1) sequence. A MEF2-binding site (M) and two E-boxes (E) located in the promoter are indicated. The TSS (+1) is marked by a bent arrow. Locations of ChIP primers for the promoter region (prom, black arrowheads) and the distal region (dist, empty arrowheads) are shown. **(B)** ChIP assays were performed with antibodies to CIBZ. Distilled water and non-specific IgG, and an antibody against MyoD, were used as negative controls and as a positive control, respectively. The input DNA (2.5%), IgG-precipitated DNA and DNA immunoprecipitated with the corresponding antibodies were amplified with primers specific for the promoter region (prom) or distal region (dist) as indicated. **(C)** ChIP assays with CIBZ and MyoD antibodies in C2C12 cells treated with 1 μM 5-aza-dC or mock (DMSO)-treated for 3 days. A promoter region-specific primer (prom) was used to amplify the input and immunoprecipitated DNA. Results shown are representative of four independent experiments.

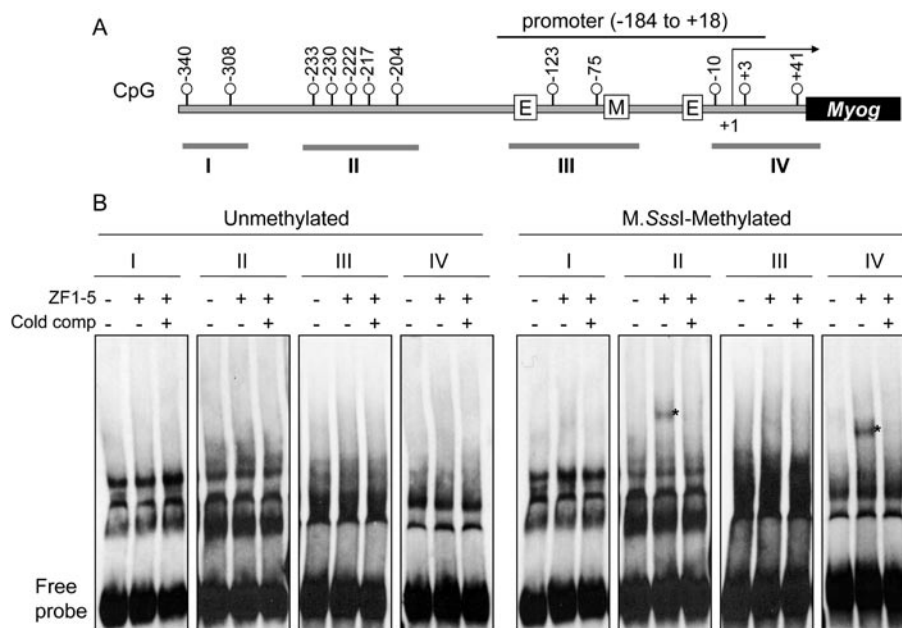


Figure 5 Electrophoretic mobility shift assay (EMSA) of CIBZ binding to the fully methylated proximal *Myog* promoter. **(A)** Schematic map showing the locations of the 12 CpG sites (open circles) in the proximal *Myog* promoter. The sequence locations of four sets of oligonucleotide probes (I to IV) covering the 12 CpG sites are indicated. The TSS is indicated by a bent arrow. **(B)** EMSA was performed using GST-tagged ZF1-5 of CIBZ with either unmethylated control (left panels) or M.SssI-methylated probes (right panels). Excess (150-fold) amounts of the corresponding unlabeled probes were used as specific cold competitors (Cold comp). Asterisks represent methyl-CpG-binding complexes. Results shown are representative of three independent experiments.

(GST-ZF1-5) and purified as described previously [21]. To determine whether CIBZ also binds to sites besides those within the *Myog* promoter (four CpG sites), eight additional CpG sites were examined, including the -340 CpG site and a CpG site located at +41 (Figure 5A). Four DNA probes (I, II, III and IV), which harbor 2, 5, 2 and 3 CpG sites, respectively, were designed and methylated *in vitro* by CpG methyltransferase *SssI* (*M.SssI*) (Figure 5A). Neither GST alone (data not shown) nor GST-ZF1-5 (Figure 5B) could bind to any of the unmethylated probes. By contrast, GST-ZF1-5 produced gel-retarded complexes with methylated probes II (five CpG sites) and IV (three CpG sites). The corresponding unlabeled cold competitors eliminated the formation of these two CIBZ-DNA complexes, indicating that the binding of CIBZ to these two probes is specific. These data demonstrate that CIBZ can bind directly and specifically to methylated CpG dinucleotides both within the *Myog* promoter and in its vicinity *in vitro* (Figure 5A).

