THE SKELETAL MUSCLE MOLECULAR CLOCK REGULATES THE TIMING OF SUBSTRATE METABOLISM AND THE CIRCADIAN EXPRESSION OF TITIN-CAP

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THE SKELETAL MUSCLE MOLECULAR CLOCK REGULATES THE TIMING OF SUBSTRATE METABOLISM AND THE CIRCADIAN EXPRESSION OF TITIN-CAP.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By
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ABSTRACT OF DISSERTATION

THE SKELETAL MUSCLE MOLECULAR CLOCK REGULATES THE TIMING OF SUBSTRATE METABOLISM AND THE CIRCADIAN EXPRESSION OF TITIN-CAP

Skeletal muscle is a major contributor to whole-body metabolism as it serves as a depot for both glucose and amino acids, and is a highly metabolically active tissue. An intrinsic molecular clock mechanism exists within skeletal muscle that regulates the timing of physiological processes. A key function of the clock is to regulate the timing of metabolic processes to anticipate time of day changes in environmental conditions. The purpose of this study was to identify metabolic genes that are expressed in a circadian manner and determine if these genes are regulated downstream of the intrinsic molecular clock by assaying gene expression in an inducible skeletal muscle-specific Bmal1 knockout mouse model (iMS-Bmal1−/−). The skeletal muscle circadian transcriptome we analyzed was highly enriched for metabolic processes. Acrophase (time of peak expression) analysis of circadian metabolic genes revealed a temporal separation of genes involved in substrate utilization and storage over a 24-h period with many differentially expressed in the skeletal muscle of the iMS-Bmal1−/− mice compared to wildtype. However, the iMS-Bmal1−/− mice displayed circadian behavioral rhythms indistinguishable from iMS-Bmal1+/+ mice. We also observed a gene signature indicative of a fast to slow fiber-type shift and a more oxidative skeletal muscle in the iMS-Bmal1−/− model. These data provide evidence that the intrinsic molecular clock in skeletal muscle temporally regulates genes involved in the utilization and storage of substrates independent of circadian activity. Disruption of this mechanism caused by phase shifts (that is, social jetlag) or night eating may ultimately diminish skeletal muscle's ability to efficiently maintain metabolic homeostasis over a 24-h period.

The molecular-clock targets genes for circadian expression in a tissue-specific manner, possibly through interactions with tissue-specific factors. In order to identify novel mechanisms responsible for driving circadian gene expression of
muscle-specific genes we focused our study on the molecular regulation of the *Titin-cap* gene. We choose this gene as it was highly circadian in the skeletal muscle circadian transcriptome, and has previously been shown to be modulated by the clock factor BMAL1 in heart-tissue, and the myogenic regulatory factor MYOD1 in skeletal-muscle. Promoter-reporter experiments demonstrated that BMAL1:CLOCK and MYOD1 work in a synergistic fashion to transactivate the *Titin-cap* gene in skeletal-muscle. Circadian expression of *Titin-cap* relied on the normal function of MYOD1 as mutant vectors altered the rhythmic oscillation and expression. We provided evidence that BMAL1 and MYOD1 bind to a tandem E-Box element in the proximal promoter element, and that this element is required for the circadian expression of *Titin-cap* in skeletal-muscle. These data provide a novel mechanism in which the molecular-clock works with a tissue specific transcription factor to drive circadian gene expression.

KEYWORDS: Circadian; Molecular-clock; Skeletal muscle; Metabolism; Bmal1; Myod1

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THE SKELETAL MUSCLE MOLECULAR CLOCK REGULATES THE TIMING OF SUBSTRATE METABOLISM AND THE CIRCADIAN EXPRESSION OF TITIN-CAP

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Chapter 1: Introduction

Throughout the history of life on Earth, organisms have experienced the diurnal periodicity of light and dark caused by the axial rotation of the Earth around the sun.[1-3] Pressures caused by daily oscillations in the environment have favored the evolution of organisms that have adopted 24-hour rhythmic cycles, or circadian rhythms.[4-6] The term ‘circadian’ stems from the Latin words ‘Circa’ and ‘diem’, and can be roughly translated to “about a day”. Numerous circadian descriptors are commonly used to define the patterns of these rhythms, and most common of which include ‘phase’, ‘amplitude’, and ‘period' (Figure 1).[7] The phase of the rhythm denotes a particular time within the rhythm and is most often associated with the peak or highest point of the rhythm. Classically, phase is often used to describe ‘circadian time’ (CT) where ‘CT 0’ denotes the beginning of the inactive period, while ‘CT 12’ denotes the beginning of the active period. The amplitude of the rhythm describes the difference between the peak/trough of the rhythm and the calculated mean value. Typically, the amplitude of the rhythm is most often interpreted as the strength or robustness of the rhythm.[8-12] Lastly, the period of the rhythm describes the length in time it takes to complete one full cycle.[9]

1.1 Circadian Rhythms and Health

Growing evidence supports the association between circadian rhythms and human health.[13] Multiple physiological processes display robust circadian oscillations throughout the day and include fluctuations in core-body temperature, metabolic flux, hormone release, immune function, sleep-wake cycles, and athletic
**Figure 1.** Representative diagram of the circadian descriptors from a typical rhythm.
Disruptions in circadian rhythms and/or mutations in the genes that regulate the body's circadian clock have been linked to sleep disorders, obesity, diabetes, psychological disorders (e.g. sleep disorders and bipolar disorder), and others.[20-25] Recent advances in circadian bioinformatics have led to the identification of the circadian transcriptional landscape in a variety of mammalian tissues. These studies have identified many circadian transcripts that encode proteins that are known drug targets. These findings suggest that the efficacy of many drugs may fluctuate throughout the day.[26-28] Surprisingly, Hogenesch et al. reported that 56 of the 100 top-selling drugs target proteins encoded by circadian genes.[27] Identifying ways to promote normal circadian rhythms hold promise in treating disease and furthering human health.

1.2 Molecular-Clock Background
Circadian rhythms are generated by an intrinsic molecular clock mechanism that has remained highly conserved throughout evolution, and is present in all most all cellular life-forms on earth including unicellular protozoa, insects, vertebrates, plants, and mammals.[29-35] These clocks serve to align behavior and physiological processes with the rising and setting of the sun, and thus allow organisms to more efficiently anticipate, and adapt to environmental changes (nutritional availability, predation, etc.) that occur throughout the day.[36] A fundamental component of circadian rhythms is the cell-autonomous nature in which they exist. In other words, circadian rhythms are intrinsic, and do not require an external stimulus to provoke a response, as is most often observed in physiological systems, but exist in
robust fashion even in the absence of environmental stimuli.[1, 37, 38] The notion that circadian rhythms are not a mere byproduct of exogenous time-cues was a long-standing topic of debate in the field of chronobiology, originating from what some claim to be the very first circadian study. In 1792 a French astronomer, Jean-Jacques d'Ortous de Mairan, found himself very interested in the heliotrope plant, because of the daily opening and closing of its leaves.[39] With no general knowledge of circadian rhythms, Mairan hypothesized that the leaves opened everyday in response to sunlight. He then tested this idea by recording the leaf activity when the plant was placed in constant darkness. To his surprise, the leaves opened during the day then closed again at night in the absence of light cues. Mairan was not convinced that all possible confounding environmental factors were properly controlled for, and suggested that light-leak was the most likely scientific explanation. Chronobiologists have since repeated this experiment with proper controls, and put to rest the notion that circadian rhythms are a direct response of exogenous time-cues, although light and other environmental cues help to entrain circadian rhythms. The birth of modern molecular genetic approaches and the use of forward-genetic screens have allowed investigators to identify the molecular components responsible for keeping biological time and has transformed the field of circadian rhythms.
1.2.1 Molecular-Clock Mechanism

Circadian rhythms in behavioral and physiology are generated by what is commonly referred to as the ‘molecular-clock’. [40] This machinery is genetically programmed within the majority of cells in the body, and exist in a highly organized and hierarchal manner. [41] The master-clock, located in the region of the brain known as the suprachiasmatic nucleus (SCN), processes light information from the retinohypothalamic tract to synchronize its internal clock to the light:dark cycle. [42-44] The SCN serves to relay light information to the clocks in the peripheral tissues via multiple entrainment pathways by temporally regulating hormone release, the autonomic nervous system, core body temperature, and behavior (feeding, activity). [45] In effect, the SCN synchronizes the peripheral clocks so they run in concert, and loss of a functional SCN results in the di-synchrony of the peripheral clocks. [46] The peripheral clocks can sense environmental and systemic cues and adjust (i.e. phase-shift) their rhythms to temporally align with the environment. [47] For instance, the clocks in skeletal muscle can be entrained by time of feeding and/or exercise. Social jet-lag, or misaligning the body’s internal clock with social time, has become increasingly common in our modern society and has been associated with detrimental health effects. [48] The molecular-clock’s ability to phase-shift provides additional flexibility to the system, and allows organisms to quickly adjust to alterations in the timing of the external light-cycle (i.e. travel across time zones).

The molecular-clock mechanism is composed of two interconnected transcriptional-translational feedback loops that generate molecular rhythms of gene expression
with an approximate 24-hour periodicity.[49] The ‘core-loop’ is composed of the positive-limb Period-Arnt-Sim (PAS) domain basic helix-loop-helix (PAS-bHLH) family of transcription factors Brain and Muscle Arnt-like protein-1 (Bmal1) and Circadian Locomotor Output Cycles Kaput (Clock) and the negative-limb Period (Per1,2,3) and Cryptocrome (Cry1,2) gene families (Figure 2).[50, 51] BMAL1 and CLOCK protein, oscillate in a circadian fashion, reach peak expression near the middle of the inactive phase, heterodimerize, and target E-box elements (5’-CACGTG-3’) within the proximal promoters of the Per and Cry genes.[52] Inversely, PER and CRY proteins reach peak expression toward the middle of the active phase, heterodimerize, and inhibit BMAL1:CLOCK activity.[53] Upon ubiquitin mediated degradation of PER and CRY proteins the 24-hour cycle is complete, and ready to begin again. The ‘alternative’ interlocking loop is comprised of the Rev-erb and Rora gene families that are also targets of BMAL1:CLOCK.[54, 55] When REV-ERB and RORA proteins reach peak expression they target Rev-erb Response Elements (ROREs) within the promoters of the Bmal1 and Clock genes, and repress (REV-ERBs) or activate (RORAs) gene expression.[56, 57] This alternative loop is thought to provide stability to the rhythmic expression of Bmal1 and Clock, while also serving as an integrator of environmental information (i.e. nutrient status) that temporally aligns the clock to the local environment. Genetic disruption of the core interlocking loop genes (Bmal1, Clock, Per, Cry) results in behavioral arrhythmias, while disruption of the alternative loop genes (Rev-erb, Rora) alters the periodicity of the rhythm.[49]
Figure 2. Model of the core and alternative interlocking loops of the molecular clock. BMAL1:CLOCK heterodimers target E-box elements in proximal promoter regions of Period and Cryptochrome gene families to drive expression of the negative limb transcription factors. Upon transcription/translations Period and Cryptochrome proteins heterodimerize and translocated into the nucleus where they inhibit BMAL1:CLOCK activity at the protein level. BMAL1:CLOCK also drive the expression of the alternative limb composed of the Rev-erb and RORA gene families that target ROREs in the promoters of Bmal1 and Clock to repress and activate gene expression, respectively.
1.2.2 Clock-Controlled Genes

The molecular-clock temporally modulates gene expression to generate a protein expression pattern that peaks at a single time-point throughout a 24-hour period. The convergence of expression of functionally related proteins ensures that peak rates of biochemical and physiological processes are temporally aligned with predictable changes in the environment. Genes that are direct targets of the molecular-clock, but lack the ability to modulate the timing mechanism are referred to as ‘clock-controlled genes’ (CCG). The molecular-clock promotes circadian gene expression through multiple distinct mechanisms. The most commonly studied mechanism is the transactivation of genes by the positive-limb transcription factors BMAL1:CLOCK. These transcription factors target E-box elements in proximal promoter elements, and drive gene expression to peak at the transition from the inactive to active-phase (~CT 12.0).\textsuperscript{[58, 59]} Protein expression typically follows that of the mRNA so that the proteins encoded by genes directly targeted by BMAL1:CLOCK are most highly expressed during the active-phase. Chromatin-immunoprecipitation sequencing (ChIP-Seq) studies in peripheral tissues have led chronobiologists to estimate that roughly 10% of the actively transcribed genes are target by BMAL1:CLOCK in their promoter regions.\textsuperscript{[60, 61]} Additionally, substantial portions of CCGs are transcriptional activators/repressors themselves providing an additional layer of circadian clock output. In addition to their role in stabilizing the molecular-clock, the \textit{Rora} and \textit{Rev-erb} gene families, regulate CCGs by targeting ROREs in the promoters of genes to rhythmically activate or repress gene expression, respectively.\textsuperscript{[62]} REV-ERBs have an important role in regulating
metabolic timing as they have been shown to repress lipogenic genes during the active-phase.[63, 64] By repressing gene express during the active-phase, many REV-ERB targets display expression patterns that peak during the inactive-phase (anti-phasic to that of BMAL1:CLOCK targets). Many additional mechanisms of clock-output have been observed, but will not be discussed here as they are outside the scope of this dissertation. Interestingly, temporal gene expression experiments in peripheral tissues have revealed that the molecular-clocks promote the circadian expression of CCGs in a tissue specific manner.[41, 61, 65] It is clear that the tissue-specific output of the molecular-clock allows for the temporal control of tissue-specific genes in peripheral tissues, many of which are rate-limiting enzymes in biochemical pathways, but currently little is understood about the molecular basis for this phenomenon.

