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Xiping Zhang
University of Kentucky, xiping.zhang@uky.edu

Samir P. Patel
University of Kentucky

John J. McCarthy
University of Kentucky, john.mccarthy@uky.edu

Alexander G. Rabchevsky
University of Kentucky, alexander.rabchevsky@uky.edu

David J. Goldhamer
University of Connecticut

See next page for additional authors

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Authors

Xiping Zhang, Samir P. Patel, John J. McCarthy, Alexander G. Rabchevsky, David J. Goldhamer, and Karyn A. Esser

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A non-canonical E-box within the *MyoD* core enhancer is necessary for circadian expression in skeletal muscle

Xiping Zhang[1], Samir P. Patel[1], John J. McCarthy[1], Alexander G. Rabchevsky[1], David J. Goldhamer[2] and Karyn A. Esser[1,*]

[1]Center for Muscle Biology, Department of Physiology, University of Kentucky, Lexington, KY 40536 and [2]Department of Molecular and Cell Biology, Center for Regenerative Biology, University of Connecticut Stem Cell Institute, University of Connecticut, Storrs, CT 06269, USA

ABSTRACT

The myogenic differentiation 1 (*MyoD*) gene is a master regulator of myogenesis. We previously reported that the expression of *MyoD* mRNA oscillates over 24 h in skeletal muscle and that the circadian clock transcription factors, BMAL1 (brain and muscle ARNT-like 1) and CLOCK (circadian locomotor output cycles kaput), were bound to the core enhancer (CE) of the *MyoD* gene *in vivo*. In this study, we provide *in vivo* and *in vitro* evidence that the CE is necessary for circadian expression of *MyoD* in adult muscle. Gel shift assays identified a conserved non-canonical E-box within the CE that is bound by CLOCK and BMAL1. Functional analysis revealed that this E-box was required for full activation by BMAL1/CLOCK and for *in vitro* circadian oscillation. Expression profiling of muscle of CE$^{loxP/loxP}$ mice found approximately 1300 genes mis-expressed relative to wild-type. Based on the informatics results, we analyzed the respiratory function of mitochondria isolated from wild-type and CE$^{loxP/loxP}$ mice. These assays determined that State 5 respiration was significantly reduced in CE$^{loxP/loxP}$ muscle. The results of this work identify a novel element in the *MyoD* enhancer that confers circadian regulation to *MyoD* in skeletal muscle and suggest that loss of circadian regulation leads to changes in myogenic expression and downstream mitochondrial function.

INTRODUCTION

The *MyoD* (myogenic differentiation 1) gene is the founding member of the myogenic regulatory factor family of transcription factors and is known to have a central role in myogenesis (1). In adult skeletal muscle, *MyoD* is thought to function primarily in the myogenic differentiation of satellite cells during muscle regeneration (2,3). Recently, however, *MyoD* expression has been found to oscillate over a 24 h period in both mouse and rat skeletal muscle (4,5). Andrews and colleagues (4) also determined that *MyoD* was a direct transcriptional target of the molecular clock. Both BMAL1 and CLOCK were found to bind to the *MyoD* core enhancer (CE) 20 kb upstream (6). The molecular link between the core clock and *MyoD* implicated a role for the circadian expression of *MyoD* in skeletal muscle homeostasis. This was supported by phenotypic analysis of muscle from two different genetic mouse models in which expression of the core circadian genes, *Bmal1* or *Clock*, were disrupted (4). Andrews and colleagues (4) reported that in skeletal muscle of clock-compromised mice *MyoD* expression did not oscillate and this was associated with a disruption of muscle structure and diminished mechanical function.

The CE is a relatively small enhancer of 258 bp and resides ~20 kb upstream of the *MyoD* transcription start site. Previous studies have shown that loss of the CE affects expression of *MyoD* in all embryonic skeletal muscle compartments, including somitic myotomes, limb buds and branchial arches (6,7). The CE appears to be primarily regulated by a positive control mechanism operating through multiple, discrete *cis*-elements that are scattered throughout the enhancer (8,9). Kablar *et al.* (7) reported that deletion of the CE in the mouse caused a reduction in *MyoD* mRNA expression in both embryos and neonates and resulted in a delay in muscle differentiation. In contrast to its role during development, the CE was found to be dispensable for expression during late fetal stages and was assumed to be inactive in adult muscle (10). Thus, these studies showed that the CE was critical for initiation of *MyoD* transcription during development but was argued to be not necessary for expression of *MyoD* in adult skeletal muscle (8,11).

The molecular clock that governs circadian rhythms can be described as a transcription–translation feedback loop (12). The forward arm of the molecular clock is comprised of CLOCK (circadian locomotor output cycles kaput) and BMAL1 (brain and muscle ARNT-like 1) which are bHLH-PAS transcription factors that bind to an E-box motif (5′-CACGTG-3′) as a heterodimer. As part of the molecular clock, CLOCK and BMAL1 activate transcription of factors that constitute the negative loop of the molecular clock, including the *Period* (*Per*) and *Cryptochrome* (*Cry*) gene isoforms *Per1*, *Per2*, *Cry1* and *Cry2*. In turn, CRY and PER proteins act together to inhibit the *trans*-activation function of CLOCK and BMAL1 and this mechanism oscillates over an approximate 24 h cycle (12,13). Consistent with the feedback mechanism, the circadian phase of *Bmal1* expression was in anti-phase to circadian phase of *Per1/2* and *Cry1/2* expression (14–17).

The purpose of the current study was to determine whether the CE is the module through which the molecular clock is regulating the circadian expression of *MyoD*. In addition, we wanted to identify the *cis*-acting sites [E-box(es)] within the CE that bind CLOCK and BMAL1. In this study, we provide evidence showing that the *MyoD* CE is necessary for circadian oscillation of *MyoD* expression *in vivo*. We also show that the CE is sufficient to confer circadian expression *in vitro*. We identify a previously unknown non-canonical E-box in the *MyoD* CE that mediates CLOCK and BMAL1 binding and using an *in vitro* circadian assay we demonstrate that this non-canonical E-box is required for oscillation of a *MyoD* reporter. Expression profiling of muscle from adult $CE^{loxP/loxP}$ mice identified mis-expressed genes that overlap with *MyoD* target genes from ChIP-Seq studies and also disrupted genes identified from analysis of muscle from the $Clock^{\Delta 19}$ mouse (18). Further experiments indicated that mitochondrial respiration was impaired in the $CE^{loxP/loxP}$ muscle. These findings support a model in which the molecular clock factors directly regulate *MyoD* expression in skeletal muscle through regulation of a non-canonical E-box in the CE and that loss of circadian expression of *MyoD* contributes to downstream changes in the expression of *MyoD* target genes with effects on mitochondrial function.

MATERIALS AND METHODS

Animals

All animal procedures were conducted in accordance with institutional guidelines for the care and use of laboratory animals as approved by the University of Kentucky Institutional Animal Care and Use Committee. Mice (C57BL/6J strain) were purchased from The Jackson Laboratory. The *MyoD* core enhancer knockout strain ($CE^{loxP/loxP}$ mice) was originally developed and described by Chen and Goldhamer in 2004 (10). These mice are germline knock-outs for the CE so no further breeding is required. We bred the original $CE^{loxP/loxP}$ mice onto a C57BL/6J background by backcrossing for 10 generations. The $CE^{loxP/loxP}$ mice pups were genotyped by

PCR as described by (10). The $CE^{loxP/loxP}$ mouse was also crossed with *Per2::Luc* knock-in mice (19) to produce homozygous mice harboring both $CE^{loxP/loxP}$ and *Per2::Luc*.