CIBZ negatively regulates the activity of a 451-bp proximal Myog promoter in a methylation-dependent manner

To elucidate the mechanism underlying transcriptional

regulation of *Myog* by CIBZ, a luciferase assay was performed. A 1609-bp *Myog* 5'-regulatory fragment containing the promoter region was cloned into the promoterless luciferase reporter pGL3-Basic (pGL3-*Myog*1609). As shown in Supplementary information, Figure S4, ectopic MyoD [26] activated pGL3-*Myog*1609 luciferase expression in 293T cells, while CIBZ overexpression failed to suppress pGL3-*Myog*1609, suggesting that the negative regulation of *Myog* by CIBZ is methylation-dependent.

To determine whether CIBZ indeed modulates *Myog* transcription in a methylation-dependent manner, we cloned a 451-bp proximal *Myog* promoter fragment (*Myog*451: -406 to +45) into the pCpGL luciferase vector, which is devoid of CpG dinucleotides [28]. *Myog*451 was chosen because it contains not only the promoter fragment to which CIBZ binds preferentially (Figure 4B and 5B), but also nine additional CpG sites including the -340 *HpaII* recognition site (Figure 6A). The resultant reporter plasmid pCpGL-*Myog*451 was methylated *in vitro* by *M.SssI*, which methylates all CpG sites, and methylation of this construct was confirmed using *HpaII* (Figure 6B). As shown in Figure 6C, the luciferase activity of methylated pCpGL-*Myog*451 in C2C12 cells