1.3 The Skeletal Muscle Molecular-Clock

Multiple studies have demonstrated the existence of a robust molecular-clock mechanism in skeletal muscle.[66-75] To identify genes and processes regulated downstream of the molecular-clock investigators have studied genetic molecular-clock mutant mouse models.[60, 76, 77] Initial studies relied on the utilization of “germ-line” knockout models in which core-clock genes were disrupted or ablated in every cell throughout the body.[78-81] Characterization of the skeletal muscle of the germ-line Bmal1 knockout (BMAL1−/−) and ClockΔ19 mutant mice revealed significant decreases in maximal specific-force generation in both models.[66, 69, 74] The ClockΔ19 mice contain an antimorphic mutation that results in the deletion
of exon-19 of the Clock gene that encodes for the activation domain of the protein.[82] Electron microscopy imaging of skeletal muscle from these germ-line models displayed disruptions in myofilament organization, suggesting a possible role of the molecular-clock in regulating muscle structure and function.[66] Multiple studies have demonstrated that Bmal1 is required for skeletal muscle development.[83, 84] Interpretation of the phenotypes present in the germ-line mouse models is troublesome since these mice lack a functional molecular-clock throughout all tissues, and at all developmental stages. Therefore, it is difficult to discern whether the disruption of the molecular-clock is directly responsible for the skeletal muscle phenotypes observed in these germ-line knockout models. To address the confounding factors associated with the germ-line knockout mice, our lab and others have recently generated inducible skeletal muscle specific Bmal1 knockout mouse models.

1.4 Scope of Thesis: Part 1 (Chapter 2)

The intrinsic molecular-clock is most known for its role in regulating the timing of cellular metabolism in peripheral tissues, even under constant conditions. [19-26] These studies have shown that the molecular-clock temporally regulates the rhythmic activation and/or repression of rate-limiting metabolic genes to help the cell anticipate changes in environmental conditions and metabolic demand. [27][85] The capacity of the molecular-clock to regulate metabolism is highlighted by the metabolic phenotypes observed in genetic clock mutant models.[63, 64, 81, 86-89] The ClockΔ19 mice develop obesity as well as elevated blood-glucose levels, while
the Bmal1−/− mice display impairments in glucose handling and gluconeogenesis.[90] Chip-Seq analysis of REV-ERBα in liver tissue has demonstrated REV-ERBα binding on a large number of lipid-metabolic genes.[91, 92] Double knockout models in which Rev-erbα and Rev-erbβ are ablated display disruptions in circadian behavioral patterns and core-clock gene expression.[92] The Rev-erb double-knockout mice display hyperglycemia and increased circulating triglycerides. Interestingly, these mice display a decrease in the respiratory exchange ratio indicative of a shift from carbohydrate to lipid metabolism.[64] The authors suggested that the shift in metabolism to a more oxidative-state was caused by an increase in lipid metabolic gene expression due to the loss of REV-ERB mediated repression.[64]

Identifying the circadian gene expression profile in skeletal muscle is fundamental to understanding how the molecular-clock regulates physiological processes.[45] Miller et al. were the first to identify the circadian skeletal muscle transcriptome.[71] This group performed a time-course micro-array analysis by isolating RNA from skeletal muscle every 4-hours for 48-hours (2 full circadian cycles), and subsequently performed micro-array analysis at each time-point. To accurately distinguish bona fide circadian genes from that of outliers within inherently noisy time-course micro-array datasets requires the utilization of powerful circadian statistical approaches. Miller et al. used the circadian statistical algorithm known as COSOPT that fits temporal gene expression profiles to a cosine waveform. By comparing the temporal expression pattern of a circadian guide gene (e.g. Per2), Miller et al. established a ‘goodness of fit’ score they used to set a statistical cut-off for identifying circadian genes. The goodness of fit score was
calculated by employing a multiple-measures corrected-B (MMC-β), and was set to MMC-β≤0.2 for this study. 267 genes of the 7,824 genes expressed in wild-type (C57Bl6) skeletal muscle were identified as circadian (MMC-β≤0.2). Functional gene-ontology analysis of the Miller circadian muscle transcriptome demonstrated that the largest circadian gene clusters included genes involved in biosynthesis/metabolism, transcriptional regulation, and cell-cycle regulation. McCarthy et al. were the next group to generate a skeletal muscle circadian transcriptome.[70] Their experimental approach was very similar to that of Miller et al., as they performed micro-array analysis every 4-hours for 48-hours and used the COSOPT algorithm to identify circadian transcripts in skeletal muscle. In contrast to Miller et al., McCarthy et al. considered transcripts to be circadian if they displayed a 20-28 hour period length and were under the goodness of fit score: MMC-β≤0.15. This analysis resulted in the identification of 215 circadian genes in skeletal muscle. Notable gene-ontology clusters included regulation of protein/lipid metabolism as well as transcriptional regulation. Interestingly, McCarthy et al. identified a handful of muscle-specific circadian genes including: Myod1, Ucp3, Atrogin1, Pdk4, and Myh1. Andrews et al. validated the myogenic regulatory factor, Myod1, as a bona fide BMAL1:CLOCK target, providing additional evidence that the molecular-clock plays a role in skeletal muscle development and function.[66]

These initial transcriptome studies provided a fundamental framework to our understanding of circadian gene regulation in skeletal muscle, but vastly underrepresented the number of oscillating transcripts. The circadian genes identified in these studies amounted for only ~3% of the total skeletal muscle transcriptome, a
number that is lower than one would predict from the BMAL1 ChIP Seq studies (10-15% coverage of expressed genes).[60] This discrepancy may be explained by the technological and/or experimental limitations of these initial transcriptome studies. The Miller and McCarthy studies utilized gene arrays that are less sensitive than the Affymetrix gene Chips now available. In addition, these groups employed the COSOPT statistical algorithm to identify circadian genes, which has since been demonstrated as less sensitive and specific for the detection of rhythmic transcripts in comparison to the JTK_CYCLE algorithm. The JTK_CYCLE algorithm is a fusion of two powerful, non-parametric statistical tests called Jonckheere-Tepstra (JT) and Kendall’s Tau. JTK_CYCLE employs the JT statistic to detect monotonic orderings within independent groups. This test compares the expression at of each circadian time-point in relation to each other, and assigns a positive or negative sign. In effect, a matrix is generated for each microarray probset depending on the pattern of expression throughout the experimental time series. Previous statistical algorithms, such as COSOPT, identify rhythmic gene expression by curve fitting the experimental time-series to a cosine waveform. This was accomplished by determining the inter-group differences between the experimental time-series and the cosine waveforms. In contrast, JTK_CYCLE utilizes a ‘look-up’ function to compare the Kendall’s tau rank correlation coefficient between the experimental time series matrix, and the matrices from all possible cosine waveforms within the range of user-defined period lengths. Through the use of the Harding algorithm, JTK_CYCLE can rapidly and efficiently determines the optimal combination of phase and period length that provides the lowest possible Kendall’s Tau correlation. Since
the Kendall’s tau depends only on the sign of the inter-group differences between circadian time-points this algorithm is very resistant to outliers, and the analysis of logarithmically transformed data does not alter its ability to efficiently determine the optimal period and phase. The ability to provide permutation-based p-values and q-values makes JTK_CYCLE more statistically powerful and computationally efficient than the previous circadian statistical algorithms. Quite possibly the biggest limitation of the initial circadian transcriptome studies is the low sampling frequency (every 4-hours for 48-hours). The most recent skeletal muscle circadian transcriptome generated by Hogenesch et al. has an improved statistically power for the detection of circadian transcripts due to a doubling in the sampling frequency.[27] Hogenesch et al. performed micro-array analysis every 2-hours for 48-hours. As is described in section 2.2.1 below, we employed the use of JTK_CYCLE for our analysis of the Hodgenesch skeletal muscle transcriptome and identify approximately 1,628 genes (8x that of the previous transcriptomes).[93]

An additional benefit of the JTK_CYCLE algorithm is its ability to determine the acrophase of each circadian gene.[94] We hypothesized that genes involved in metabolic processes in skeletal muscle would be temporally clustered over a 24-hour period. We sought to test this idea by identifying the acrophase of genes with common ontologies, to assist in predicting the potential timing of metabolic processes in skeletal muscle. As will be shown in chapter 2, the acrophase analysis of the metabolic genes within the Hodgenesch skeletal muscle transcriptome showed that catabolic and anabolic processes are temporally separated. To our knowledge, we are the first to propose an in-depth molecular mechanism to describe how
metabolic processes are temporally regulated. These findings will be of particular interest to the skeletal muscle and metabolism fields since skeletal muscle is such a large tissue and is the main sight of post-prandial glucose uptake, as well as amino-acid storage.

To further elucidate genes and physiological processes regulated by the molecular-clock many investigators have performed transcriptional analyses of peripheral tissues with genetically disrupted clocks. Miller et al. and McCarthy et al. performed micro-array analyses comparing gene expression in wild-type and ClockΔ19 skeletal muscle.[70, 71] Miller et al. showed that approximately 78% of the genes they identified as circadian displayed significantly different expression values in the ClockΔ19 mouse model with the majority of genes exhibiting significantly decreased expression. This group did not provide a detailed description of the circadian genes that were down-regulated in the ClockΔ19 skeletal muscle, although they did note that previously identified CCGs such as Dbp and Per2 were significantly down-regulated. McCarthy et al. observed similar findings with the ClockΔ19 skeletal muscle as they reported changes in the expression pattern of approximately 30% of the circadian transcriptome, but do not provide a detailed analysis of these genes. Although these studies demonstrate large scale disruptions of the circadian genes in the ClockΔ19 skeletal muscle, it is now widely accepted that deletion/mutation of the Clock gene does not completely ablate molecular and behavioral rhythms as redundancy in the molecular-clock mechanism functions in the absence of the CLOCK protein. The only single knockout model that completely ablates both molecular and physiological rhythms is the Bmal1−/− knockout model.[50]
Additionally, the use of the ClockΔ19 mouse model makes it hard to discern whether the transcriptional changes observed in the skeletal muscle are truly caused by the intrinsic molecular-clock as these animals lack a functional CLOCK protein throughout the body and therefore display behavioral arrhythmias.