Circadian tissue collection

We followed the established protocol in the circadian field for collection of tissues for circadian analysis (18,20). Briefly, mice (10–12 weeks of age) were entrained to a 12 h light/12 h dark (12L:12D) cycle for 2 weeks and then placed in total darkness for 30 h prior to the start of the tissue collection to remove any light cues. Tissues were collected in darkness with red light from mice (n = three/genotype) every 4 h for 32 h (nine time points). By circadian convention, the onset of activity for a nocturnal animal is defined as circadian time 12 (CT12) so the first collection was performed at CT18.

Cell culture

The mouse myogenic cell line C2C12 was obtained from ATCC (CRL 1722). Myoblasts were grown in Dulbecco's modified Eagles medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and penicillin–streptomycin in a humidified incubator at 37°C under 5% CO_2. To establish stable clones, C2C12 cells (5×10^4) were co-transfected with 1 µg of a particular reporter gene plasmid and 1 µg of pCI-neo plasmid (Promega, Madison, WI, USA). Stable clones (n = 3/construct) were selected following treatment with G418 at 600 µg/ml and maintained in culture with 200 µg/ml.

Transient transfection

C2C12 cells were transiently transfected with expression vectors for *Clock* and *Bmal1* (200 ng/vector) and one of the *MyoD* reporter genes (50 ng) using Fugene 6 reagent according to the manufacturer's instructions (Roche, Indianapolis, IN, USA) (21). Five ng of pRL Renilla Luciferase vector (Promega) was co-transfected to control for transfection efficiency. When necessary, an empty pGEM plasmid was used to equalize the total amount of DNA transfected. Cells were collected 24–48 h after transfection and luciferase activity was determined using Dual Luciferase Assay System according to the manufacturer's instructions (Promega).

In vitro circadian assay

Serum shock was used to synchronize molecular clock in C2C12 myotubes *in vitro* using a method previously described (22,23). Briefly, C2C12 myoblasts were grown to confluence and then switched to differentiation medium (DM) consisting of DMEM supplemented with 2% horse serum. DM was changed every 48 h. After 5 days in DM, C2C12 myotubes were treated with 50% horse serum for 2 h, washed with phosphate buffered saline (PBS) and switched to DMEM supplemented with 2% horse serum for the duration of the experiment. At selected time points following serum stimulation, cells were washed with cold PBS and total RNA was isolated from cells using TRIzol reagent (Invitrogen).

MyoD reporter genes

Based on our finding that the CE and not the DRR were bound by CLOCK:BMAL1 we generated a *MyoD* luciferase reporter gene lacking the DRR (CE-4.7*MyoD*) using the CE-6.8*MyoD*:luciferase as a template (4). Approximately 4.7 kb of *MyoD* upstream 5′-flanking sequence was cloned into the pGL3 basic vector (Promega) to replace the 6.8 kb fragment in the CE-6.8MyoD:luciferase reporter. The CE region was amplified from mouse genomic DNA and was subsequently inserted upstream of 4.7 kb sequence to produce CE-4.7*MyoD*:luciferase reporter gene. The primers used for amplification of the 4.7 kb flanking sequence (−4566 to +103) were 5′-CACAAGTGGTTTGAAGTGCTTC-3′ (forward) and 5′-AGGTTCTGTGGGTTGGAATG-3′ (reverse). The primers for amplifying the CE were 5′-AATGCCCAAAGAGTGGCAGA-3′ (forward) and 5′-CCAATCTCAAAGCCCTGGA-3′ (reverse). The DNA sequence of the reporter gene was verified by sequencing.

Mutagenesis

To create the mutant CE-4.7*MyoD*:luciferase reporter gene (CEmt-4.7*MyoD*), mutagenesis was performed with plasmid CE-4.7*MyoD*:luciferase using GeneEditor *in vitro* Site-Directed Mutagenesis System (Promega). The sequence of the oligonucleotide used for mutagenesis of the non-canonical E-box of the *MyoD* CE was 5′-ATCTCCCAGAGTGCTGCGCTTAAAACCCGTG-3′ with the underlined sequence indicating the position of the E-box and the bold letters indicating the mutated bases. The successful mutation of the non-canonical E-box was verified by sequencing.

Real-time PCR

To analyze gene expression levels, 1 μg of RNA was reverse-transcribed to cDNA using Superscript III First Strand Synthesis system (Invitrogen). Real-time PCR was performed using Power SYBR Green PCR Master mix (Applied Biosystems, Carlsbad, CA, USA). The PCR conditions were as follows: 10 min at 95°C, 15 s at 95°C, 60 s at 60°C and 15 s at 95°C. The mRNA levels of target genes were normalized to the corresponding level of *Rpl26* mRNA. The following primers were used: *Rpl26*, 5′-CGAGTCCAGCGAGAGAAGG-3′ and 5′-GCAGTCTTTAATGAAAGCCGTG-3′; *Bmal1*, 5′-GCAGTGCCACTGACTACCAAGA-3′ and 5′-TCCTGGACATTGCATTGCAT-3′; *Per2*, 5′-GGCTTCACCATGCCTGTTGT-3′ and 5′-GGAGTTATTTCGGAGGCAAGTGT-3′; *MyoD*, 5′-ATGGATTACAGCGGCCCC-3′ and 5′-TGTGGAGATGCGCTCCACTA-3′. The specificity of each PCR product was analyzed by electrophoresis and melting curve.

DNA mobility shift assay

Bmal1 and *Clock* expression plasmids were transfected into C2C12 cells and nuclear extracts were prepared as previously described (24). DNA duplexes were synthesized for each of the five E-boxes in the *MyoD* CE. The sequence for each oligonucleotide was as follows:

E-box-1, 5′-GAGCCCCACAGCA TTTGGGGGCATT TATG-3′; E-box-2, 5′-GTAT CCTCCTCCAGCAGCTG GTCACAAAGC-3′; E-box-3, 5′-CACAACACAGCCAG TTGGGGGAAGGGG-3′; E-box-4, 5′-GCGCCCAGA GTCAGCT GTTCCTGGGTCTTC-3′; the non-canonical E-box, 5′-CCCAGAGTGCTCAGCTTAAAACCCG TGAC-3′; the non-canonical E-box mutant, 5′-CCCAG AGTGCTGCTAGGAAAACCCGTGAC-3′ (25). The underlined sequences indicate the position of E-boxes. Duplexes of these DNA oligonucleotides were 5′-end labeled with ^{32}P-γ-ATP using T4 polynucleotide kinase (Promega). DNA mobility shift assays were performed as previously described (26). For super-shift experiment, CLOCK (Abcam ab3517) and BMAL1 Ab (Abcam, ab3350) were used. The protein–DNA complexes were resolved on a 5% polyacrylamide gel with 0.4 × Tris/Borate/EDTA. The images were obtained using a PhosphoImager and scanned using Storm Scanner 860.