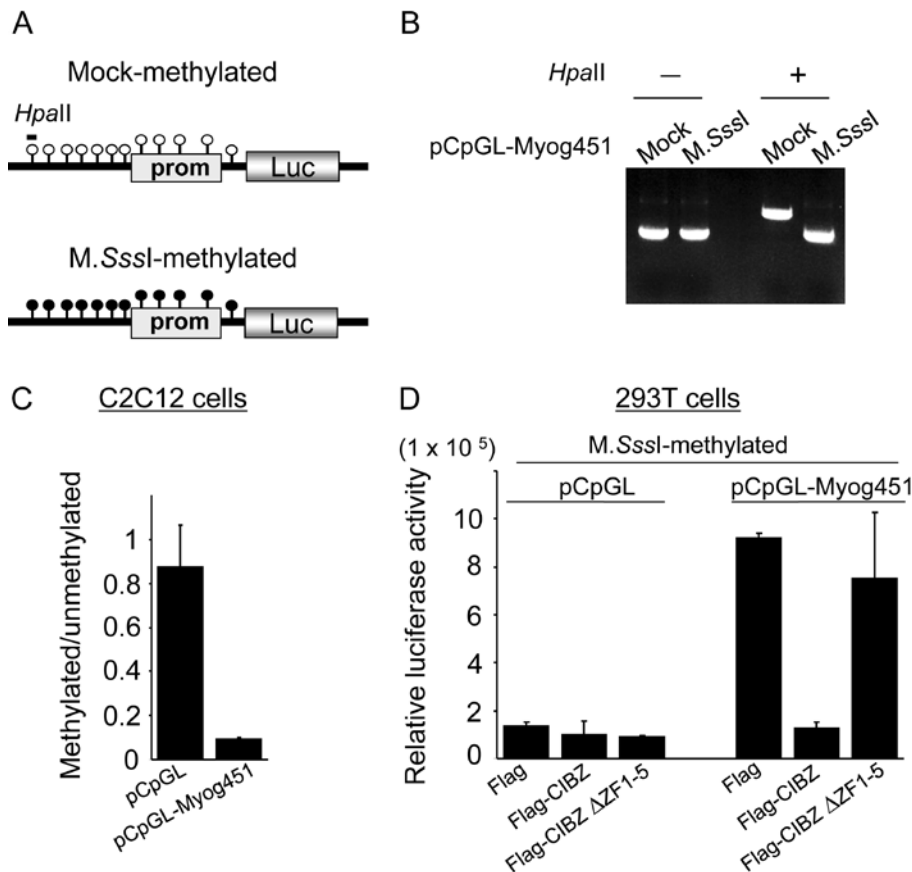


Figure 6 CIBZ negatively regulates the Myog gene in a methylation-dependent manner. **(A)** Schematic representation of the M.SssI-methylated pCpGL-Myog451 luciferase reporter construct (lower panel), and the promoter (prom). The mock-methylated control (upper panel) consisted of all components required for *in vitro* methylation except M.SssI. Methylated and unmethylated CpGs are marked by filled and open circles, respectively. The *Hpa*II site in Myog451 is indicated in the upper panel. **(B)** M.SssI-methylated pCpGL-Myog451 or a mock-methylated control was digested with methylation-sensitive *Hpa*II and analyzed on a 0.8% agarose gel to verify the efficiency of *in vitro* methylation. **(C)** C2C12 cells were transiently transfected with M.SssI-methylated or mock-methylated pCpGL and pCpGL-Myog451, together with an internal control pRL-TK, for 2 days. Luciferase activity expressed from M.SssI-methylated pCpGL and pCpGL-Myog451 was calculated relative to that from unmethylated plasmids, which was normalized to 1. **(D)** M.SssI-methylated pCpGL and pCpGL-Myog451 were cotransfected with Flag, Flag-tagged CIBZ and CIBZ ΔZF1-5 into 293T cells for 2 days. pRL-TK was cotransfected as an internal control. Relative luciferase activity was determined by normalization to internal control activity. Each bar represents mean ± standard deviation (SD) derived from three experiments.

was much lower than that of its unmethylated form, indicating that methylation of Myog451 accounts for its transcriptional repression. In 293T cells, Flag-CIBZ significantly decreased the luciferase activity of methylated pCpGL-Myog451 (Figure 6D and Supplementary information, Figure S4D), whereas CIBZ ΔZF1-5 lacked this repressive effect (Figure 6D). We also observed that ectopic expression of CIBZ in 293T cells did not inhibit the luciferase activity of pGL3-Myog451 (data not shown). These data indicate that suppression of *Myog* promoter activity by CIBZ is methylation-dependent.

Methylation of CpG sites in Myog 451 is inversely correlated with Myog expression

The methylation status of all 12 CpG sites in Myog451 was evaluated by sodium bisulfite sequencing (SBS). As shown in Figure 7A, sequence analysis of individual clones (10 clones per sample) revealed that Myog451 was moderately methylated (42.5%) in proliferating C2C12 cells. Upon DM induction, CpG methylation of Myog451 decreased slightly (40.0%) on day 3, but it dropped to 32.5% on day 5. Significantly, treatment of C2C12 cells with 1 μM 5-aza-dC for 3 days led to a dramatic demethylation of Myog451(8.3%). Notably, CpG

sites at -308, -233 and -230 were more demethylated during myogenic differentiation (Supplementary information, Figure S5B-S5C). In parallel, semiquantitative PCR showed that the expression of *Myog* was induced by 5-aza-dC or by DM in a time-dependent manner (Figure 7B).

We next examined the methylation status of *Myog451* *in vivo*. As seen in Figure 7C, bisulfite mapping using genomic DNA from adult tissues showed that *Myog451* was highly methylated in non-muscle samples (brain, 62.5%; kidney, 68.5%), but less so in skeletal muscle (39.2%). The methylation level of *Myog451* in the limb

of postnatal day 1 (P1) mice was even lower (24.2%). In particular, demethylation at -340, -308, -230, -123, -75, -10 and +3 CpG sites correlated with the up-regulation of *Myog* mRNA expression in adult muscles as compared with non-muscle tissues (Supplementary information, Figure S5D-S5E, Figure 7D). Notably, -340, -308, -230, -75 and -10 CpG sites were more demethylated during skeletal muscle development (Figure 7D). These data imply that demethylation at specific CpG sites may be responsible, in part, for *Myog* transcription *in vivo*.

siRNA-mediated knockdown of CIBZ does not significantly affect the methylation status of Myog451

We next asked whether CIBZ abrogation affected the methylation status of *Myo451*. To address this, genomic DNA extracted from *CIBZ* siRNA-transfected C2C12 cells (described in Figure 2) was analyzed by SBS. As shown in Supplementary information, Figure S7, siRNA-mediated *CIBZ* knockdown in C2C12 cells did not greatly influence CpG methylation, either overall (Supplementary information, Figure S7A) or at individual sites (Supplementary information, Figure S7B) in *Myog451*. These data indicate that induction of *Myog* transcription by CIBZ knockdown is independent of demethylation of CpG sites in *Myog451*.