On-going studies are aimed at utilizing organ-specific molecular-clock mutant models to determine the function of the clock in each tissue. These studies also serve to assess the role(s) the peripheral clocks play in regulating whole body metabolism [39-43]. Dyar et al. have recently developed two skeletal muscle specific Bmal1 knockout models to study metabolic changes.[68] The two models consist of a Bmal1 knockout model from birth (Bmal1flox/flox x Mlc1f-Cre), called mKO, and a tamoxifen inducible Bmal1 knockout model (Bmal1flox/flox x Acta1-Cre), called imKO. The mKO mice displayed decreases in specific force generation while the imKO mice did not show any significant decrease in specific force generation, and this discrepancy may be explained by developmental differences caused by the loss of Bmal1 at birth. This group demonstrated a significant decrease in insulin-stimulated glucose uptake in isolated soleus muscle in both the mKO and imKO models compared to age-matched wildtype controls (basal levels unaffected). Dyar et al. attributed the impairments in insulin-stimulated glucose uptake to the marked reduction in the expression of the muscle specific glucose transporter GLUT4, and a reduction in the activity of Pyruvate Dehydrogenase (PDH) responsible for regulating the flux of carbohydrates into the TCA-cycle. Since the mKO and imKO models display normal circadian activity patterns, the impairments in glucose handling can be attributed to the intrinsic skeletal muscle clock. In agreement with
the results reported by Dyar et al., analysis of our inducible muscle specific Bmal1 knockout model resulted in circadian genes involved in glucose and lipid handling being down and up-regulated, respectively.

1.4.1 Scope of Thesis: Part 2 (Chapter 3)

Previous studies have sought to elucidate the transcriptional mechanisms regulating the Titin-cap (T-cap) gene given its functional importance in striated muscle, and its roles in Limb-Girdle muscular dystrophy type 2G and dilated cardiomyopathy.[95-99] The T-cap protein forms one of the strongest known non-covalent protein-protein interactions in the body through hydrogen binding of the Z1-Z2 N-terminal domains of adjacent Titin molecules to effectively link two sarcomeres at the Z-line.[95] T-cap is highly expressed in both skeletal muscle and the heart.[98] The proximal promoter region of the T-cap gene contains multiple E-box elements that are highly conserved between humans and mice and suggests the regulation of this gene by bHLH transcription factors.[99] The myogenic regulatory factor MYOD1 has been shown to promote the expression of T-cap in both porcine and mouse skeletal muscle.[97, 99] Podobed et al. showed that T-cap is regulated down-stream of the molecular-clock in mouse heart tissue as its mRNA and protein oscillate in wild-type mice, and are down-regulated in ClockΔ19 heart tissue.[96] This group demonstrated the ability of BMAL1:CLOCK to promote T-cap promoter:luciferase activity and observed BMAL1 binding on the T-cap proximal promoter at a highly conserved tandem E-box element (E-box-xxxxxx-E-box). Further analysis is required to determine if the Titin-bound portion of T-cap oscillates over time of day.
As stated earlier, there is a very limited knowledge of the mechanisms responsible for tissue-specific regulation of CCGs. In an attempt to study this phenomenon in skeletal muscle, we sought to first identify a muscle specific CCG by performing a bioinformatics analysis that ultimately led to the identification of $T$-$cap$ as a circadian gene in skeletal muscle. Since previous studies have validated $T$-$cap$ as a BMAL1:CLOCK target (heart tissue) as well as its regulation by MYOD1, we decided to investigate the transcriptional regulation of $T$-$cap$ as a model for CCG regulation in skeletal muscle. Our study validates $T$-$cap$ as a CCG in skeletal muscle and reveals a previously unidentified mechanism in which MYOD1 works synergistically with the molecular-clock to promote the circadian expression of $T$-$cap$.

2 Chapter 2: The endogenous molecular clock orchestrates the temporal separation of substrate metabolism in skeletal muscle

Text and figures in this chapter are adapted from the first author manuscript: The endogenous molecular clock orchestrates the temporal separation of substrate metabolism in skeletal muscle. Hodge et al., Skeletal Muscle 2015.[93]

Author Contributions: BAH participated in the time-course collection and RNA extraction from iMS-$Bmal1$ muscle tissue, contributed to the recombination assay, carried out the bioinformatics analysis, and drafted the manuscript. YW participated in the bioinformatics analysis, contributed to the interpretation of the data, generated the heat-map, and helped with the writing of the manuscript. LAR helped with the bioinformatics analysis and figure production. JHE participated in the time-course collection and preparation of the RNA used in the microarray assays. BDH
and EAS participated in the time-course collection and completed the mouse activity data collection and analysis. EAS also carried out the real-time PCR analysis and participated in the recombination assay. XZ participated in the time-course collection and performed the Western blot analysis. KAE was involved in the design and coordination of the study and helped with the drafting of the manuscript.

2.1 Introduction

Skeletal muscle plays a large role in whole-body metabolism as it constitutes approximately 40% of body mass and is a highly metabolically active tissue.[100, 101] Basal metabolic rate is dependent on both the size and activity of skeletal muscle as cross-bridge cycling and calcium handling associated with contraction are energetically expensive processes.[101-104] Skeletal muscle is a principle contributor to whole-body glucose handling as it is responsible for approximately 80% of postprandial glucose uptake.[105, 106] It has been widely reported that skeletal muscle has regulatory mechanisms that modulate substrate utilization and storage in response to varying metabolic demands and environmental conditions (for example, nutrient status).[102, 107-111] For instance, skeletal muscle rapidly modulates rates of glucose uptake and utilization in response to contraction and/or insulin stimulation.[112-114] While the fluctuations in the role for muscle to store vs. use is commonly linked with the fed/fasted and active/inactive behaviors, these changes in storage and use are also aligned with the 24-h (circadian) light/dark
cycles attributed to the rising and setting of the sun and feeding/activity behavior.[115]

Utilizing high-resolution temporal transcriptome data coupled with circadian statistics has proved to be an effective method for identifying genes expressed in a circadian manner.[116, 117] In the present study, we employ a bioinformatics approach with a publically available high-resolution circadian data set collected under constant dark conditions to analyze the skeletal muscle circadian transcriptome (gastrocnemius muscle) with a focus on the temporal phase of gene expression. We reveal that skeletal muscle circadian genes are highly enriched for metabolic processes, and furthermore, we identify the temporal pattern of peak expression for different key metabolic genes separating catabolic vs. anabolic processes over 24 h. To identify which circadian-metabolic genes are regulated downstream of the intrinsic molecular clock, we generated an inducible muscle-specific Bmal1 knockout (iMS-Bmal1−/−) mouse and performed a time series transcriptome analysis. Mice lacking Bmal1 in skeletal muscle displayed no apparent changes in circadian behavior, yet we observed significant decreases in the expression of circadian genes involved in glucose utilization and adrenergic signaling, while observing significant increases in lipogenic genes. Consistent with a substrate shift from carbohydrate to lipid utilization, we observed a concomitant shift from a fast to slow fiber-type gene expression profile indicative of a more oxidative muscle in iMS-Bmal1−/−. These findings demonstrate that the endogenous molecular clock in skeletal muscle contributes significantly to the time of day shifts in carbohydrate/lipid metabolism.
2.2 Materials and Methods

2.2.1 High-resolution circadian microarray

Microarray data for the high-resolution circadian time-course are from gastrocnemius muscles of male C57Bl6 mice collected every 2 h for 48 h under constant dark conditions and ad libitum food availability.[27] The data were downloaded from NCBI GEO datasets (GSE54652) and consist of 24 individual arrays, one for each time point from circadian time 18 to 64.[117] Expression intensities from the series matrix file for all probesets at all time points were used as input for JTK_CYCLE analysis, with period length set to 24 h.[94] We defined circadian genes as having a JTK_CYCLE adjusted P value of less than 0.05. We utilized the Bioconductor package to identify mapped probesets on the Affymetrix Mouse Gene 1.0ST chip that represent unique genes, thus eliminating control probesets from further analyses. Genes with median expression intensities of at least 100 were considered as expressed in skeletal muscle. We entered the list of circadian genes into Gene Ontology Consortium online tools to identify enriched biological processes.[118, 119] Enrichment P values were adjusted for multiple testing using Bonferroni correction.

2.2.2 Inducible skeletal muscle-specific Bmal1 inactivation mouse model

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. The floxed Bmal1 mouse
[B6.129S4(Cg)-Arnttm1Wir]] was purchased from The Jackson Laboratory and has no reported breeding, physical, or behavioral abnormalities.[120] The skeletal muscle-specific Cre-recombinase mouse, [human skeletal actin (HSA)-MerCreMer] has been previously characterized.[121] The floxed Bmal1 mouse has loxP sites flanking exon 8 and is indistinguishable from wild-type littermates. Breeding with the skeletal muscle-specific Cre-recombinase mouse generates offspring in which selective deletion of the bHLH domain of Bmal1 in skeletal muscle can be induced upon tamoxifen administration. Inducible skeletal muscle-specific Bmal1 knockout mice were generated as follows: the Bmal1^lox/lox female was crossed with the skeletal muscle-specific Cre-recombinase male. This yielded an F1 generation of skeletal muscle-specific Cre^+/−;Bmal1^+/flox mice. Breeding the F1 generation males to the Bmal1^lox/lox females resulted in the skeletal muscle-specific Cre^+/−;Bmal1^lox/lox mice (referred to as iMS-Bmal1^lox/lox) needed for this study. Mouse genotypes were determined by PCR using genomic DNA isolated from tail snips. Activation of Cre-recombination was done by intraperitoneal injections of tamoxifen (Sigma-Aldrich, St. Louis, MO, USA; Cat. No. T5648) (2 mg/day) for five consecutive days when the mice reached 12 weeks of age. This age was chosen to eliminate any effects that the lack of Bmal1 might have on skeletal muscle development and postnatal maturation. Controls were vehicle (15% ethanol in sunflower seed oil)-treated iMS-Bmal1^lox/lox mice.
2.2.3 Recombination specificity

The iMS-Bmal1 mice were injected (intraperitoneal) with either vehicle (iMS-Bmal1+/+) or tamoxifen (iMS-Bmal1−/−) between 12 and 16 weeks of age. Five weeks postinjection, mice were anesthetized with isoflurane, and the heart, diaphragm, liver, lung, abdominal aorta, brain, tibialis anterior, soleus, gastrocnemius, brown fat, white fat, and cartilage were collected and immediately frozen in liquid nitrogen for DNA analysis. Genomic DNA was extracted from the tissues using the DNeasy Blood and Tissue Kit (Qiagen, Venlo, Netherlands). To assess recombination specificity, PCR was performed with tissue DNA and primers for the recombined and non-recombined alleles as described in Storch et al.[120] The forward and reverse primers for the non-recombined allele were the same as the genotyping primers and yielded a 431-bp product. A second forward primer 5′-CTCCTAACTTGTCTGGTTCTGT-3′ was included to detect the recombined allele, which showed a band at 572 bp [50]. The PCR reaction was run on a 1.5% agarose gel (0.0005% ethidium bromide) to visualize the DNA products.

2.2.4 RNA isolation and real-time PCR

Total RNA was prepared from frozen gastrocnemius tissue samples using TRIzol (Invitrogen) according to the manufacturer’s directions. RNA samples were treated with TURBO DNase (Ambion, Austin, TX, USA) to remove genomic DNA contamination. Isolated RNA was quantified by spectrophotometry (λ = 260 nm). First-strand cDNA synthesis from total RNA was performed with a mixture of oligo(dT) primer and random hexamers using SuperScript III First-Strand Synthesis
SuperMix (Invitrogen, Waltham, MA, USA). All isolated RNA and cDNA samples were stored at −80°C until further analysis. Real-time quantitative PCR using TaqMan (Applied Biosystems, Waltham, MA, USA) assays was used to examine the gene expression of *Bmal1* (Mm00500226_m1), *Rev-erbα* (Mm00520708_m1), *Dbp* (Mm00497539_m1), *Hk2* (Mm00443385_m1), *Pdp1* (Mm01217532_m1), *Fabp3* (Mm02342495), and *Pnpla3* (Mm00504420_m1). The ΔΔCT method was used for the quantification of real-time PCR data in the circadian collections.