Bioluminescent analysis of circadian expression *in vitro*

Stable clones of C2C12 cells ($n = 3$/construct) were established with luciferase based reporter genes containing either the *Bmal1* promoter, CE-4.7*MyoD* or the CE non-canonical E-box mutant CEmt-4.7*MyoD* promoter. The mouse *Bmal1* promoter–luciferase reporter vector was kindly provided by Dr J. Hogenesch (University of Pennsylvania) (27). These reporter genes were individually co-transfected with pCI-neo plasmid into C2C12 myoblast cells. Three selected stable clones for each reporter gene were grown in 35 mm dishes to confluence and differentiated for 5 days in DM. Serum shock is a standard technique used in the circadian field to synchronize the molecular clocks *in vitro* (28–31). In this study, myotubes were serum shocked and then incubated in recording medium which contained 0.1 mM luciferin in phenol red free DMEM with 2% horse serum as previously described (32). Bioluminescence was measured at 10 min intervals for 4 days using the Lumicycle (Actimetrics Inc., Evanston IL) (32). Both raw and baseline subtracted data were analyzed for circadian characteristics including period and phase using Lumicycle Analysis software. The period length of luminescence for each reporter gene was calculated based on the distances between peaks or troughs over the 24–60 h post-serum shock from the same three clones. For these studies, the phase of expression is defined as the time of the first peak 24 h after serum shock. For the bioluminescent studies of muscle tissue, mPER2::LUC homozygous mice were sacrificed by cervical dislocation. Intact soleus muscles were dissected out and incubated in recording medium which contained 0.1 mM luciferin in phenol red free DMEM with 5% fetal bovine serum. Results from the luminescence data (period, phase) were analyzed as described above.

Microarray

For the microarray experiments, we collected gastrocnemius muscles from the CE knockout and wild-type mice ($n = $ three/genotype/time point) at four time points (CT26, CT30, CT38 and CT42). Total RNA was isolated from each muscle using the TRIzol reagent

(Invitrogen) as previously described (18,20). RNA integrity was analyzed using Agilent 2100 Bioanalyzer. For this study, we wanted to determine differences in gene expression and not circadian expression so samples for the array were generated with equal amounts of total RNA/ genotype pooled from the CT26 and CT30 samples, or from the CT38 and CT42 samples with each pooled sample analyzed by a separate Affymetrix chip (430 2.0). For each probe set, we calculated the average expression for the two chips from each genotype. To identify differentially expressed genes we set a cutoff of more than 400 intensity and either a >2-fold increase or >50% decrease in expression between the CE$^{loxP/loxP}$ and wild-type probe sets.

Mitochondrial preparation and measurement of mitochondrial respiration

Skeletal muscle mitochondria were isolated using isolation buffer (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM EGTA and pH is adjusted to 7.2 with KOH) at 4°C as describe earlier (33). Mitochondrial respiration was performed using a miniature Clark-type electrode (Hansatech Instruments, Norfolk, UK) in a sealed, thermostatically controlled chamber at 37°C as described previously (34). Briefly, oxymetric traces were taken by addition of mitochondria to yield a final concentration of ~200–300 μg protein/ml respiration buffer (125 mM KCl, 2 mM MgCl$_2$, 2.5 mM KH$_2$PO$_4$, 20 mM HEPES and 0.1% BSA, pH 7.2). Mitochondrial respiration is initiated by addition of the Complex I, Nicotinamide adenine dinucleotide (NADH)-linked oxidative substrates pyruvate and malate (State 2 respiration). This is followed by addition of ADP which causes flow of protons through the ATP synthase (State 3 respiration) as the added ADP is phosphorylated to ATP. Mitochondria enter State 4 respiration as all the added ADP has been phosphorylated (oligomycin, an inhibitor of the ATP synthase is added to ensure that no protons cross the inner membrane via the ATP synthase). Complex I-State 5 respiration is initiated by the addition of carbonyl cyanide-p-trifluoromethoxyphenylhydrazone, which acts as a protonophore that collapses the proton gradient and result in maximum electron transport and NADH-driven respiration (Complexes I–IV assessed); to assess Complex II, FADH-linked respiration, the Complex I inhibitor rotenone is added to the chamber which completely stops Complex I electron transport and NADH-driven respiration, and the Complex II substrate succinate is added to the chamber to assess Complexes II, III and IV (Succ-State 5). Respiration rate was calculated as nanomoles of oxygen consumed/ min/mg of mitochondrial protein. Two independent oxymetric traces for each sample were taken and respiration rates were averaged to yield *n* = 1/sample.

Statistical analysis

Data are reported as mean ± SEM. To determine if a significant difference existed between treatment and/or groups, one-way ANOVA was performed followed by the Tukey *post-hoc* test to identify specific significant differences between means. Differences between groups were considered significant if $P \leq 0.05$. For circadian analysis of gene expression, we used the JTK_CYCLE program (35). JTK_CYCLE is a non-parametric algorithm developed to determine whether a gene expression pattern can be considered circadian and if so, it will provide an objective analysis of phase and period length.

RESULTS

The *MyoD* CE is required for circadian expression of *MyoD* in vivo

Previously, we showed that BMAL1 and CLOCK bind to the *MyoD* CE but not the *MyoD* DRR, suggesting the CE might be necessary for circadian expression of *MyoD* (4). To test this prediction, we determined the expression of *MyoD* in adult skeletal muscle of the CE$^{loxP/loxP}$ mouse (10). To allow for comparison with our initial study describing the circadian expression of *MyoD*, the CE$^{loxP/loxP}$ mice were backcrossed to C57BL/6J for 10 generations to produce a congenic strain that is considered to be 99.9% C57BL/6J background. These backcrosses were necessary to ensure any differences observed in the temporal pattern of *MyoD* expression could be attributed to the loss of the CE and not genetic background. Since little is known about the behavior of the adult CE$^{loxP/loxP}$ mice, we carried out a series of experiments to characterize the strain before analyzing circadian gene expression. There was no difference in either body weight or muscle weight in comparison to wild-type C57BL/6J mice (data not shown). Analysis of circadian behavior found that adult CE$^{loxP/loxP}$ mice exhibited normal behavior as assessed by wheel running activity (36) under 12L:12D and free running conditions 12D:12D (Supplementary Figure S1). Under free running conditions, CE$^{loxP/loxP}$ mice had an average period length of 23.75 ± 0.28 h compared to 23.57 ± 0.37 h period length for wild-type C57BL/6J mice (Supplementary Figure S1).