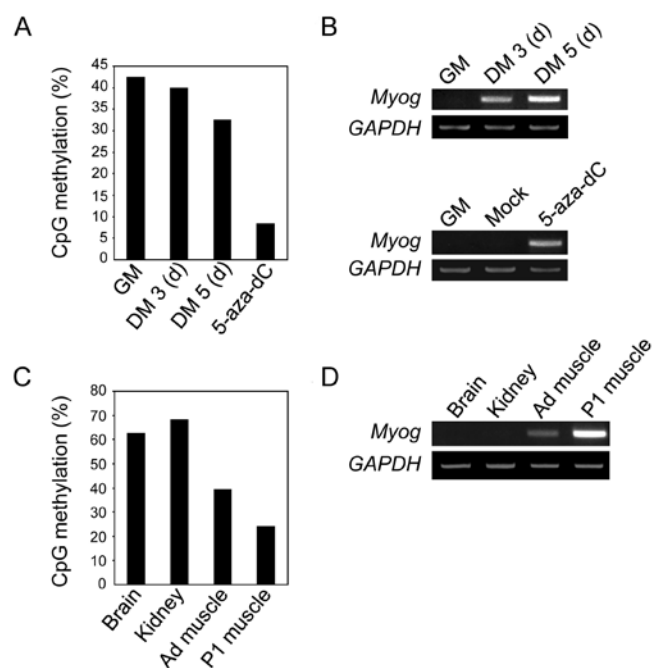


Figure 7 The methylation status of the 451-bp proximal *Myog* promoter is inversely correlated with *Myog* expression. **(A, C)** Genomic DNA was extracted and assayed by bisulfite sequencing analysis, and overall cytosine methylation percentages for *Myog451* are shown in the black bars. The percentage values indicate the total methylation level of the 12 CpG sites in *Myog451*; methylation of each CpG dinucleotide was assessed for 10 individual clones. **(A)** Methylation status of *Myog451* in C2C12 cells under growth medium (GM), DM treatment for 3 or 5 days, and 1 μ M 5-aza-dC treatment for 3 days. **(B)** Semiquantitative RT-PCR analysis shows the expression of *Myog* in DM-treated C2C12 cells at the time points indicated (upper panel) or in cells treated with 1 μ M 5-aza-dC for 3 days (lower panel). Mock, same volume of DMSO. **(C)** Methylation status of *Myog451* in 2-month-old mouse tissues (brain, kidney and skeletal limb muscle (Ad muscle)) and postnatal day 1 limb muscle (P1 muscle). **(D)** Semiquantitative RT-PCR for *Myog* expression in the indicated 2-month-old mouse tissues and in postnatal day 1 limb muscle (P1 muscle). *GAPDH* served as a loading control for **B** and **D**.

Discussion

We previously reported that CIBZ plays an important role in the negative regulation of apoptosis in murine cells [21]. Interestingly, genome-wide association data demonstrate that the gene most frequently associated with human stature is *ZBTB38* [29], although the reasons behind this are unclear. We show here that CIBZ is down-regulated both in differentiating C2C12 cells and in regenerating skeletal muscle. Consistent with this, gain- and loss-of-function data indicate that CIBZ suppresses myogenic differentiation in cultured cells. Our data strongly suggest that these effects are attributable, at least in part, to CIBZ suppressing its direct target, *Myog*. We also reveal that demethylation in and around the *Myog* promoter region facilitates *Myog* transcription *in vitro* and *in vivo*.

DNA methylation is hypothesized to act as an on-off switch for developmentally regulated and/or tissue-specific gene expression. Two current models are considered to account for the transcriptional repression of a methylated gene, and it is likely that both are biologically relevant [13, 30]. The first is that methylation of CpG dinucleotides impairs the binding of some transcriptional factors to their cognate DNA sequences. The second is that methylated cytosines at CpG dinucleotides recruit

Table 1 Summary of methylation level of Myog451, CIBZ protein expression, and Myog transcription

	Methylation status of Myog451	CIBZ expression	Myog transcription
C2C12 cells			
GM		High	Off
DM-early stage		Low	Weak
DM-late stage		Very low	Very strong
5-aza-dC		High ^a	Strong
Non-muscle tissues		Moderate~High	Off
Adult skeletal muscle		Very high	Weak
Postnatal (day 1) skeletal muscle		Moderate	Very strong

The black portions of the pie chart represent the proportion of all CpG dinucleotides in Myo451.