2.2.5  **Wheel activity monitoring**

One cohort of mice was used for analysis of circadian behavior (gene expression not analyzed in this cohort). A total of 20 mice (mixed genders) were analyzed with 11 receiving tamoxifen treatment and the remaining 9 receiving vehicle treatment. The mice were maintained in individual cages with a running wheel under 12 L:12D (LD) conditions for 4 weeks. The wheel running of the vehicle (iMS-*Bmal1*+/+) or tamoxifen (iMS-*Bmal1*−/−) mice were continuously recorded and monitored throughout the experiment using ClockLab software.[122] To determine the free-running period of the mice, we released them into total darkness (DD) for 3 weeks. Activity was evaluated using voluntary running wheel rotations plotted in 1-min bins. The free-running period (*tau*) during the 3-week DD period was calculated using periodogram analysis in the ClockLab software.
2.2.6  **Circadian collections**

Forty-eight iMS-Bmal1<sup>Rox/fox</sup> mice were housed in individual cages in light boxes, entrained to a 12-h LD cycle for 14 days, and had *ad libitum* access to food and water. Following the 2-week entrainment period, 24 mice were injected with vehicle and 24 with tamoxifen for five consecutive days, generating 24 iMS-Bmal1<sup>+/+</sup> and 24 iMS-Bmal1<sup>−/−</sup> mice, respectively. The light schedule was kept the same during injections and for the subsequent 5 weeks. Five weeks after the last day of injections, mice were released into constant darkness for 30 h following protocols established in the circadian field.[27, 70] Mice were sacrificed in darkness (dim red light), and skeletal muscle s were collected every 4 h for 20 h (six time points) and frozen for RNA and protein analysis.

2.2.7  **Microarray analysis of iMS-Bmal1<sup>+/+</sup>, iMS-Bmal1<sup>−/−</sup>, and MKO (Dyar et al.)**

We pooled equivalent amounts of total RNA from four mice for each time point (circadian time 18, 22, 26, 30, 34, 38) and treatment (vehicle or tamoxifen). Pooled RNA samples were used to construct cDNA libraries that were hybridized to Affymetrix Mouse Gene 1.0ST microarrays (Affymetrix, Santa Clara, CA, USA) (1 sample/time point). Intensity data for iMS-Bmal1<sup>+/+</sup> and iMS-Bmal1<sup>−/−</sup> gastrocnemius muscles are quantile normalized, and a low pass median intensity filter of greater than or equal to 100 is applied to both iMS-Bmal1<sup>+/+</sup> and iMS-Bmal1<sup>−/−</sup> datasets separately. Nine thousand one hundred eighty-four non-redundant, mapped genes (9,988 probesets) are considered to be expressed in one or both datasets. Gene expression changes in iMS-Bmal1<sup>−/−</sup> muscle tissue were
calculated by averaging the change in expression for each gene throughout the circadian time course (CT18-38, n = 6). Tibialis anterior and soleus gene expression values for control and muscle-specific knockout model (MKO) from Dyar et al.[68] were downloaded from NCBI GEO datasets (GSE43071) and consists of 18 individual arrays, three for each time point from circadian time 0 to 20. To compare temporal gene expression changes for the TA and SOL, we averaged Affymetrix ST 1.0 expression values for each gene at circadian times 0, 4, 8, 12, 16, and 20. Student's t test was used to identify differentially expressed probesets at a significance of P ≤ 0.05.

2.2.8 Western blot

Whole cell lysates were prepared from the liver and gastrocnemius of iMS-\textit{Bmal1}\textsuperscript{+/+} and iMS-\textit{Bmal1}\textsuperscript{+/-} mice (n = 3/strain). SDS-PAGE (4-15% separating gel, Bio-Rad, Hercules, CA, USA) and immunoblotting were carried out with routine protocols. Affinity-purified \textit{Bmal1} polyclonal antibody (Sigma-Aldrich, SAB4300614) was visualized with IRDye-conjugated secondary antibody using the Odyssey system (Li-Cor, Lincoln, NE, USA). Each lane contained 50μg total protein.
2.3 Results and Discussion

2.3.1 Cellular metabolic processes are highly enriched in the circadian transcriptome of skeletal muscle

To identify circadian gene expression in skeletal muscle, we used a publicly available, high-resolution, circadian time-course microarray dataset from gastrocnemius muscles of male C57BL/6 mice.[27, 117] These mice were housed in constant darkness, and food was provided ad libitum to eliminate the influence of external environmental cues. We chose this dataset because it has double the sampling frequency of previously published circadian muscle transcriptomes, and this allows for greater precision for circadian analysis.[27, 123] Using the JTK_CYCLE statistical algorithm [94] for the reliable detection of oscillating transcripts with a 24-h periodicity, we identified 1,628 circadian mRNAs (adjusted \( P < 0.05 \)). An unbiased Gene Ontology enrichment analysis of these circadian genes revealed a significant overrepresentation of cellular metabolic processes, with approximately 1,004 (62%) genes directly involved in skeletal muscle metabolic processes as well as the regulation of metabolism (Figure 3).

Herein, we report the acrophase according to their respective circadian times (CT), which is standardized to the free-running period of the mice under constant conditions. For the array studies, the mice were only in DD for 30 h so CT 0 denotes the start of the inactive period, while CT 12 denotes the start of the active period. To identify the timing of gene expression and its relationship to metabolic processes in skeletal muscle, we annotated a subset of circadian genes by their known functions,
**Figure 3.** Gene ontology analysis of the skeletal muscle circadian transcriptome. Top 15 enriched GO processes listed from left to right in order of significance.
timing of peak expression, and involvement in key metabolic pathways. We focused our analysis on metabolic functions that involve substrate (carbohydrate and lipid) utilization as well as storage and biosynthetic processes.

2.3.1.1 Lipid metabolism: genes involved in fatty-acid uptake and β-oxidation peak in the mid-inactive/light phase

Skeletal muscle expresses specialized membrane transporters to facilitate the transport of lipids into the cell.[124-126] Two lipid transport genes that encode for fatty-acid binding proteins, *Fabp4* (CT 24.0) and *Fabp3* (heart/muscle isoform, CT 6), are expressed in a circadian manner with the highest mRNA expression in the early- and mid-inactive periods, respectively. Acrophase of circadian genes involved in lipid metabolism are illustrated in Figure 4. Normalized expression traces for each gene are located in Appendix Files 1 and 2. Previous studies have demonstrated oscillations in plasma fatty acid concentrations in mice with peak levels occurring during the inactive/light period.[92, 127, 128] Further functional analysis is required to validate the prediction that the rate of fatty-acid uptake in skeletal muscle peaks during the mid-late inactive period. Upon uptake into the cell, fatty acids can be stored as triglycerides or be converted to acetyl-CoA through β-oxidation.[129] *Slc25a20* encodes for an acyl-carnitine translocase that transfers fatty acids into the inner-mitochondrial matrix and reaches peak expression in the middle of the inactive period (CT7.5).[130] We identified multiple genes that encode for β-oxidation enzymes to be circadian and also reach peak expression around the mid-inactive phase. These include the enoyl CoA hydratase *Ech1* (CT 7.0), the tri-
Figure 4. Schematic acrophase diagram of circadian genes involved in lipid metabolic processes. The relative location of the circadian genes (italicized) in respect to the x-axis indicates acrophase or time of peak expression calculated by the JTK_CYCLE algorithm. Location of substrates and pathways does not represent peak substrate concentrations and/or rates of individual pathways as these were not measured in our analysis. White/grey shading is representative of the inactive and active phases, respectively.
functional enzyme subunits *Hadha* (CT 8.0) and *Hadhb* (CT 8.0), and the acetyl-CoA acyltransferase *Acaa2* (CT 9.0). Malonyl-CoA, an intermediate formed during *de novo* fatty acid synthesis, is a potent inhibitor of β-oxidation. The striated muscle enriched gene *Mlycd* (CT 7.5) encodes for the malonyl-CoA decarboxylase that promotes β-oxidation by reducing cytosolic concentrations of malonyl-CoA and reaches peak expression during the mid-inactive period similar to that of the circadian β-oxidation genes. These observations suggest that rates of β-oxidation are modulated over time of day and potentially through the endogenous molecular clock in skeletal muscle.[109, 131, 132]

Nuclear receptors are known to be potent transcriptional regulators of metabolism as they sense changes in environmental conditions and induce appropriate changes in the expression of metabolic genes.[133-137] The nuclear receptor Estrogen-related receptor alpha (*Esrra*, CT 7.5) and the nuclear co-activator PPARγ coactivator-1 beta (*Ppargc1b*, CT 7.0) are both circadian genes in skeletal muscle with peak expression occurring at the mid-inactive phase. These factors have been shown to promote mitochondrial biogenesis, fatty-acid uptake (targets *Fabp3*), and β-oxidation.[138, 139] The nuclear co-repressor *Nrip1*, also known as *Rip140*, is a potent negative regulator of skeletal muscle oxidative metabolism and has been shown to suppress expression of the fatty-acid transporter, *Fabp3*, in skeletal muscle.[140-142] NRIP1 suppresses gene expression by binding nuclear receptors (including PPARs and estrogen-related receptors) and recruiting histone deacetylases.[143] Interestingly, peak expression of *Nrip1* occurs during the beginning of the active period (CT 13.0) and may therefore act as a molecular brake
to oxidative metabolism as the muscle transitions from lipid to carbohydrate utilization during the early active phase.

2.3.1.2 Lipid metabolism: lipogenic genes reach peak expression at the end of the active/dark phase

The lipogenic genes *Acly* (CT23.0), *Acaca* (CT 23.0), and *Fasn* (CT 22.5) involved in *de novo* fatty-acid synthesis, or the conversion of excess carbohydrates into fatty acids, reach peak expression at the end of the active phase (Figure 4).[129, 144] *Scd1* (CT 24.0) encodes the enzyme that catalyzes the rate-limiting reaction of monounsaturated fatty-acid formation to promote lipid bilayer fluidity and lipogenesis.[145, 146] The genes *Srebf1* (CT 24.5), *Srebf2* (CT 24.0), and *Mlxip* (CT 23.5) encode transcription factors that target carbohydrate response elements within lipogenic gene promoter regions (*Acly*, *Acaca*, and *Fasn*) and are also circadian with peak expression at the end of the active phase.[147, 148] Consistent with our results, *Srebf1* oscillations have been reported in the liver and genome-wide binding studies have shown a circadian recruitment pattern of SREBF1 to the promoters of lipogenic genes with maximal binding during the active (fed) stage.[149-152]

The gene *Pnpla3* (CT 21.0), also known as adiponutrin, promotes lipogenesis by converting LPA to phosphatidic acid (PA).[153] The gene *Lpin1* (CT 24.0) which encodes for the lipin-1 enzyme is responsible for converting phosphatidic acid (PA) to diacylglycerol (DAG), which is the upstream metabolite required in phospholipid biosynthesis.[154, 155] The highly regulated, committing step in triacylglycerol
(TG) synthesis, addition of a fatty-acyl-CoA to DAG, is performed by the product encoded by *Dgat1* (CT 24.5), which is also expressed in a circadian manner.[156] Once a TG molecule is formed, it can be elongated by enzymes encoded by *Acsl5* (CT 23.0) or *Elov5* (CT 22.5).[157, 158] The observation that circadian lipogenic genes reach peak expression levels around the end of the active phase suggests that skeletal muscle promotes storage of excess energy at the end of the active/absorptive phase.

2.3.1.3 Carbohydrate metabolism: genes involved in carbohydrate catabolism peak in the early active/dark phase

Glycolysis, the breakdown of glucose to form pyruvate, is primarily regulated at two enzymatic reactions catalyzed by the hexokinase and phosphofructokinase enzymes.[159] We observe that the hexokinase-2 (*Hk2*) gene is circadian with peak expression occurring at the beginning of the active phase (CT 12.0). Acrophase of circadian genes involved in carbohydrate metabolism are illustrated in Figure 5. Normalized expression traces for each gene are located in Appendix Files 3 and 4. *Hk2* is responsible for the first step in glycolysis by phosphorylating glucose to make glucose-6-phosphate, thereby trapping glucose within the cell.[160] The rate-limiting step of glycolysis involves the catalysis of fructose-6-phosphate to the highly unstable fructose-1,6-bisphosphate by the enzyme phosphofructokinase-1 (PFKM).[161, 162] A potent allosteric activator of PFKM is fructose-2,6-bisphosphate, which is the product of the other phosphofructokinase isozyme phosphofructokinase-2 (PFK2).[163] Three genes (*Pfkfb-1,3,4*) that encode
Figure 5. Schematic acrophase diagram of circadian genes involved in carbohydrate metabolic processes. The relative location of the circadian genes (italicized) in respect to the x-axis indicates acrophase or time of peak expression calculated by the JTK_CYCLE algorithm. Location of substrates and pathways does not represent peak substrate concentrations and/or rates of individual pathways as these were not measured in our analysis. White/grey shading is representative of the inactive and active phases, respectively.
phosphofructokinase-2 subunits are circadian with peak expression occurring during the mid- and late-inactive phases (CT 10.0, CT 4.5, and CT 12.0, respectively).