Next, we compared the circadian expression of core clock factors, *Bmal1* and *Per2* in tibialis anterior (TA) muscles of the CE$^{loxP/loxP}$ and wild-type mice. To be consistent with previously published circadian gene expression studies, skeletal muscle collection started 30 h after the lights had been turned off, corresponding to CT18 (18,20). First we examined expression of *Bmal1* and *Per2* mRNA in the muscles of the wild-type mice every 4 h over 32 h (9 time points). As shown in Figure 1A, *Bmal1* mRNA (empty circle) levels oscillated, peaking at CT22 and CT50, and *Per2* mRNA (filled triangle) oscillated anti-phase to *Bmal1* with peak expression at CT38. Next we determined *Bmal1* and *Per2* mRNA in the muscle of the CE$^{loxP/loxP}$ mice (Figure 1C). As shown in Figure 1C, expression of *Bmal1* (empty circle) and *Per2* (filled triangle) mRNAs are oscillating across time points however, the amplitude of expression was slightly lower. To statistically evaluate the circadian pattern of gene expression in the muscle of wild-type and CE$^{loxP/loxP}$ mice, we used the JTK_CYCLE
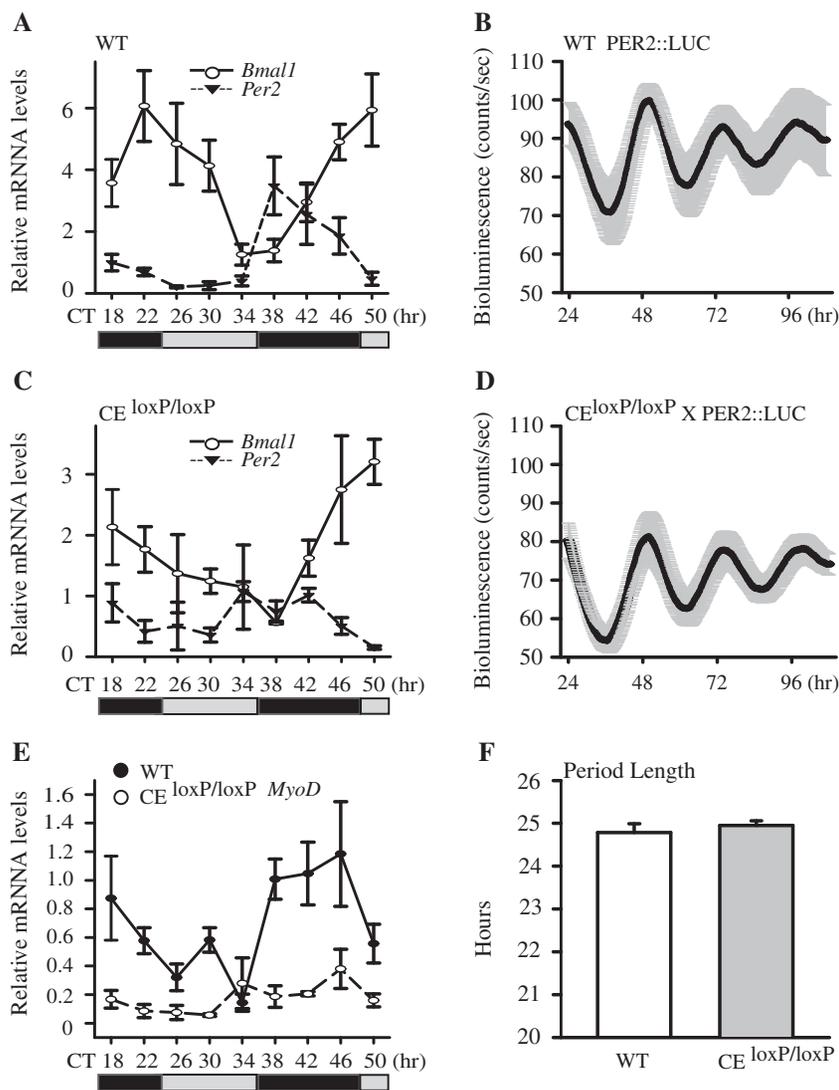
Figure 1. Circadian oscillation of *MyoD* mRNA is dampened in skeletal muscle tissues of the CE$^{loxP/loxP}$ mouse. Adult C57BL/6 J and CE$^{loxP/loxP}$ mice were entrained to 12 h light/12 h dark conditions for 2 weeks. Thirty hours after lights were turned off (CT18), TA muscles were collected every 4 h from CT18 to CT50. Transcript levels were determined by RT–PCR. *Bmal1*, *Per2* and *MyoD* mRNA levels were normalized to *Rpl26* mRNA levels. (**A**) *Bmal1* and *Per2* expression in wild-type mice TA muscle; (**B**) Luminescence recording from *Per2::Luc* soleus muscles. The data for the graphs represent the mean values ± SEM of raw data for five animals. (**C**) *Bmal1* and *Per2* expression in CE$^{loxP/loxP}$ mice TA muscle; (**D**) Luminescence recordings from *Per2::Luc* × CE$^{loxP/loxP}$ soleus muscles. The data for the graphs represent the mean values ± SEM of raw data for five animals. (**E**) *MyoD* expression in wild-type and CE$^{loxP/loxP}$ mice TA muscle; (**F**) Average period length data of bioluminescence from *Per2::Luc* and *Per2::Luc* × CE$^{loxP/loxP}$ soleus muscles. The graphs in (A, C and E) display the results as the mean value ± SEM for three independent samples for each genotype. The gray bars indicate the subjective circadian light period and the black bars indicate subjective circadian dark period. For each respective gene, one-way ANOVA followed by Tukey's *post-hoc* test was performed to determine significant differences across all time points. For circadian analysis of gene expression, the JTK_CYCLE program was used.

program (35). Using this analysis, we confirmed that *Bmal1* and *Per2* in both wild-type and the CE KO mice muscle were expressed in a circadian manner with $P < 0.05$ (Supplementary Table S1).

To more rigorously evaluate molecular clock function in the muscle of CE$^{loxP/loxP}$ mice we employed real-time bioluminescent analysis using the Lumicycle (Actimetrics). Previous studies have shown that the bioluminescence rhythm obtained from tissue explants from the circadian reporter *Per2::Luc* mouse matches endogenous PER2 protein *in vivo* (19,32,37). We crossed the CE$^{loxP/loxP}$ mice with *Per2::Luc* mice and produced homozygous

mice harboring both the CE$^{loxP/loxP}$ and *Per2::Luc*. For these studies both *Per2::Luc* and the *Per2::Luc* × CE$^{loxP/loxP}$ mice were entrained for 2 weeks. At that time, the mice were sacrificed and soleus muscles were collected and placed in culture with recording media plus luciferin and real-time bioluminescence was recorded every 10 min for 4 days. The power of this experimental approach for circadian analysis is that it allows us to follow PER2 expression for each sample over a longer period of time with much higher temporal frequency (once every 10 min versus tissue collections every 4 h). The increased sampling frequency and longer duration permits a more robust

quantitative analysis of circadian expression with the capacity to more precisely define important characteristics including period length and phase.

Raw data are presented in Figure 1B (*Per2::Luc*) and Figure 1D (*Per2::Luc* × $CE^{loxP/loxP}$) represent the mean ± SEM luminescence recordings averaged for all five explants per genotype. Compared to *Per2::Luc*, the average level of *Per2::Luc* × $CE^{loxP/loxP}$ bioluminescence is slightly reduced. However baseline subtracted data are basically the same for *Per2::Luc* and the *Per2::Luc* × $CE^{loxP/loxP}$ tissues (Supplementary Figure S2). Analysis of circadian parameters determined that there were no significant differences for either the period length (Figure 1F) or the phase between the muscles from *Per2::Luc* × $CE^{loxP/loxP}$ and the *Per2::Luc*.