^aIndicates that highly expressed CIBZ cannot bind to demethylated Myog451.

MBPs that specifically recognize these sites. Our data suggest that the latter model is valid (see Table 1). In proliferating C2C12 myoblasts, where Myog451 is moderately methylated and CIBZ is highly expressed, *Myog* transcription is turned off (Figures 1A, 7A and 7B). In the early stage of differentiation following DM induction, down-regulation of CIBZ protein, together with the demethylation of several CpG sites in Myog451, correlates with induction of *Myog* transcription (Figures 1A, 7A and 7B, Supplementary information, Figure S5C). In the late stage of differentiation, progressive hypomethylation of Myog451 and a profound decrease of CIBZ protein correlate with further up-regulation of *Myog* (Figures 1A, 7A and 7B, Supplementary information, Figure S5C). Upon treatment of C2C12 cells with 5-aza-dC, the almost complete demethylation of Myog451 coincides with elevated expression of *Myog* (Figure 7A and 7B, Supplementary information, Figure S5C); ChIP data indicated that CIBZ could not bind to demethylated Myog451 (Figure 4C). Notably, the induction of *Myog* transcription by CIBZ knockdown is independent of demethylation of CpG sites in Myog451 (Supplementary information, Figure S7). Taken together, these data suggest that either demethylation of CpG sites in Myog451 or CIBZ down-regulation leads to the induction of *Myog* transcription, and that these two events together result in a further up-regulation of *Myog*, contributing to the terminal differentiation of C2C12 myoblasts.

We previously showed that CIBZ is ubiquitously expressed in adult mouse tissues [20]; its expression is high in kidney, and especially so in skeletal muscle (Supplementary information, Figure S6). In non-muscle tissues, such as brain and kidney, hypermethylation of Myog451 correlates with the silencing of *Myog* (Figure 7C and 7D). Conversely, partial demethylation of Myog451 correlates with the induction of *Myog* in adult skeletal muscle (Figure 7C and 7D). Moreover, the dramatic de-

crease in CIBZ protein level and the demethylation of Myog451 coincide with the remarkable up-regulation of *Myog* in postnatal day 1 muscle relative to adult muscle (Figure 7C and 7D, Supplementary information, Figure S6). These data suggest that CIBZ protein participates in the regulation of postnatal growth of skeletal muscle. The precise reason for CIBZ protein degradation during skeletal muscle growth and regeneration remains unclear. The proteasome inhibitor MG132 can efficiently prevent CIBZ degradation during myogenic differentiation in C2C12 cells [21], suggesting that the ubiquitin-proteasome pathway is involved in the regulation of CIBZ protein *in vivo*. Collectively, these observations lead us to conclude that the silencing of the *Myog* locus is coordinately regulated by the levels of CIBZ expression and of Myog451 CpG methylation in tissues, and during postnatal growth of skeletal muscle.

Although the *Myog* promoter is CpG island-free, multiple CpG dinucleotides occur within and in close proximity to this promoter (Figure 5A). During revision of this manuscript, Steffens *et al.* reported that exposure of C2C12 cells to sodium arsenite delayed DM-induced differentiation [31]. Increased methylation of the same 12 CpG sites at the *Myog* promoter that we describe here correlated with a reduction in *Myog* transcription, which may consequently lead to a delay in myogenic differentiation [31]. Generally, the number of methylated CpGs and the methylation levels of CpG sites appear to coordinately control gene transcription in cells and tissues, although the molecular mechanism underlying this phenomenon remains poorly understood [13, 30]. We observed that the 12 CpGs in Myog451 are important to mediate methylation-dependent silencing of *Myog* (Figure 6C), and that demethylation of these sites correlates with transcriptional activation of *Myog* (Figure 7B, Supplementary information, Figure S5B-5C). Since some specific CpG sites are more demethylated during

C2C12 myoblast differentiation (Supplementary information, Figure S5C), as well as in adult mouse tissues and in muscle development (Supplementary information, Figure S5E), we suppose that, under various circumstances, these CpG sites might collectively be critical for the regulation of *Myog* transcription.