Glycolytic flux through the Kreb’s cycle is controlled by pyruvate dehydrogenase complex (PDH).[164, 165] PDH decarboxylates pyruvate to form acetyl-CoA, which is a substrate for the Kreb’s cycle. The activity of PDH is regulated at the posttranslational level. Phosphorylation by kinases (PDKs) inhibits PDH activity, while dephosphorylation by phosphatases (PDPs) activates the complex.[166, 167] The Pdk4 gene, which encodes for a PDH kinase that inhibits PDH, reaches maximal expression at the mid-inactive phase (CT 6.0). This expression pattern is similar to that of the β-oxidation genes and suggests that skeletal muscle substrate preference is pushed toward utilization of lipids over carbohydrates during the mid- to late-inactive phase. Conversely, the PDH phosphatase gene, Pdp1, peaks at the beginning of the active phase (CT 10.0) in a similar temporal fashion compared to the glycolytic enzymes described above. This temporal regulation of Pdp1 may therefore help increase glycolytic flux during the active phase. Dyar et al. observed similar expression patterns of Pdk4 and Pdp1 in skeletal muscle and were first to report a shift to carbohydrate utilization at the beginning of the active phase.[68]

Adrb2 encodes for the β2-adrenergic receptor (β2AR) involved in the fight-or-flight response in peripheral tissues.[168, 169] Agonist (that is, catecholamine) binding is well established to evoke a cell-signaling cascade that promotes glucose uptake, glycogenolysis, and lipolysis to provide a readily available source of energy for skeletal muscle.[170-172] Adrb2 is expressed in a similar pattern to that of the
glycolytic activating genes as it peaks at the beginning of the active phase. Interestingly, the expression of Adrb2 coincides with that of oscillating epinephrine concentrations in mammals, which has previously been identified as peaking at the beginning of the active phase in mouse models.[173] The G-protein receptor kinase, encoded by Adrbk1, phosphorylates the β2AR, thereby rendering it susceptible to receptor-mediated endocytosis via β-arrestin proteins encoded by Arrdc3 and Arrb1.[174-176] Adrbk1, Arrdc3, and Arrb1 are all expressed in a circadian manner and antiphase to the expression of Adrb2. These observations suggest there is a time of day difference in adrenergic signaling and that sensitivity to epinephrine may be highest in skeletal muscle during the active period while being desensitized prior to the inactive period.

2.3.1.4 Carbohydrate metabolism: genes involved in carbohydrate storage peak at the mid-active/dark phase

Excess carbohydrates are stored as glycogen in skeletal muscle which accounts for approximately 70 to 80% of whole body stores.[177] Unlike the liver, skeletal muscle glycogen content is not responsible for maintaining blood glucose concentrations but serves as a rapidly accessible energy depot for active contractions.[170] Glycogenesis is regulated by both glucose-6P concentrations and the enzymatic activity of glycogen synthase.[178, 179] The gene Ppp1r3c (CT 20.0) reaches peak expression around the mid-inactive phase and encodes a regulatory subunit of the protein phosphatase-1 (PP-1) responsible for activating glycogen synthase while also inhibiting glycogen breakdown (Figure 5).[180] Enzymatic
activity of PP-1, and subsequent activation of glycogen synthase, is regulated downstream of the insulin signaling pathway.[181]

Insulin promotes an anabolic signaling cascade that works in opposition to that of adrenergic signaling to drive glycogen and lipid storage. Previous reports have identified a ‘counter-regulatory’ role of insulin receptor to selectively inhibit β2AR signaling through phosphorylation and subsequent internalization of the receptor.[169, 182] Interestingly, the genes that encode the insulin receptor substrate-1, Irs1 (CT 22.0), and its downstream PI3-kinase target, Pik3r1 (CT 19.0), are both circadian with peak expression occurring at the late-active phase while the genes involved in suppressing PI3-kinase, Pik3ip1 (CT 8.0), and the insulin-receptor substrate-1, Fbxo40 (CT 5.0), reach peak expression during the inactive phase.[183, 184] These data suggest that the molecular clock may act to prime skeletal muscle to store excess glucose during the mid- to late-active phase. This prediction is further supported by previous studies that report skeletal muscle glycogen content as having a diurnal rhythm with the highest levels occurring during the mid-active phase.[185-187] Skeletal muscle glucose uptake is primarily controlled via the presence/absence of the glucose transporter GLUT4/Slc2a4 in the plasma membrane (sarcolemma) and transverse tubules. A t-SNARE syntaxin-4 interacting protein, encoded by Stxbp4, has previously been shown to repress GLUT4 insertion into the plasma membrane in the absence of insulin signaling.[188-190] The gene Tbc1d1 encodes for Rab-GTPase that represses GLUT4 translocation in the absence of insulin- or contraction-induced signaling cascades.[191-193] Interestingly, Tbc1d1 and Stxbp4 are both expressed in a circadian manner and reach peak
expression in the middle of the active phase (CT 19.0). Previous reports have identified \textit{Tbc1d1} as a circadian gene in skeletal muscle and other tissues.[68, 194] Together, these gene products may play a role in reducing glucose uptake at the end of the active phase by repressing GLUT4 translocation and/or insertion into the plasma membrane. This temporal separation of anabolic and catabolic signaling processes in skeletal muscle may be vital for maintaining a tight regulation of serum glucose levels, and disruption of which may contribute to the metabolic phenotypes often reported in clock-mutant mice models.

2.3.2 \textbf{Generation of an inducible skeletal muscle-specific mouse model of Bmal1 inactivation}

Use of the high-resolution microarray data set allowed for the identification of mRNAs expressed in a circadian pattern, but this could be due to the intrinsic molecular clock or could be a response to external behavioral (feeding/activity) or neural/humoral cues.[195-197] To determine the role of the intrinsic skeletal muscle molecular clock in the temporal regulation of metabolic gene expression, we generated an inducible mouse model to inactivate \textit{Bmal1} specifically in adult skeletal muscles. Upon treatment with tamoxifen in 12-week-old adult mice, we detect recombination of exon-8 (that is, DNA binding region) of the \textit{Bmal1} gene specifically in skeletal muscle (\textbf{Figure 6A}), confirming the tissue specificity of the mouse model. We waited until 12 weeks of age to limit possible developmental effects as BMAL1 has been shown to promote myogenesis.[66, 84] As seen in \textbf{Figure 6A}, recombination was not detected in the skeletal muscle or non-muscle tissues of
Figure 6. Characterization of iMS-Bmal1−/− mice. Recombination assay (A) of genomic DNA isolated from muscle and non-muscle tissues from tamoxifen-treated (iMS-Bmal1−/−) and vehicle-treated (iMS-Bmal1+/+) mice at 17 to 18 weeks of age (5 weeks post-injection). Recombination of the Bmal1 gene (exon 8) yields a 572-bp PCR product. The non-recombined allele is detected at 431 bp. Western blot (B) analysis of BMAL1 expression in iMS-Bmal1−/− and iMS-Bmal1+/+ liver and gastrocnemius samples. Note that the original blot containing both muscle and liver samples was cut, and brightness/contrast was altered to enhance the visibility of Bmal1 in the muscle samples. (C) Real-time PCR results of time-course expression values for Bmal1, Rev-erbα, and Dbp in the iMS-Bmal1−/− (black) and iMS-Bmal1+/+ (red). Representative wheel running rhythms (D) of iMS-Bmal1−/− and iMS-Bmal1+/+ mice. White and black bars (top) indicate light and dark phases. 12 L/12D represents the 12-h light/12-h dark cycle. 12D/12D represents constant darkness conditions. Tick marks indicate wheel running activity. Representative chi-squared periodograms (E) of iMS-Bmal1−/− and iMS-Bmal1+/+ mice indicating approximate 24-h period lengths in both mice.
vehicle-treated mice (iMS-\textit{Bmal1}^{+/+}). Western blot analysis confirmed the depletion of BMAL1 protein in the skeletal muscle of the iMS-\textit{Bmal1}^{−/−} mice with no effect on the liver (\textbf{Figure 6B}). Tamoxifen-induced loss of \textit{Bmal1} in adult skeletal muscle resulted in significant and expected gene expression changes of genes involved in the core clock mechanism. In particular, genes directly activated by the BMAL1/CLOCK heterodimer, such as \textit{Rev-erba} and \textit{Dbp}, are markedly down-regulated in iMS-\textit{Bmal1}^{−/−} but not in the iMS-\textit{Bmal1}^{+/+} samples (\textbf{Figure 6C}). Collectively, these results demonstrate the effective loss of BMAL1 protein and disruption of core-clock gene expression in the iMS-\textit{Bmal1}^{−/−} muscle tissue.

2.3.2.1 iMS-\textit{Bmal1}^{−/−} display normal circadian activity rhythms

We used voluntary wheel running to assess circadian behavior in the iMS-\textit{Bmal1} mice 22 to 29 weeks post-treatment. We did not detect any significant differences in entrainment to light under 12-h light/12-h dark conditions between iMS-\textit{Bmal1}^{+/+} and iMS-\textit{Bmal1}^{−/−}, and analysis of activity rhythms under constant darkness did not reveal any changes in circadian behavior (\textbf{Figure 6D, E}). Clock-lab analysis indicates that both iMS-\textit{Bmal1}^{+/+} and iMS-\textit{Bmal1}^{−/−} exhibit approximate 24-h period lengths (23.85 ± 0.083 and 23.77 ± 0.138 h, respectively) with no differences in amplitude, the relative strength of the rhythm. These data are consistent with other studies and confirm that inactivation of BMAL1 in skeletal muscle does not directly alter circadian activity patterns.[68, 198] Therefore, gene expression changes observed in this model are more likely to be downstream of the endogenous molecular-clock mechanism in skeletal muscle.
2.3.3 Expression of key circadian metabolic genes are significantly altered in iMS-Bmal1−/− skeletal muscle

Gene expression analysis of iMS-Bmal1+/+ and iMS-Bmal1−/− muscle tissue reveals that the intrinsic molecular clock, even in constant conditions, plays a role in temporally regulating carbohydrate and lipid metabolism. We performed our transcriptome analysis at 5 weeks post-recombination to identify early gene expression changes caused by the loss of the clock mechanism in skeletal muscle. Analyzing gene expression at this time point also limits potential off-target effects of tamoxifen treatment by allowing for a sufficient wash-out period. We found that the circadian genes involved in carbohydrate metabolism were most affected by loss of Bmal1. The expression of the glycolytic enzymes, Pfkfb1, Pfkfb3, and Hk2 as well as the PDH phosphatase, Pdp1 were all significantly down-regulated in the gastrocnemius (Figure 7A). In addition, expression of the adrenergic receptor, Adrb2, was also significantly decreased. These genes are convincing clock-controlled candidates in skeletal muscle as they have circadian expression patterns similar to that of known clock-controlled genes (peak expression during inactive to active phase transition), and their loss of expression following Bmal1 inactivation is indicative of direct transcriptional regulation by the clock. By targeting these genes, the molecular clock mechanism can precisely regulate the timing of carbohydrate utilization to occur during the active phase. The observation that circadian genes involved in glucose utilization are diminished in our model is in agreement with the muscle-specific Bmal1 knockout model generated by Dyar et al. in which they report
Figure 7. Differentially expressed circadian, metabolic genes in iMS-Bmal1 −/− skeletal muscle. Average expression changes of the circadian carbohydrate (A) and lipid (B) genes in iMS-Bmal1 −/− gastrocnemius averaged over circadian times 18, 22, 26, 30, 34, and 38. Tibialis anterior and soleus gene expression changes (Dyar et al.) averaged over circadian times 0, 4, 8, 12, 16, and 20. The red line denotes control (iMS-Bmal1+/+) gene expression values. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.
significant decreases in glucose oxidation and insulin stimulated glucose uptake in their muscle tissues.[68]