Once we confirmed the molecular clock functions properly in the muscle of the $CE^{loxP/loxP}$ mice we tested whether loss of the CE affects expression of *MyoD* mRNA. Consistent with our previous findings in the gastrocnemius muscle, *MyoD* mRNA levels oscillated in the TA muscle of wild-type mice with peak expression from CT38 to CT46 (Figure 1E, filled circle) (4). We then examined expression of *MyoD* mRNA in the TA muscle of the $CE^{loxP/loxP}$ mice. As illustrated in Figure 1E (empty circle), the expression pattern of *MyoD* mRNA was significantly dampened. Analysis of *MyoD* expression using JTK_CYCLE confirmed that expression was oscillating in a circadian manner in the wild-type mice but was not circadian in the muscle of the $CE^{loxP/loxP}$ mice. These results confirm that the CE is necessary for circadian expression of *MyoD in vivo*. We also noted that while expression of *MyoD* in the $CE^{loxP/loxP}$ mice was reduced compared to wild-type across most time points there was no difference in expression between the two genotypes at the trough of *MyoD* expression, CT34 suggesting that the lower expression of *MyoD* reported previously maybe more related to time of analysis.

The non-canonical E-box in the *MyoD* CE is required for BMAL1 and CLOCK Binding and activation

The nucleotide sequence of the CE is highly conserved across mouse, rat and human genomes (∼89–98%). There are four canonical E-boxes (5′-CANNTG-3′) in the *MyoD* CE as previously described (13,38). The canonical E-box sequences from upstream to downstream are CATTTG (E1), CAGCTG (E2), CAGTTG (E3) and CAGCTG (E4) (Supplementary Figure S3). All four E-boxes are identical in the rat and mouse whereas only E-boxes E2 and E4 are conserved in human. In addition to these four canonical E-boxes, there was one non-canonical E-box (5′-CAGCTT-3′) that is located between E2 and E3 (indicated by hash). The sequence of the non-canonical E-box and its flanking sequences are conserved across mouse, rat and human *MyoD* CE sequences (Supplementary Figure S3). Interestingly, the non-canonical E-box is similar to the BMAL1:CLOCK binding site (5′-CACGTT-3′) found within the promoter of the core clock gene *Per2* (39).

Our previous work demonstrated that BMAL1 and CLOCK can bind to the *MyoD* CE using chromatin

immunoprecipitation assays with nuclear extracts from mouse adult skeletal muscle (4). In order to determine if CLOCK and BMAL1 proteins were capable of binding any of the aforementioned E-boxes we performed electrophoretic mobility shift assay (EMSA) experiments. The binding reaction contained one of four different E-box oligonucleotide probes and nuclear extracts derived from C2C12 myoblasts that were transfected with *Bmal1* and *Clock* expression plasmids. Figure 2A shows representative images from EMSA using each of the canonical E-box probes. For each probe, protein:DNA complexes were detected and shown to be specific as indicated by successful competition with excess cold oligonucleotide competitor (Figure 2A). While the protein:DNA complexes were specific, none of these complexes contained CLOCK as incubation with a CLOCK antibody failed to deplete or super-shift the observed protein:DNA complexes.

We next tested whether the non-canonical E-box located between E2 and E3 within the CE was capable of binding CLOCK and/or BMAL1. Figure 2B shows that the non-canonical E-box formed a protein:DNA complex that contained both CLOCK and BMAL1. The addition of either CLOCK (left panel) or BMAL1 (middle panel) antibody to the binding reaction was able to prevent formation of the protein:DNA complex whereas addition of control IgG did not alter binding. The specificity of the E-box for CLOCK and BMAL1 binding was further confirmed by mutagenesis. EMSA performed with a mutated form of the non-canonical E-box resulted in two binding complexes that were not altered by addition of either the CLOCK antibody or the BMAL1 antibody to the binding reaction (Figure 2B, right panel). Taken together, these results demonstrate that CLOCK and BMAL1 bind to the non-canonical E-box in the *MyoD* CE.

The EMSA results demonstrated that both CLOCK and BMAL1 bind to the non-canonical E-box (5′-CAGCTT-3′) so our next set of experiments tested whether this E-box was functional *in vitro*. We generated the reporter construct, CEmt-4.7*MyoD*:luciferase and performed transient co-transfection experiments in C2C12 cells. We found that transfection of the CE-4.7*MyoD*:luciferase reporter gene with *Clock* plus *Bmal1* expression vectors resulted in a 2.5-fold increase of CE-4.7*MyoD*:luciferase expression (Figure 2C). In contrast, over-expression of both *Clock* and *Bmal1* did not result in an activation of the CEmt-4.7*MyoD*:luciferase reporter gene. We concluded from these transfection experiments that the non-canonical E-box within the CE is functional and required for full activation of *MyoD* reporter by BMAL1 and CLOCK.

The *MyoD* CE is required for circadian expression of the *MyoD* reporter gene in C2C12 myotubes

Having established that the CE is required for *MyoD* oscillation *in vivo*, and that the non-canonical Ebox binds CLOCK and BMAL1, we next wanted to determine whether this E-box could confer circadian oscillation using an *in vitro* assay. This required the validation of a serum shock cell culture model for evaluating molecular
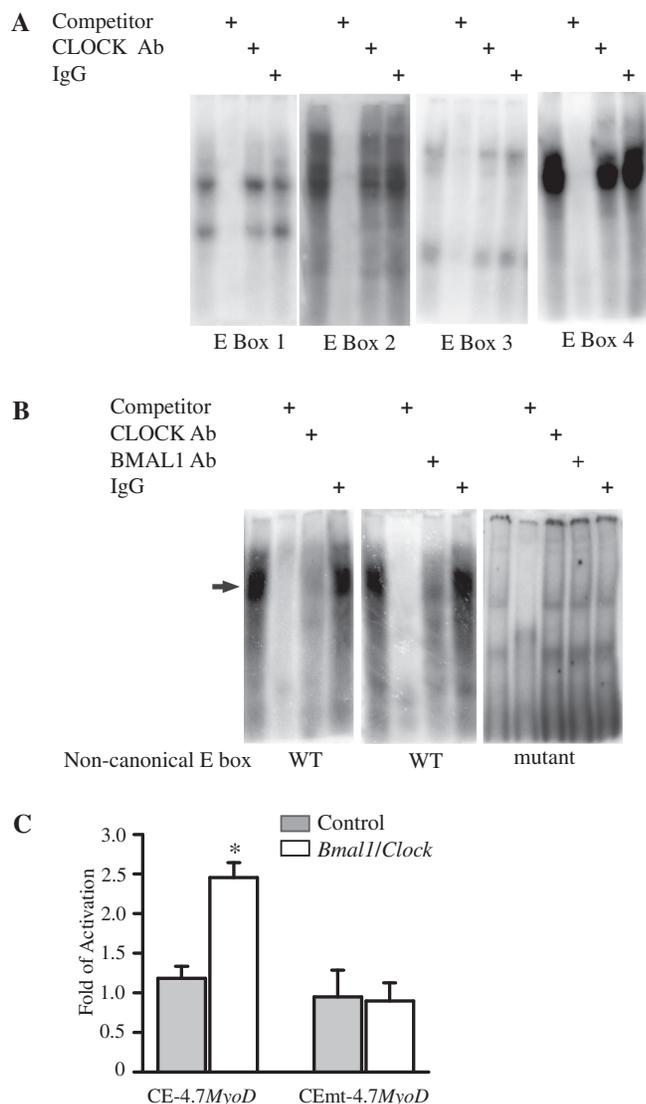
Figure 2. The non-canonical E-box of the *MyoD* CE is required for CLOCK and BMAL1 binding and activation of the *MyoD* promoter by BMAL1: CLOCK. EMSA analysis of CLOCK and BMAL1 binding at the E-boxes located within the *MyoD* CE was performed with nuclear extract derived from C2C12 cells over-expressing *Clock* and *Bmal1* and an oligonucleotide probe containing one of the five CE E-boxes. (**A**) The DNA:protein complexes formed using a probe containing one of the canonical E-boxes (E1, E2, E3 or E4). (**B**) The DNA:protein complexes formed using the non-canonical E-box and the mutant of the non-canonical E-box. The arrow indicates the specific DNA:protein complex that contained CLOCK and BMAL1. (**C**) A wild-type (CE-4.7*MyoD*:luciferase) or mutant (CEmt-4.7*MyoD*:luciferase) reporter gene construct was transiently co-transfected with *Clock* plus *Bmal1* expression plasmids into C2C12 myoblasts. Luciferase activity was measured 24–48 h later. The histogram displays the results from three independent experiments with values presented as mean ± SEM. A one-way ANOVA test indicated CE-4.7*MyoD*:luciferase reporter gene activation by CLOCK plus BMAL1 (empty bar) was significantly greater than control (gray bar) denoted by asterisks.