Currently, two major families of MBPs are known in vertebrates: methyl-CpG-binding domain (MBD) proteins and C₂H₂ zinc finger proteins, all of which bind preferentially to methylated CpG and repress transcription. The MBD-containing proteins include MBD1, MBD2, MBD4 and MeCP2 [32, 33]; Kaiso, ZBTB4 and ZBTB38 (CIBZ in mouse) use zinc fingers to bind methylated CpG [34]. Compared to the loss of DNA methyltransferases, the consequence of loss of MBD-containing proteins in animals is quite mild, with the exception of *Kaiso* depletion in *Xenopus laevis*, where \times Kaiso is linked with the maintenance of transcriptional silencing before the onset of zygotic transcription [35]. Interestingly, MBD1, Kaiso and ZBTB4 have the ability to bind, albeit weakly, unmethylated DNA: MBD1 binds unmethylated CpG through its CXXC3 motif, while Kaiso and ZBTB4 recognize CTGCNA sites via zinc fingers [19, 36]. These findings raise questions of whether these MBPs bind preferentially to a particular site (methylated or non-methylated), and of what the biological significance is of such binding. By analyzing Kaiso-binding sites in HEK293T cells, Ruzov *et al.* found that Kaiso associates with methylated CpGs but not CTGCNA sites, and importantly, their data show that only the methylated DNA-binding function of Kaiso is required in early *Xenopus laevis* development [37]. It should be noted that binding of MBPs to methylated DNA appears to be sequence-specific: additional DNA sequence surrounding the CpG site(s) is important [38, 39].

So far, no MBP has been reported to directly regulate the transcription of *Myog*. Among the MBD-containing MBPs, the expression of MBD2 and MeCP2 is very low in proliferating myoblasts but increases during myogenic differentiation [40], suggesting that their main role is in terminal muscle differentiation. In support of this hypothesis, up-regulation of these two proteins leads to the reorganization of heterochromatin, and contributes to the terminal differentiation of myotubes [40]. Upon oxidative stress induction, the nuclear translocation of focal adhesion kinase and its consequent interaction with MBD2 have been reported to induce skeletal muscle differentiation [41]. However, it remains unclear whether MBD2 acts alone to influence early myogenic differentiation, or regulates *Myog* transcription directly. Of the C₂H₂ zinc finger MBPs, CIBZ and ZBTB4 can bind to a single methylated CpG, whereas Kaiso only recognizes

DNA sequences that contain at least two adjacent methyl-CpGs [19, 42]. Since *Myog451* contains 12 CpG sites but lacks two consecutive CpGs, Kaiso probably cannot bind to the dispersed methylated CpGs in *Myog451*. Most recently, artificial selection of ZBTB4 binding sites *in vitro* demonstrated that ZBTB4 binds specifically to methylated C₁/_ACGC₁/_TAT, a sequence to which Kaiso and ZBTB38 can also bind [39]. The absence of such a consensus sequence in *Myog451* suggests that ZBTB4 does not bind to *Myog451*. Further studies are required to determine whether other MBPs, including MBD1 and MBD4, contribute to early myogenic differentiation, and directly regulate *Myog* transcription through interactions with the *Myog451* region, both in cells and in tissues.

In conclusion, our data demonstrate that CIBZ negatively regulates C2C12 myoblast differentiation. This finding leads us to hypothesize that CIBZ may also affect satellite cell differentiation *in vivo*, one of the crucial steps of skeletal muscle regeneration. The observation that CIBZ down-regulation may be required for skeletal muscle regeneration in mice (Figure 1B) suggests that suppression of CIBZ would promote muscle regeneration in myodegenerative diseases. Drugs that antagonize CIBZ may offer opportunities for the treatment of skeletal muscle disorders.

Materials and Methods

Cell culture, DM and 5-aza-dC treatment

C2C12 and 293T cells were cultured in DMEM supplemented with 15% or 10% fetal bovine serum for C2C12 cells and 293T cells, respectively, as described previously [21]. To induce myogenic differentiation of C2C12 cells, subconfluent cells were cultured with DM consisting of DMEM supplemented with 2% horse serum. Demethylation studies were performed with 5-aza-2'-deoxycytidine (5-aza-dC) (Sigma-Aldrich) at a concentration of 0.5–1 μ M. Cells treated with equivalent amounts of DMSO served as a negative control. The medium for cells cultured with 5-aza-dC or DMSO was replaced every 24 h. Cells were harvested at different time points after 5-aza-dC treatment.