Lipid metabolic processes appear to be elevated as the nuclear co-repressor, Nrip1, involved in repressing β-oxidation was significantly decreased with loss of Bmal1 (approximately 21% decrease, Student's t test P value = 0.019). Previous studies have shown that knockout of Nrip1 results in an increase in succinate dehydrogenase staining of gastrocnemius muscle consistent with a shift to slow oxidative fiber types.[140] Interestingly, the fatty-acid transporter, Fabp3, and the β-oxidation genes, Hadha and Hadhb, were significantly elevated in the iMS-Bmal1−/− gastrocnemius tissues (Figure 7B). Two circadian genes involved in triacylglycerol elongation, Pnpla3 and Elovl5, were also increased in the iMS-Bmal1−/−. Altogether, we report significant expression changes in circadian genes that are key regulators of metabolism in skeletal muscle. We think that the gene changes observed in iMS-Bmal1−/− are either directly or indirectly regulated downstream of BMAL1/molecular clock in skeletal muscle and not due to changes in external cues as circadian activity patterns in iMS-Bmal1−/− are indistinguishable from vehicle-treated controls. The observation that circadian genes involved in carbohydrate and lipid metabolism are disrupted in iMS-Bmal1−/− highlights a fundamental importance of the intrinsic molecular clock in temporal regulation substrate utilization and storage in skeletal muscle in the absence of external cues.
2.3.3.1 iMS-Bmal1−/− gene expression changes reveal a fast to slow fiber-type shift

Skeletal muscle is comprised of different fiber types that are differentiated based on contractile function as well as predominant substrate utilization.[199-202] For example, fast-type skeletal muscle s (type IIIX/IIB) primarily rely on ATP generated from anaerobic metabolism (glycolysis/lactic-acid fermentation) to provide quick energy sources required for short bursts of activity, while slow-type skeletal muscles and fast-type IIA muscles rely on oxidative metabolism to promote a more sustained and less fatigable bout of contractions. We analyzed changes in gene expression related to fiber type following Bmal1 ablation in adult skeletal muscle and included both circadian and non-circadian transcripts. We identified a selective increase in slow-type sarcomeric genes in the gastrocnemius muscles with a limited effect on fast-type sarcomeric genes (Figure 8A, B). We chose the list of ‘slow’ and ‘fast’ sarcomeric genes, because these have been shown to be significantly enriched in either slow-soleus or fast-EDL myofiber preparations.[203] Additionally, calcium handling genes and nuclear receptors common in slow-fiber muscles (for example, Casq2, Atp2a2, Ankrd2, Csrp3.) were significantly increased in iMS-Bmal1−/− (Table 1). Similar to the changes observed for the circadian metabolic genes, we see that non-circadian metabolic genes involved in carbohydrate metabolism are significantly decreased, while genes involved in lipid metabolism are increased (Tables 2 and 3). This switch from a fast to a slow fiber-type mRNA profile is in agreement with the observed metabolic changes as slow fiber-type muscles rely more heavily on oxidative metabolism compared to fast-type skeletal muscle.
Figure 8. Increase in slow type sarcomeric genes in iMS-Bmal1−/−. Average gene expression changes of slow (A) and fast (B) type sarcomeric genes in iMS-Bmal1−/− compared to control values (red line). *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.
<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Fast or slow</th>
<th>Gene description</th>
<th>ΔExpression (tam/veh)</th>
<th>Student’s T-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atp2a1</td>
<td>Fast</td>
<td>Calcium handling</td>
<td>0.99</td>
<td>ns</td>
</tr>
<tr>
<td>Atp2a2</td>
<td>Slow</td>
<td>Calcium handling</td>
<td>1.06</td>
<td>ns</td>
</tr>
<tr>
<td>Calm3</td>
<td>Fast</td>
<td>Calcium handling</td>
<td>0.84</td>
<td>***</td>
</tr>
<tr>
<td>Casq1</td>
<td>Fast</td>
<td>Calcium handling</td>
<td>1.00</td>
<td>ns</td>
</tr>
<tr>
<td>Casq2</td>
<td>Slow</td>
<td>Calcium handling</td>
<td>2.89</td>
<td>***</td>
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<td>ns</td>
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<td>Nuclear receptor</td>
<td>1.66</td>
<td>***</td>
</tr>
<tr>
<td>Csrp3</td>
<td>Slow</td>
<td>Nuclear receptor</td>
<td>2.13</td>
<td>**</td>
</tr>
<tr>
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<td>Slow</td>
<td>Nuclear receptor</td>
<td>0.88</td>
<td>ns</td>
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<td>Pdim1</td>
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<td>Nuclear receptor</td>
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<td>***</td>
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<td>Ppara</td>
<td>Slow</td>
<td>Nuclear receptor</td>
<td>1.23</td>
<td>*</td>
</tr>
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<td>Fast</td>
<td>Nuclear receptor</td>
<td>0.83</td>
<td>*</td>
</tr>
<tr>
<td>Sos2</td>
<td>Fast</td>
<td>Nuclear receptor</td>
<td>0.84</td>
<td>***</td>
</tr>
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</table>

Average gene expression changes of calcium handling and nuclear receptor genes in iMS-Bmal1-/-: ns, non-significant; * ≤ 0.01; *** ≤ 0.001.
### Table 2 Metabolic genes upregulated in iMS-Bmal1-/-

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene function</th>
<th>ΔExpression (tam/veh)</th>
<th>Student's T-test</th>
</tr>
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<tr>
<td>Agpat3</td>
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<td>***</td>
</tr>
<tr>
<td>Acadm</td>
<td>Lipolysis</td>
<td>1.31</td>
<td>***</td>
</tr>
<tr>
<td>Acot7</td>
<td>Lipolysis</td>
<td>1.18</td>
<td>***</td>
</tr>
<tr>
<td>Acot9</td>
<td>Lipolysis</td>
<td>1.44</td>
<td>**</td>
</tr>
<tr>
<td>Acsl1</td>
<td>Lipolysis</td>
<td>1.24</td>
<td>**</td>
</tr>
<tr>
<td>Cd36</td>
<td>Lipid transport</td>
<td>1.18</td>
<td>**</td>
</tr>
<tr>
<td>Cox5a</td>
<td>Electron transport chain</td>
<td>1.24</td>
<td>***</td>
</tr>
<tr>
<td>Cox6a1</td>
<td>Electron transport chain</td>
<td>1.30</td>
<td>*</td>
</tr>
<tr>
<td>Cpt2</td>
<td>Lipolysis</td>
<td>1.11</td>
<td>*</td>
</tr>
<tr>
<td>Fabp1</td>
<td>Lipid transport</td>
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<td>*</td>
</tr>
<tr>
<td>Fabp5</td>
<td>Lipid transport</td>
<td>1.29</td>
<td>**</td>
</tr>
<tr>
<td>Fads2</td>
<td>Lipogenesis</td>
<td>1.29</td>
<td>*</td>
</tr>
<tr>
<td>Ldhb</td>
<td>Lactate metabolism</td>
<td>1.33</td>
<td>***</td>
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<tr>
<td>Ndufa8</td>
<td>Electron transport chain</td>
<td>1.24</td>
<td>***</td>
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<tr>
<td>Ndufb8</td>
<td>Electron transport chain</td>
<td>1.18</td>
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<tr>
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<td>Lipogenesis</td>
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<td>***</td>
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<tr>
<td>Sdhc</td>
<td>Electron transport chain</td>
<td>1.18</td>
<td>***</td>
</tr>
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<td>Electron transport chain</td>
<td>1.21</td>
<td>**</td>
</tr>
<tr>
<td>Uqcr10</td>
<td>Electron transport chain</td>
<td>1.14</td>
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Average gene expression changes of metabolic genes that are significantly upregulated in iMS-Bmal1-/- skeletal muscle. *P ≤0.05; **P ≤0.01; ***P ≤0.001.
<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene function</th>
<th>ΔExpression (tam/veh)</th>
<th>Student's T-test</th>
</tr>
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<tr>
<td>Agl</td>
<td>Glycogenolysis</td>
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</tr>
<tr>
<td>Akt1</td>
<td>Glucose uptake</td>
<td>0.84</td>
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</tr>
<tr>
<td>Il15</td>
<td>Glucose uptake</td>
<td>0.86</td>
<td>*</td>
</tr>
<tr>
<td>Pak1</td>
<td>Glucose uptake</td>
<td>0.79</td>
<td>*</td>
</tr>
<tr>
<td>Pfkm</td>
<td>Glycolysis</td>
<td>0.81</td>
<td>***</td>
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<tr>
<td>Pgm2</td>
<td>Glycogenolysis</td>
<td>0.87</td>
<td>***</td>
</tr>
<tr>
<td>Phka1</td>
<td>Glycogenolysis</td>
<td>0.81</td>
<td>**</td>
</tr>
<tr>
<td>Prkab2</td>
<td>Glucose uptake</td>
<td>0.85</td>
<td>*</td>
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<tr>
<td>Prkag2</td>
<td>Glucose uptake</td>
<td>0.83</td>
<td>***</td>
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<tr>
<td>Prkag3</td>
<td>Glucose uptake</td>
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<td>Rab10</td>
<td>Glucose uptake</td>
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</tr>
<tr>
<td>Slc2a3</td>
<td>Glucose uptake</td>
<td>0.35</td>
<td>***</td>
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Average gene expression changes of metabolic genes that are significantly downregulated in iMS-Bmal1-/- skeletal muscle. *P≤0.05; **P≤0.01; ***P≤0.001.
2.3.4 Global Analysis of iMS-Bmal1⁻/⁻ transcriptome

The ablation of Bmal1 in the iMS-Bmal1⁻/⁻ skeletal muscle resulted in the large-scale disruption of circadian gene expression patterns well beyond that of the core-clock genes. The disruption in circadian rhythmicity is visually apparent in the heat-maps of the circadian genes in the iMS-Bmal1⁺/+ compared to iMS-Bmal1⁻/⁻ (Figure 9). These maps were generated by plotting the temporal expression patterns of the circadian genes in order of their acrophase. Due to the limited time-points within our transcriptome, we were unable to use JTK_CYCLE to statistically identify which genes were circadian or not in the iMS-Bmal1⁻/⁻. By analyzing the differential expression of the circadian genes, we observed approximately 297 and 205 genes either significantly up or down-regulated in iMS-Bmal1⁻/⁻, respectively. Phase analysis of these genes revealed a temporal component to the genes that were either up or down-regulated. The majority of genes that were down-regulated in iMS-Bmal1⁻/⁻ had a phase of gene expression in wild-type muscle around the transition from the inactive to active-phase (Figure 10). Inversely, the majority of genes that are up-regulated had a phase of gene expression around the transition from the active to inactive-phase. This unique pattern remains consistent when observing the core-clock gene changes in iMS-Bmal1⁻/⁻ as genes that are known BMAL1:CLOCK targets with phases around CT 12.0 are significantly decreased (e.g. Dbp, Per2, Rev-erb, Rora, etc.) (Appendix File 7). Interestingly, three core-clock genes (Clock, Nfil3, and Npas2) are up-regulated in the iMS-Bmal1⁻/⁻ tissue and reach peak expression in an antiphasic manner to that of the other core-clock genes (~CT 24.0) (Figure 11). Analysis of the gene expression profile in the iMS-Bmal1⁻/⁻ shows that Clock, Nfil3,
Figure 9. Heat-maps of the muscle circadian transcriptome genes in iMS-\textit{Bmal1}+/+ (left) and iMS-\textit{Bmal1}−/− (right) muscle tissue. Probsets are plotted by phase with high expression in yellow and low expression in blue over the circadian time-course from CT 18.0 to 36.0.
Figure 10. Diagram of the differentially expressed genes in iMS-\textit{Bmal1-/-} muscle tissue plotted according to the acrophrase of gene expression. Red bars indicate the total number of genes that were significantly up-regulated at least 20%, while black bars indicate the total number of genes that were significantly down-regulated 20% in the iMS-\textit{Bmal1-/-} time-course microarray data set compared to iMS-\textit{Bmal1+/+}. 
Figure 11. Average expression changes of the core molecular-clock genes in iMS-\textit{Bmal1}⁻/⁻ muscle tissue. The red line denotes control (iMS-\textit{Bmal1}⁺/+\) gene expression values. *\(P \leq 0.05\); **\(P \leq 0.01\); ***\(P \leq 0.001\).
and Npas2 are not only up-regulated at all time-points, but also seem to flat-line which may suggest a common upstream transcriptional regulator. Interestingly, all three of these genes contain RORE’s within their promoter regions and have been shown to be target for repression by REV-ERBα during the active phase. The expression of Rev-erbα is markedly decreased in the iMS-Bmal1 −/− as this gene is a direct BMAL1:CLOCK target. The loss of Rev-erbα expression in other genetic models have been shown to result in a de-repression of REV-ERBα target genes resulting in an increase in their expression similar to the up-regulated circadian genes in our analysis.[64, 204] The loss of Rev-erbα has also been associated with an increase in an oxidative phenotype.[204] From these observations we propose a model in which both limbs of the core-molecular-clock, BMAL1:CLOCK and REV-ERBα, function to rhythmically activate or repress gene expression during the active period (Figure 12). Further analysis involving a time-course ChIP-seq analysis for both BMAL1 and REV-ERBα would help to further elucidate potential mechanisms.