clock factor expression in C2C12 muscle cells. Serum shock is commonly used to synchronize molecular clocks across cells *in vitro*. This is necessary to allow for objective analysis of circadian gene expression taking into account

the proper pattern (cosinar) and temporal oscillations (~24 h). To determine whether serum stimulation is sufficient to synchronize circadian gene expression in C2C12 myotubes we evaluated expression of *Bmal1* mRNA using real-time PCR. Myotube cultures were serum shocked and samples were collected every 6 h for 54 h. As shown in Figure 3A, *Bmal1* mRNA expression varies over time following serum stimulation. We found that the peak and trough expression of *Bmal1* mRNA was separated by 12 h (18 versus 30 h, 30 versus 42 h) and the difference in expression between peak and trough was statistically significant ($P < 0.05$). Likewise, endogenous *MyoD* mRNA expression showed an oscillation in expression with the peak and trough levels occurring 12 h apart (Figure 3B). In addition, the pattern of *Bmal1* and *MyoD* oscillations were anti-phase and this is similar to the pattern observed *in vivo* and consistent with *MyoD* expression being regulated by the clock factors, BMAL1 and CLOCK (4). Use of JTK-CYCLE software also confirmed these observations that expression of endogenous *Bmal1* and *MyoD* mRNAs in myotubes following serum shock were circadian (Supplementary Table S2).

To more quantitatively evaluate the necessity of the non-canonical E-box for the circadian expression of *MyoD*, we employed real-time bioluminescent analysis using the Lumicycle. For this set of experiments, we generated stable C2C12 cells lines containing either the *Bmal1*:luciferase reporter gene, CE-4.7*MyoD*:luciferase reporter gene or CEmt-4.7*MyoD*:luciferase reporter gene. C2C12 stable clones (n = three/reporter) were differentiated into myotubes for 5 days and serum shocked as previously described. The medium was then replaced with non-phenol red DMEM containing luciferin plus 2% horse serum. Light emission was captured at 10 min intervals in real time using the Lumicycle for 4 days. Raw data for each clone was baseline subtracted and the data for the graphs in Figure 4 represent the mean values ± SEM for three independent clones for the reporter gene.

We first evaluated expression of the *Bmal1* reporter gene in C2C12 myotubes. Consistent with our real-time PCR analysis (Figure 3A), the results of these experiments showed that the *Bmal1* promoter directs circadian expression of luciferase (Figure 4A). As seen in Figure 4, the circadian amplitude damped gradually with time resulting from the depletion of nutrients and luciferin as replacement with fresh medium was able to restore the amplitude (data not shown).

Next, we tested whether the CE-4.7*MyoD*:luciferase reporter gene directed circadian expression. As shown in Figure 4B, there was a robust oscillation. In contrast, the CEmt-4.7*MyoD*:luciferase reporter gene expression (Figure 4C) was greatly dampened. JTK analysis determined that *Bmal1*:luciferase and CE-4.7*MyoD*: luciferase were circadian, but CEmt-4.7*MyoD*:luciferase was not expressed in a circadian manner (Supplementary Table S2). These results provide further evidence that the non-canonical E-box is the *cis*-regulatory element within the CE that confers circadian rhythmicity to the *MyoD* promoter.
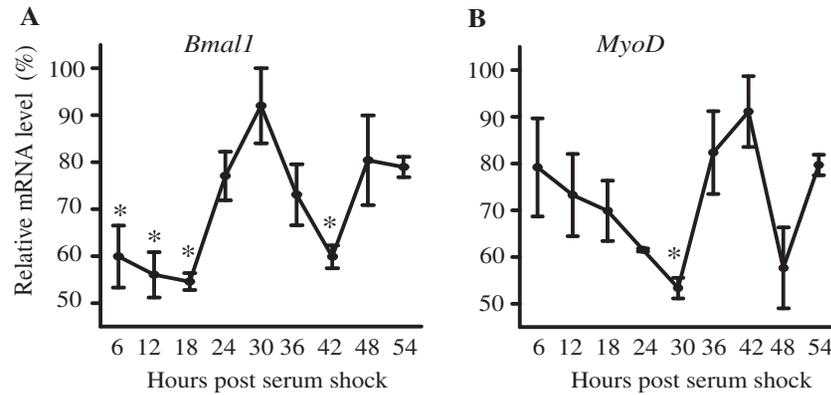
Figure 3. Serum shock is sufficient to synchronize circadian gene expression in C2C12 myotubes. Stable clones of C2C12 cells (CE-4.7*MyoD*) were differentiated for 5 days prior to serum shock. RNA was isolated at the indicated time post-serum shock and used to determine the expression level of *Bmal1* and *MyoD* by RT-PCR. The target transcript levels were normalized to *Rpl26* transcript levels and then normalized to the highest point across all time points. The data for the graphs represent the mean values ± SEM for three independent clones for the reporter gene. The expression of endogenous *Bmal1* (**A**), endogenous *MyoD* (**B**) all displayed a circadian oscillation as indicated by significant differences in peak and trough expression. For each respective gene, one-way ANOVA followed by Tukey *post-hoc* test identified significant difference ($P < 0.05$) between peak expression and other time points as indicated by an asterisk. For circadian analysis of gene expression the JTK_CYCLE program was used.
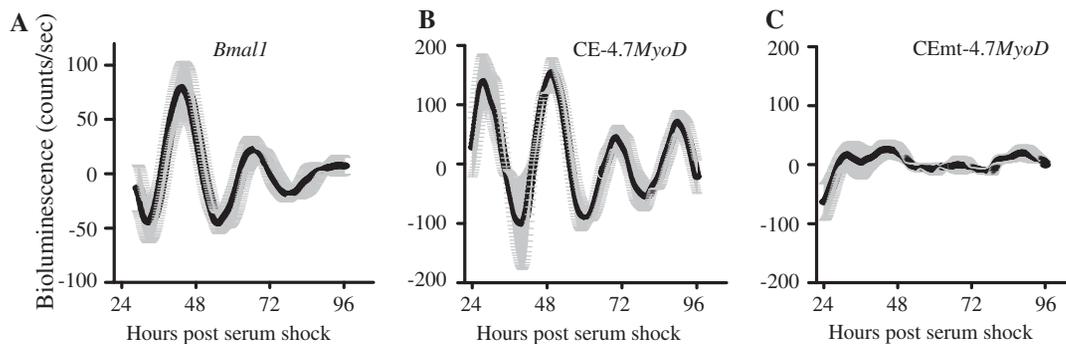
Figure 4. Mutation of the *MyoD* CE non-canonical E-box disrupts circadian oscillation in bioluminescent assay. Stable clones of C2C12 cells (carrying the *Bmal1* promoter:luciferase, CE-4.7*MyoD*:luciferase or CEmt-4.7*MyoD*:luciferase reporter) were differentiated for 5 days prior to serum shock. Then the cultures were incubated in Lumicycle at 37°C and bioluminescence was recorded. The graphs shown here are baseline subtracted results as the mean value ± SEM from three independent clones for the reporter gene. (**A**) *Bmal1* promoter:luciferase reporter. (**B**) CE-4.7*MyoD*:luciferase reporter. (**C**) CEmt-4.7*MyoD*:luciferase reporter. The graphs display the results as the mean value ± SEM from three independent clones for each reporter. For circadian analysis of gene expression, the JTK_CYCLE program was used.