Semiquantitative RT-PCR

Semiquantitative RT-PCR was conducted as described previously [21]. The primers designed were confirmed as unique in the nonredundant NCBI database. Primers for the mouse genes are shown in Supplementary information, Data S1. The annealing temperature was 58 °C for reactions using all primer sets. Reaction products were separated in a 2% agarose gel and visualized by ethidium bromide staining. To confirm the identity of PCR products, single bands of the expected size were excised from the gels and sequenced. *GAPDH* mRNA expression served as an internal control.

siRNA and transient transfection

C2C12 cells were transfected with 1 nM CIBZ-specific Dicer substrate siRNA duplexes, or with DS scrambled negative control

(Integrated DNA Technologies), as described previously [21]. Transfection with siRNA was carried out with INTERFERin reagent (Polyplus Transfection) according to the manufacturer's instructions. The siRNA-transfected cells were used 24–48 h after transfection for RT-PCR and western blotting. Transient transfections of C2C12 cells with CIBZ constructs were described previously [20]. Briefly, cells were transfected with Myc-tagged *CIBZ* and its deletion mutant using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Reporter constructs, in vitro methylation and luciferase assay

CpG-free vector pCpGL was a kind gift from Dr Michael Rehli [28]. For the pCpGL-Myog451 construct, the Myog451 (–406 to +45 bp) fragment was generated by PCR with primers (forward: 5'-CCAGGGATCCCTTGTCCCTTCCTGCCCTGTC-3'; reverse: 5'-CCCAAGCTTGGTCGGAAAAGGCTTGTTCCTG-3') that incorporated restriction sites (*Bam*HI and *Hind*III), and subsequently ligated into pre-digested pCpGL vector. DNA sequences were verified using BigDye terminator and an automated sequencer (ABI PRISM310). pCpGL and pCpGL-Myog451 were methylated *in vitro* by *M.Sss*I (New England Biolabs) according to the manufacturer's recommendations, or mock-methylated in the absence of *M.Sss*I. The extent of pCpGL-Myog451 methylation was verified by digesting the methylated CpG with *Hpa*II. Transfections were carried out with Lipofectamine 2000 and luciferase assays were performed using a previously described protocol [43]. The thymidine kinase promoter-*Renilla* luciferase reporter plasmid pRL-TK was used as an internal control. All experiments were performed in triplicate, and the data shown are representative of at least three independent experiments with similar results.

Electrophoretic mobility gel shift assay (EMSA)

GST-CIBZ ZF1-5 was constructed and purified as described previously [21]. Oligonucleotides used for probes covering the 12 CpG sites were purchased from Greiner bio-one (Tokyo, Japan). Oligonucleotide sequences are shown in Supplementary information, Data S1 about primers. EMSA was performed with digoxigenin-11-dUTP (DIG)-labeled probes using a DIG gel shift kit (Roche) according to the manufacturer's instructions. Sense-strand oligonucleotides were annealed with their complementary strands and 3'-end-labeled with DIG by terminal transferase. The binding reaction was performed with 20 fmol of DIG-labeled probes incubated with GST-CIBZ ZF1-5 in binding buffer containing 50 ng of poly(dI-dC), in a final volume of 10 μ l, on ice for 20 min. The DNA-protein complex was resolved in a 6% polyacrylamide gel, and then electroblotted onto a Nylon membrane and UV-crosslinked. The blot was then blocked and incubated with anti-DIG antibody conjugated with alkaline phosphatase at room temperature for 30 min, washed, subjected to chemiluminescent detection with CSPD (disodium 3-(4-methoxyphosphoryl)-1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan-4-yl)phenyl phosphate) as the substrate, and exposed to an X-ray film. CpG methylation of the probe was catalyzed by *M.Sss*I as described above. For competition assays, a 150-fold excess of unlabeled probe was preincubated with the binding mixture.

CIBZ antibody production and western blotting

A new CIBZ polyclonal antibody (C2) was produced by JBios

(Saitama, Japan). Briefly, a KLH-conjugated peptide corresponding to amino acids 1184–1197 (EQKDDIKAFÄENVL) of mouse CIBZ was synthesized. Two rabbits were immunized with this peptide according to standard protocols. Blood taken at two and three months after immunization was pooled, and the antibodies were affinity-purified. The specificity of C2 was confirmed by immunoblotting and comparison with an anti-CIBZ antibody described previously [20] (data not shown). C2 specifically immunoprecipitated endogenous CIBZ from C2C12 cells (data not shown). Western blotting was performed as described previously [20]. Briefly, cells were sonicated and lysed in lysis buffer. Proteins were separated by 8–15% SDS-PAGE, transferred to PVDF membranes and probed with the following antibodies: anti-CIBZ (C2), anti-MyoD (C-20, sc-304, Santa Cruz Biotechnology), anti-Myog (5FD, sc-52903, Santa Cruz Biotechnology), anti- α -tubulin (clone DM 1A, Sigma) and anti-MHC (clone MF20, Developmental Studies Hybridoma Bank). HRP-conjugated anti-mouse or anti-rabbit IgG (GE Healthcare) was used as the secondary antibody.