2.4 Conclusion

Here, we report that the intrinsic molecular-clock regulates the timing of genes involved in substrate catabolic and anabolic processes in skeletal muscle. We have identified the mid-inactive period as the time of peak expression of genes involved in fatty-acid breakdown, possibly serving as the main energy source to skeletal muscle during the overnight fasting period. The temporal expression pattern of genes that regulate glycolysis and glycolytic flux into the TCA’s cycle suggests a shift in substrate utilization during the early active period from lipids to carbohydrates,
Figure 12. Proposed model demonstrating how BMAL1:CLOCK promote the expression of glycolytic genes during the active phase while REV-ERBs repress lipogenic genes during the active phase. Loss of BMAL1 expression results in a decrease in the circadian and overall expression of glycolytic genes and REV-ERBs resulting in a de-repression of REV-ERB target genes.
which has previously been documented in other muscle-specific *Bmal1* knockout models.\[68\] Genes involved in glucose and lipid storage were observed as reaching peak expression toward the end of the active phase, where we predict excess energy is stored for usage during the post-absorptive phase. Expression analysis of time-course data from iMS-*Bmal1*−/− skeletal muscle revealed the differential expression of a number of key circadian metabolic genes in the absence of BMAL1. These findings suggest that the temporal regulation and circadian rhythmicity of these genes is directly downstream of the intrinsic skeletal muscle molecular-clock mechanism. We observe a gene expression profile that is indicative of a glycolytic to oxidative fiber type shift with loss of *Bmal1* in adult muscle tissue. These findings suggest a potential unidentified role of *Bmal1* in the maintenance of fast-type muscle fibers, possibly via direct transcriptional regulation of glucose handling. It is widely reported that aging is associated with a selective loss of fast-type skeletal muscle fibers.\[205, 206\] In addition, aging is also associated with decreases in the robustness of the molecular clock.\[69, 207\] These observations raise the possibility that fast to slow fiber-type shifts may be a result of dampening of the molecular clock with age. Lastly, our observations from the temporal phase analysis of the differentially expressed genes has led us to propose a potential mechanism in which the molecular-clock both activates and represses gene transcription during the active phase.
Chapter 3: The molecular-clock and MYOD1 promote the circadian expression of *Titin-cap* in skeletal muscle

3.1 Introduction

The molecular-clock is an endogenous, transcriptional-translational feedback mechanism responsible for the circadian regulation of genes and physiological processes in peripheral tissues.[4-6] In addition to its vital role in regulating metabolism, there is a growing recognition that the molecular-clock plays fundamental roles in both the development and maintenance of skeletal muscle.[66, 84] *Bmal1* and *CLOCK* are PAS-bHLH transcription factors that form the positive-limb of the molecular-clock.[45] *Bmal1*, in particular, has been shown to promote myogenesis via the activation of the myogenic regulatory factors (*MyoD1, Myf5, Myogenin*) and the Wnt-signaling cascade.[84] *Bmal1* is also involved in the regeneration of skeletal muscle, the process in which resident satellite cells are activated and fuse to form new muscle.[83] The myogenic and regenerative functions of *Bmal1* are even more so apparent in the skeletal muscle phenotypes present in *Bmal1−/−* and include: Significant decreases in specific-force generation, disruptions in myofibrillar organization, and decreases in total muscle-mass.[66] Although we did not observe significant decreases in muscle mass, our lab has observed similar decreases in specific force in an inducible skeletal muscle specific model suggesting that this effect is intrinsic to the molecular-clock in adult muscle (Manuscript Submitted JAP).

To understand how the molecular-clock regulates skeletal muscle physiology investigators have sought to identify CCGs and to understand the mechanisms in
which the clock regulates these genes.[208-211] It is currently unknown how the molecular-clock targets genes in a tissue specific fashion, although some have proposed this may occur through interactions between the clock machinery and resident tissue specific factors.[49, 212] The validation of the myogenic regulatory factor, *MyoD1*, as a direct target of the molecular-clock has raised the possibility that oscillations in this protein could be responsible for rhythmic regulation of muscle-specific processes. *Myod1*, along with the other myogenic regulatory factors *Myf6*, *Myogenin*, and *Mrf5* are considered ‘master-regulators’ of the transcriptional program that promotes skeletal muscle differentiation.[213] These proteins, like *Bmal1* and *Clock*, are bHLH factors that homo- and heterodimerize with themselves and other transcription factors and bind to canonical E-box elements (CANNTG) to promote myogenic gene expression.[214, 215] The activity of *MYOD1* to promote gene expression depends on the DNA-binding stability, intramolecular interactions that influence *MYOD1*’s conformation-state, and the presence/absence of co-factors that recruit additional transcriptional machinery and/or alter chromatin structure.[216-219]

In the present study, we test whether the molecular-clock and *MYOD1* work together to promote the circadian expression of the muscle-specific gene *T-cap*. We chose *T-cap* as a model gene to test this potential transcriptional mechanism in skeletal muscle as this gene has previously been identified as BMAL1:CLOCK target gene in heart-tissue, and is activated by *MYOD1* during myogenesis.[96, 99] Using a bioinformatics approach we first identify *T-cap* as a circadian transcript that is significantly down-regulated in our iMS-*Bmal1*−/− model. We demonstrate in C2C12
muscle-cells that the proximal promoter of T-cap is directly targeted by both the molecular-clock and MYOD1. Our study provides evidence of a novel mechanism in which that molecular-clock factors, BMAL1:CLOCK, work cooperatively with the myogenic regulatory factor MYOD1 to promote the circadian expression of T-cap.

3.2 Materials and Methods

3.2.1 Generation of iMS-Bmal1+/+ and iMS-Bmal1/- mice and protein isolation

Thirty-two adult iMS-Bmal1^floxfloxfloxflox mice (12-14 weeks old) were treated with Vehicle or Tamoxifen as described in section 2.4.3. Five weeks post injection 17 tamoxifen and 15 vehicle treated mice were entrained to a 12-h LD cycle for 14 days, and had ad libitum access to food and water. Following the entrainment period, the mice were euthanized at either CT2 or CT14 and gastrocnemius tissues from both hind limbs were flash frozen in liquid nitrogen and stored at -80°C. Tissues were weighed and homogenized mechanically in RIPA buffer (40 w/v) containing inhibitors (Complete Mini EDTA-Free Protease Inhibitor from Sigma Aldrich, 1% NaF, and 1% Na-Orthovandate) on ice. Cell-debris were pelleted at 10,000g for 10 minutes at 4°C and supernatants were quantified for protein concentration via Lowry method.

3.2.2 Western-blot

Whole cell lysates were prepared from gastrocnemius tissue from iMS-Bmal1^-/- and iMS-Bmal1^+/+, and liver lysates were prepared from wild-type mice as a negative control. Immunoblotting were carried out with routine protocols. Briefly, proteins were denatured for 5 minutes at 95°C in 5x reducing buffer. Approximately 40μg of total protein were added to each well of a SDS-PAGE gel containing a 4% stacking and 12% running gel, and proteins were separated by electrical current. The
proteins were then transferred to a PVDF membrane via a semi-dry transfer apparatus (BioRad). The membranes were placed in blocking solution (Odyssey) for 1 hr. The blots were then cut at approximately 35Kda, and the bottom portion was incubated with a monoclonal Anti-T-CAP primary antibody produced in mouse at a 1:200 dilution (Sigma-Aldrich, WH0008557M1) while the top portion was incubated with a polyclonal Anti-γ-tubulin primary antibody produced in mouse at a 1:5000 dilution (Sigma-Aldrich). The primary antibodies were detected by goat-anti-mouse Alexa Fluor® 647 secondary (Abcam150115) and visualized using the Odyssey system (Li-Cor, Lincoln, NE, USA).

3.2.3 RNA isolation and Real-time PCR

RNA was isolated from gastrocnemius tissue via TRizol method and cDNAs prepared as described in section 2.2.4. (RNA samples prepared in the previous study were used for this study). Real-time quantitative PCR was performed using TaqMan (Applied Biosystems, Waltham, MA, USA) assays to determine the gene expression of T-cap (MM00495557_g1) and the ΔΔCT method was used for the quantification of the real-time PCR data. The gene Rpl26 was quantified for normalization as this gene is highly expressed in skeletal muscle and does not oscillate over time of day.

3.2.4 Plasmids

The T-cap luciferase promoter-reporter construct was a gift from Dr. Davie from Southern Illinois University School of Medicine and contains a 421 base pair promoter fragment that spans from -421 to +1 of the translational start site.[99] The Bmal1 mutant vector BMAL1-R91A was a gift from Dr. Takahashi from the University of Texas Southwestern. The MYOD1-3Δ56 construct was a gift from Dr.
Rudnicki at the Ottawa Hospital Research Institute. The control plasmid we used as an empty vector control was the PGEM vector from Promega. The wild-type expression vectors used in this study include: MYOD1, BMAL1, CLOCK. In addition to the T-cap reporter, we also used a Bmal1: luciferase reporter that contains the Bmal1 promoter cloned into the pGL3 reporter vector.

3.2.5 Site-directed mutagenesis

The QuikChange Site-Directed Mutagenesis Kit was used to mutate the tandem E-box element in the proximal promoter region of the T-cap:Luciferase vector according to the manufacturers instructions. The forward and reverse primer sequences are as follows: Reverse-5'-ggcacatggcagcctgcacccaccttggcactgtc-3', Forward-5'-gacagtgcacaggtgggtgcaggctgccatgtgccc-3'. The mutated T-cap sequence can be found in Appendix File 3.

3.2.6 Dual-Luciferase Assay

The Dual-luciferase assay is commonly used to quantify the activity of the reporter (Firefly luciferase), and the co-transfection control (Renilla luciferase). The presence of this internal control serves as a baseline response, so variability due to pipetting error, cell lysis, or transfection efficiency are greatly diminished. To study the transactivation of T-cap:luciferase we performed transient co-transfections of the T-cap:luciferase promoter-reporter construct with expression plasmids in C2C12 myoblasts. For each well of a 24-well tissue culture plate we mixed 50ng T-cap:luciferase reporter, 5ng pRL reporter (Renilla), and 300ng each of the expression vectors (MYOD1, BMAL1, CLOCK, BMAL1-R91A, MYOD-3Δ56) in approximately 0.5mL of DMEM (no serum) and Xtreme-gene9 transfection reagent
5μl/1,000ng DNA. The DNA/transfection mix was then mixed with free-floating myoblasts (50,000/well) and plated with fresh GM (DMEM, 10%FBS, 1%Pen/Strep). Each transfection was performed in quadruplicate. GM was changed the following day and DM (DMEM, 2%HS, 1%Pen/Strep) was added at day 3. The cells were differentiated for 48-h in DM to form myotubes. The cells were lysed with 5x Passive Lysis Buffer (Promega) for 15 minutes shaking at room-temperature and cell extracts were then measured for Luciferase and Renilla activity according to the manufacturer’s instructions (Promega).