Differentially expressed genes in skeletal muscle of the CE$^{\text{loxP/loxP}}$ mice are also mis-expressed in the *Clock*$^{\Delta19}$ mutant muscle

Similar to the CE$^{\text{loxP/loxP}}$ mice, we previously reported a loss of *MyoD* circadian expression in the *Clock*$^{\Delta19}$ mutant strain and the *Bmal1* knockout strain (4,18). The mis-expression of *MyoD* in skeletal muscle of *Clock*$^{\Delta19}$ mutant was associated with the down-regulation of muscle-specific genes, many of which are known *MyoD* target genes. To determine if a comparable response in gene expression occurred in the muscle of CE$^{\text{loxP/loxP}}$ mice, we performed a microarray analysis of RNA from gastrocnemius muscles of adult mice. Using the criteria (either 2-fold increased or 50% decreased) outlined in 'Materials and Methods' section, we identified 1271 genes that were differentially expressed between wild-type and CE$^{\text{loxP/loxP}}$ muscle with 84% of the genes showing decreased expression in the CE$^{\text{loxP/loxP}}$

(Supplementary Table S3, in black). Gene ontology classification using DAVID (http://david.abcc.ncifcrf.gov/) revealed enrichment for genes related to contractile fiber (30 genes), endoplasmic reticulum (105 genes), cytosol (70 genes) and mitochondrion (134 genes) (Supplementary Table S6A). The expression of a number of skeletal muscle-specific genes were down-regulated in the muscle of the CE$^{\text{loxP/loxP}}$ mice including alpha actinin 3 (*Actn3*), titin (*Ttn*), dihydropyridine receptor, calcium channel, voltage-dependent calcium channel (Cacnb1) and calpain 3 (*Capn3*).

We next interrogated a ChIP-Seq derived *MyoD* target database to determine whether any of the mis-expressed genes were *MyoD* target genes (40). This analysis identified 461 genes (36.3%) that were scored as *MyoD* target genes according to the ChIP-Seq database (Supplementary Table S4). Among the 461 genes, Gene ontology classification using DAVID revealed enrichment

for genes related to sarcomere (13 genes), cytosol (35 genes) and mitochondrion (59 genes) (Supplementary Table S6B).

We also interrogated the differentially expressed genes from the CE$^{loxP/loxP}$ data set versus the data set reported for the *Clock*$^{\Delta 19}$ skeletal muscle (18). This analysis revealed that 207 genes altered in the muscle of the CE$^{loxP/loxP}$ mice are also altered in *Clock*$^{\Delta 19}$ mice (Supplementary Table S5). Among the 207 genes, we found 177 (86%) genes (Supplementary Table S5, in black) are down-regulated. Gene ontology classification using DAVID revealed enrichment for genes related to sarcomere (seven genes), cytosol (17 genes) and mitochondrion (26 genes) (Supplementary Table S6C). The overlapping genes include several muscle specific genes, for example, *Myl3*, *Myo18a*, *Ttn*, *Tpm1*, *Tpm2*, *Myoz2* and *Myom2* (Supplementary Table S5). These results suggest that genes differentially expressed in the muscle of the CE$^{loxP/loxP}$ mice could be downstream of the BMAL1:CLOCK to *MyoD* circadian transcriptional loop.

Respiratory function is reduced in the mitochondria isolated from muscle of CE$^{loxP/loxP}$ mice

The gene ontology analysis indicated that many genes related to mitochondria are altered in the CE$^{loxP/loxP}$ mouse muscle. We analyzed the respiratory function of mitochondria isolated from 5- to 6-month-old wild-type versus CE$^{loxP/loxP}$ mice to determine whether the changes in gene expression were associated with any functional effect. As shown in Figure 5, we found no significant differences in States 2, 3 and 4 respiration rates between wild-type and CE$^{loxP/loxP}$ muscle. However, we found that there was a significant difference in Complexes I and II driven maximum electron transport (Complex I-State 5 and succinate State 5 respiration rates). Specifically, we found that both Complexes I and II of State 5 respiration rates were reduced. In Complex I, NADH dehydrogenase catalyzes the electron transfer from NADH to
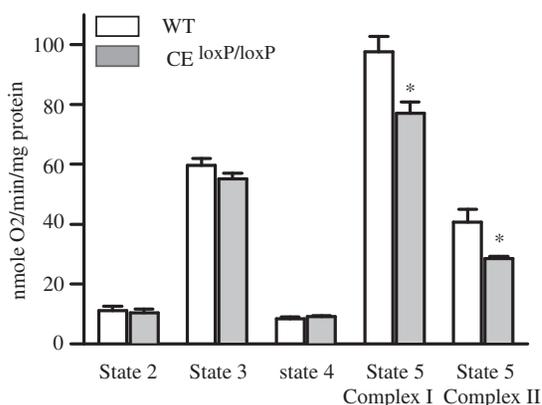
Figure 5. Respiration rates are lower in the mitochondria of the CE$^{loxP/loxP}$ than in wild-type. Gastrocnemius muscles from 5- to 6-month-old mice were isolated from wild-type (empty bars) or the CE$^{loxP/loxP}$ (gray bars) and used for the determination of respiration rates. The data for the graphs represent the mean values ± SEM for four animals.