ChIP assay

A ChIP assay was carried out with an EZ-ChIP kit (Millipore) according to the manufacturer's instructions. C2C12 cells were grown to subconfluence on 10-cm plates. After crosslinking for 30 min with 1% formaldehyde, glycine was added to a final concentration of 0.125 M and the cells were washed 3 times with ice-cold phosphate-buffered saline (PBS) and harvested with SDS-lysis buffer. The chromatin lysate was sonicated on ice to an average DNA length of 500 bp. After preclearing the lysates with Protein G Sepharose beads, anti-CIBZ antibody (C2) was used to immunoprecipitate protein-DNA complexes. Anti-MyoD antibody was used as a positive control, and preimmune IgG and distilled water served as negative controls. Amplification of the immunoprecipitated DNA was achieved using Blend Tag DNA polymerase (Toyobo, Osaka, Japan). Primers shown in Supplementary information, Data S1 were designed to evaluate the promoter region (prom) and distal region (dist). The annealing temperature for both prom and dist primers was 55 $^{\circ}$ C. Each experiment was repeated four times.

Sodium bisulfite genomic sequencing

Genomic DNA was isolated from C2C12 cells and mouse tissues (C57BL/6) with the Gentra Puregene genomic DNA purification kit (Roche) according to the manufacturer's instructions. Bisulfite reactions were performed using an EZ DNA Methylation-Gold kit (Zymo Research) under conditions that allowed for complete conversion of cytosines, but not 5-methylcytosines, to uracil. The modified DNA was amplified by nested PCR with two sets of primers listed in Supplementary information, Data S1 about primers. PCR primers were designed with MethPrimer software [44], and all primer sequences were devoid of CpG dinucleotides to avoid biased amplification of methylated alleles. Hot-start PCR was performed at 95 $^{\circ}$ C for 5 min, followed by a first-round amplification using 42 cycles of 95 $^{\circ}$ C for 30 s, 45 $^{\circ}$ C for 40 s and 72 $^{\circ}$ C for 1 min. The conditions for the second amplification were the same as those for the first round of PCR. The PCR products were electrophoresed in 2% agarose gels and visualized with ethidium bromide. Bands of the expected sizes were isolated and their DNA was extracted using a Wizard SV Gel and PCR clean-up system (Promega). The purified DNA was cloned into pGEM-T (Promega). Ten clones were picked for each sample, and their DNA

was extracted and sequenced. CpGviewer [45] was used to determine methylation profiles by comparing the sequence of bisulfite-converted DNA with that of unmodified DNA.

Muscle regeneration assay

Mice (C57BL/6, 6-week-old males) were anesthetized by intraperitoneal injection of nembutal using a 27-gauge needle. To introduce a muscle injury, 50 μ l of 0.075% CTX from *Naja mossambica mossambica* (Sigma, C-9759) in PBS was injected into the right muscle tibialis anterior (TA) using a 27-gauge needle [23]. The left side was injected with the same volume of PBS as a control. Muscles were harvested at 1 and 3 days after injection. Mice were killed by cervical dislocation, and total muscle lysates isolated from the CTX- and PBS-injected TA muscles were analyzed by immunoblotting. Expression of Myog mRNA and protein was used as a positive control to evaluate regeneration efficiency.

Acknowledgments

We are grateful to Dr Ian Smith (Nara Institute of Science and Technology) for critical reading of the manuscript. We especially thank Dr Manabu Sugai (Kyoto University) for valuable discussions about the work. We thank members of the Kawaichi laboratory for technical advice. We thank Dr Michael Rehli (University Hospital Regensburg) for providing the pCpGL plasmid. This work was supported by a grant-in-aid for Scientific Research (C), and the Global COE program, from Japan's Ministry of Education, Culture, Sports, Science and Technology.

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(Supplementary information is linked to the online version of the paper on the *Cell Research* website.)