Ubiquinone (Q) and in Complex II succinate dehydrogenase catalyzes electron transfer from succinate to Ubiquinone (Q) via FAD. The changes in respiration were consistent with our data from expression analysis as level of *Sdhc* mRNA, which encodes a subunit of succinate dehydrogenase is down-regulated by 67% in the CE$^{loxP/loxP}$ muscle. These findings demonstrate that loss of the CE of *MyoD* is associated with a disruption in mitochondrial function.

DISCUSSION

The main finding from this study was the identification of a novel non-canonical E-box in the CE of the *MyoD* promoter that is necessary for CLOCK:BMAL1 binding and confers circadian expression. The deletion of the CE abolished circadian expression of *MyoD* mRNA *in vivo* and transfection assays demonstrated that mutation of the E-box abolished the circadian expression *in vitro*. Of the E-boxes located within the CE, only the non-canonical E-box was capable of binding the core clock factors CLOCK and BMAL1. However, previous studies have shown that the canonical E-boxes in the CE are required for *MyoD* expression in developing skeletal muscle (9). Conclusions from this work were that the E-box 1 region is essential for basal *MyoD* expression while the E-box 4 region defines the CE as a target for Myf5-dependent activation of *MyoD* in myotomal muscle. Thus, our current findings expand the role of the CE in the regulation of *MyoD* expression to include BMAL1 and CLOCK binding and activation in adult skeletal muscle.

The finding that CLOCK and BMAL1 are capable of binding to a non-canonical E-box (5′-CAGCTT-3′) within the CE is not without precedent as these factors have been shown to bind a range of different E-box sequences (41,42). For instance, the CLOCK:BMAL1 heterodimer has been shown to regulate the circadian expression of *Per1* via a canonical E-box (5′-CACGTG-3′) while analysis of the promoter of the clock-controlled gene, *Dbp*, demonstrated BMAL1 and CLOCK binding at multiple E-boxes, including one non-canonical E-box important for circadian expression (26). In *Per2*, the BMAL1: CLOCK heterodimer binds to an E-box-like sequence (5′-CACGTT-3′) that was shown to be sufficient and indispensable for the circadian oscillation of *Per2* expression (39,43). The same non-canonical E-box was also shown to have circadian function in another clock-controlled gene, bHLH family member e41 (*Bhlhe41*) (44). In cryptochrome 1 (*Cry1*), a 47 bp DNA fragment containing a canonical E-box and a non-canonical E-box, both E-boxes were required and sufficient to drive *Cry1* circadian expression with the appropriate phase (38,44). Collectively, these findings clearly show that both canonical and non-canonical like E-boxes we identified in the *MyoD* CE are commonly found as targets for BMAL1 and CLOCK binding.

The loss of circadian *MyoD* expression seen in the muscle of the CE$^{loxP/loxP}$ mouse clearly demonstrated that this regulatory region was necessary for conferring

a circadian pattern of expression in adult muscle. This finding was somewhat surprising given that previous studies had shown that the DRR (distal regulatory region) was the enhancer responsible for regulating *MyoD* expression in adult skeletal muscle (8,11). The necessity of the CE for the circadian expression of *MyoD*, however, was consistent with our previous ChIP results showing that CLOCK and BMAL1 bound only the CE, not the DRR, in a diurnal manner (4). However this does not exclude the possibility that DRR and other elements within the *MyoD* promoter, which may also contribute to proper circadian *MyoD* expression. The CE and the DRR enhancers have been shown to exhibit complementary activities and together are able to recapitulate *MyoD* expression in developing and mature skeletal muscle (8,11,45,46,). The molecular mechanisms are not well defined at this time and future studies will be required to delineate all the necessary elements that cooperate to direct the proper *MyoD* circadian expression.

Since circadian *MyoD* mRNA expression is disrupted in the $CE^{loxP/loxP}$ mice, we used expression profiling to ask whether there was an alteration in muscle gene expression and if so, whether any of the changes were in common with those defined for the muscle of the $Clock^{\Delta19}$ mouse. This concept emerged from our earlier work using the $Clock^{\Delta19}$ mouse in which the loss of *MyoD* cycling, but not loss of *MyoD* expression, was associated with mis-expression of many muscle-specific genes. Furthermore, in agreement with the $Clock^{\Delta19}$ findings, the expression level of some of the same muscle-specific genes *(Ttn, Tpm1, Tpm2, Myom2)* were found to be mis-expressed in the $CE^{loxP/loxP}$ mice. While the downstream consequences of this alteration in *MyoD* target gene expression remains to be fully explored, preliminary studies indicate the muscle from the $CE^{loxP/loxP}$ mice does not phenocopy the $Clock^{\Delta19}$ in terms of mechanical function (Zhang, X., Wolff, G. and Esser, K.A., unpublished data). The reason for a lack of a muscle phenotype in the $CE^{loxP/loxP}$ mice at 4 months of age remains to be determined but may be an issue of time and/or age—it may take longer for the muscle phenotype to emerge in the $CE^{loxP/loxP}$ mice than in $Clock^{\Delta19}$ mouse. This is a reasonable hypothesis given that the muscle pathologies seen in the $Clock^{\Delta19}$ mouse occur in a system in which all cells/tissues exhibit circadian misalignment due to the mutation in *Clock*. Future studies will test whether long-term loss of *MyoD* oscillation in the $CE^{loxP/loxP}$ mice leads to downstream alterations associated with changes in the myogenic expression program.

Previously, we reported decreased mitochondrial volume and depressed respiratory function of muscle from circadian mutant *Bmal1* KO and $Clock^{\Delta19}$ mice. However in *MyoD* KO mouse muscle, we did not detect any decrease in mitochondrial volume based on analysis of electron micrographs (4). In this study, we evaluated mitochondrial respiration in the muscle of the $CE^{loxP/loxP}$ mice based on our expression profiling data. We found a significant reduction in State 5 which is consistent with the array results but was not what we expected based on our morphological analysis of mitochondria from the *MyoD* KO mouse. One explanation for this difference is

that we did not measure mitochondrial respiration in the muscle of the *MyoD* KO mouse, thus while the volume was unchanged the biochemical capacity was not assessed. Additionally, previous studies have reported that the loss of *MyoD* is compensated by other functionally redundant genes, such as *Myf5*, and this could act to preserve mitochondrial volume. In the muscle of the $CE^{loxP/loxP}$ mice we do not see any changes in expression of the other myogenic regulatory factor family. This leads us to suggest that circadian expression of *MyoD* in the adult mouse is important for the regulation of mitochondrial function.

In conclusion, we have identified a new function for the CE of *MyoD* in adult skeletal muscle. We determined that the CE was required for *MyoD* circadian expression *in vivo*. We also found that a novel non-canonical E-box located within the CE was required for the full activation of *MyoD* expression as well as its circadian expression. The loss of the *MyoD* circadian expression is associated with significant changes in expression of many *MyoD* target genes and disrupted mitochondrial function. These results provide evidence that the molecular clock in skeletal muscle is important for daily regulation of myogenic gene expression and contributes to the maintenance of mitochondrial/metabolic function of adult skeletal muscle.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1–6 and Supplementary Figures 1–3.

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