3.2.7 **Lumicycle**

Co-transfections were performed as described in the previous sections in C2C12 myoblasts. Approximately 150ng of *T-cap*:luciferase or *Bmal1*:luciferase reporter vectors were transfected per 35mm dish. Expression plasmids or negative controls (PGEM) were co-transfected with approximately 500ng per 35mm dish. C2C12 myoblasts were plated with the transfection/DNA mix at 300,000 cells per 35mm dish in fresh GM. After 48-h the GM was switched to DM, and the cells were differentiated for 5-full days (DM refreshed every other day). After differentiation, the myotubes were synchronized with dexamethasone (1μM) for 90 minutes, and then washed with PBS twice and 1.5mL of Recording buffer was added (DMEM-Phenol Red free, 10mM HEPES, luciferin 0.1mM, 1%Pen/Strep, 2% Horse Serum, sodium bicarbonate 350mg/L). Cells were then airtight sealed and placed in the lumicycle apparatus. A 32-well turn-table is fully automated so that the photomultiplier tubes captured and recorded the number of photons given off by each culture dish every five minutes. The luminometry data was stored as photon
counts/second on a computer using the lumicycle software by Actimetrix.

JTK_CYCLE analysis was performed on the lumicycle data as follows: The first 1.5 days of recording was excluded to allow the cells to equilibrate fully after changes in temperature and media that occur during setup. We condensed the data for ease of handling and improved computational efficiency by binning data-points from every 5-minutes to two hour intervals. The relative phase is a measurement of the initial peak following the beginning of the analysis (CT 1.5).

3.2.8 Chromatin Immunoprecipitation

To determine binding of BMAL1 and MYOD1 on the T-cap promoter regions we performed ChIP analysis with primers that scan the tandem-E-Box element and primers for the most proximal E-Box element on the T-cap promoter. We performed ChIP analysis as described in Andrews et al. 2010.[66] Briefly, C2C12 myotubes were synchronized with 1μM dexamethasone for 90 minutes then crosslinked with 1% formaldehyde solution at room temperature for 11 minutes. Following sonication, the fragmented chromatin was pre-cleared with protein-A/G beads and then immunoprecipitated with antibodies against IgG, BMAL1 (Abcam), and MYOD1 (Santa-Cruz). Antibodies were then pulled down with protein-A/G beads, washed, and the DNA was eluted using the QIAquick purification kit (Qiagen). Quantitative Real-Time PCR (SyberGreen method) was performed using DNA from the input reaction, serum, and pull-downs from IgG, MYOD1, and BMAL1. Primers for the T-cap promoter are as follows: Tandem-E-Box element Forward:5’-AGCAGAGGAAGACAGTGCCAA-3’, Reverse:5’-AAGATGCTCTGTGGGACCTG-3’; proximal E-Box element Forward:5’-CAGGTCCCAACAGACACATCTT-3’, Reverse:5’-
GGGGCTATTTTCAGCCCCTCTG-3’. Primers for the *Myogenin* promoter were used as a positive control for MYOD1 binding, and primers for the *Period-2* promoter were used as a positive control for BMAL1 binding. To visualize the qRT-PCR reaction, the samples were run on a 4% stacking, 10% polyacrylamide gel, stained with a fluorescent DNA stain, and imaged with a Typhoon scanner.

3.2.9 **Co-Immunoprecipitation**

To determine if BMAL1 and MYOD1 bind in a protein:protein interaction we used the Nuclear Complex Co-IP kit (Active Motif) according to the manufacturer’s instructions. To improve specificity and strength of the pull-down we transiently transfected C2C12 myoblasts with expression vectors for BMAL1-HA (Hemagglutinin) & MYOD1, or MYOD1-HA & BMAL1. We performed the pull-down reactions with an anti-HA (Sigma Aldrich) and anti-IgG antibodies. Western-blots were then performed with antibodies against BMAL1 (for MYOD-HA pull-down) or MYOD1 (for BMAL1-HA pull-down).

3.3 **Results and discussion**

3.3.1 **Circadian Bioinformatics and Identification of a Candidate Clock-Controlled Gene: Titin-Cap (Tcap)**

The decreases observed in specific-force generation in the skeletal muscle of iMS-*Bmal1*/*−−* mice suggests that the molecular-clock mechanism may be sufficient to modulate the expression of genes involved in muscle structure and/or function. We employed a circadian bioinformatics workflow to determine if *T-cap* displays characteristics of a clock-controlled gene in skeletal muscle. **Figure 13** outlines the
Figure 13. Schematic of the bioinformatics workflow to identify skeletal muscle specific clock-controlled genes.

Bioinformatics Workflow

**Circadian Expression in Gastrocnemius**
- JTK_Cycle p-value < 0.05
  - ~1,368 genes

**Expression Similar to Known CCGs**
- Dbp Spearman’s Rho p-value < 0.05
  - 161 genes

**Significant Decrease in Expression in iMS-Bmal1-/**
- T-test p-value < 0.05
  - 68 genes

**Enriched in Skeletal Muscle**
- Bio-GPS
- Fold Change > 3.0
  - 5 genes
bioinformatics workflow that resulted in the identification of *T-cap* and four other genes that adequately fit our criteria as potential skeletal muscle specific CCGs. A brief description of our analysis is as follows: To identify genes that are direct targets of BMAL1:CLOCK we chose to focus on circadian genes with similar gene expression patterns as that of the clock-controlled gene *Dbp*. To do so we utilized the Spearman’s Rho rank correlation coefficient test, which is a non-parametric test commonly used to statistically identify similar patterns. As was established in chapter 2, the majority of known BMAL1:CLOCK targets displayed decreases in overall gene expression in the iMS-*Bmal1*/*- muscle tissue, so we next choose to focus on genes that are significantly decreased with loss of *Bmal1*. At this step, 12 of the 68 genes were known targets of BMAL1:CLOCK, a finding that suggests the genes within this list are strong CCG candidates. Lastly, we choose to identify the genes that are highly enriched in skeletal muscle yielding the 5 muscle-specific CCGs including *T-cap*. **Figure 14** demonstrates the circadian pattern of T-cap mRNA expression generated from the Hogenesch circadian time-course microarray. JTK_CYCLE analysis of the *T-cap* expression profile found the transcript to be highly statistically circadian (p-value: 0.00031). *T-cap* is one of the most highly expressed transcripts in skeletal muscle, and compared to the circadian muscle transcriptome its expression ranks in the 99th percentile. Real-time PCR was performed in iMS-*Bmal1*/*+* and iMS-*Bmal1*/*- muscle-tissue (gastrocnemius) to further validate the oscillation of *T-cap* mRNA that was observed in the Hogenesch time-course microarray. We observed a circadian gene expression pattern of *T-cap* with peak expression occurring at the transition from inactive to active-phases in the iMS-
Figure 14. Temporal expression pattern of $T$-$cap$ from the Hogenesch skeletal muscle circadian transcriptome. The black circles represent the expression values of $T$-$cap$ at each circadian time-point. The dotted line is a 6-degree polynomial fitted to the $T$-$cap$ expression data. Grey bars indicated the active period, and white bars indicate the inactive period. Note that mice were in constant darkness during the time-course collection. JTK_CYCLE p-value 0.00031, period 24h, phase CT12.
Bmal1+/+ muscle (Figure 15). The temporal expression pattern of T-cap is indicative of a BMAL1:CLOCK down-stream target gene as direct CCGs typically reach peak expression in an anti-phasic manner to that of Bmal1. These findings are in agreement with that of T-cap’s circadian expression profile that has been documented in heart-tissue. [96] As expected, the circadian expression pattern of T-cap was completely abolished in the iMS-Bmal1−/− muscle tissue. Additionally, the overall gene expression of T-cap was reduced on average ~66%. Since the activity pattern of iMS-Bmal1−/− mice is indistinguishable from that of the controls, we argue that T-cap expression is regulated downstream of the molecular-clock, and that the observed expression changes are caused by the loss of Bmal1 in skeletal muscle.

3.3.2 T-cap is circadian in wild-type skeletal muscle

Although circadian transcriptome analyses are very accurate at identifying oscillatory transcripts, it is becoming increasingly evident in the field of circadian rhythms that protein expression does not always mirror that of the transcript so we quantified T-CAP protein expression over time. Western-blot analysis of T-CAP from wild-type skeletal muscle (gastrocnemius) demonstrates the circadian oscillation of T-CAP protein with peak expression occurring during the early active-phase (Figure 16). The finding that T-CAP oscillates at the protein level suggests that the relative T-CAP protein content, at any given circadian time, may be directly proportional to the relative amount of T-cap transcript levels. T-CAP protein levels seem to follow the mRNA expression pattern with a slight-lag, which is typical of a circadian gene. These observations do not however discount the possibility that T-CAP protein expression may also be temporally modulated by post-translational
Figure 15. *T-cap* real-time PCR results of time-course expression values in the iMS-*Bmal1*+/+ (black) and iMS-*Bmal1*−/− (red). Fold difference was calculated from control, Rpl-26 Real-Time PCR for each time-point. Grey bars indicated the active period, and white bars indicate the inactive period. Mice were in constant darkness during the time-course collection.
Figure 16. Representative western-blot (A) of T-CAP and γ-TUBULIN in wild-type gastrocnemius tissue from CT 18.0 to 46.0. Quantification (B) of the T-CAP to γ-TUBULIN loading control in the skeletal muscle tissue. JTK_CYCLE p-value:0.0089, period 24h, phase CT0. Wild-type liver tissue was used a negative control for the T-CAP antibody.
mechanisms. T-CAP is among a handful of sarcomeric proteins ubiquitinated by the muscle-specific E3-ubiquitin ligase, MURF1 (Trim63) and is targeted for degradation by the 26S proteasome.[220] Interestingly, we found that Trim63/Murf1 is circadian in skeletal muscle (p-value 0.0024), and is expressed in an anti-phasic manner to that of T-cap (phase CT23.0) (Appendix File 8). We predict Murf1 levels to be highest at the end of the active-phase. During the inactive-phase, in addition to lower transcript levels caused by a decrease in BMAL1:CLOCK transactivation, the skeletal muscle may also more readily target T-CAP for degradation by MURF1 to keep T-CAP protein levels low during this time period. Additional experimentation is required to determine whether T-CAP is modified post-translationally, or degraded in a circadian manner as these experiments are outside the scope of this dissertation.

3.3.3 T-cap’s expression and circadian oscillation are dampened in iMS-Bmal1−/− muscle tissue

The iMS-Bmal1−/− transcriptome analysis (section 2.3.4) revealed large-scale disruptions of the circadian transcripts. BMAL1, together with CLOCK/NPAS2, function as the positive limb of the molecular-clock.[7] To determine if T-CAP protein was also reduced in the iMS-Bmal1−/−, we compared protein expression via western-blot of muscle extracts from iMS-Bmal1+/+ and iMS-Bmal1−/− muscle tissue at CT2 and CT14 (n=8 per treatment and time-point). These times were chosen based on the temporal gene expression pattern of T-cap as CT2 represents 2 hours into the inactive-phase when mRNA expression is dampening, while CT14 is 2 hours into the
active-phase when mRNA expression is highest. Overall T-CAP protein was greatly diminished in iMS-Bmal1−/− muscle tissue as to be expected from the real-time PCR results (Figure 17). These results further argue that T-CAP protein expression is regulated by the relative amounts of T-cap mRNA present within the cell.

3.3.3.1 *T-cap*:luciferase oscillates in an anti-phasic manner to *Bmal1*:luciferase *in vitro*

It is widely known that the timing of the molecular-clock can be influenced by numerous environmental time-cues (Zietgeibers).[49] For instance, the skeletal muscle clock phase-shifts in response to external time-cues, such as time of exercise.[73] External environmental stimuli and downstream cell-signaling cascades can also influence gene expression in the absence of the molecular-clock mechanism. In this manner it is possible for genes to oscillate in a circadian manner without being directly regulated by the molecular clock. The observation that *T-cap* mRNA and protein oscillates in a circadian manner in skeletal muscle suggests that it is regulated downstream of the endogenous molecular clock, but the *in vivo* setting in which these assays were performed raises the possibilities of confounding factors such as eating or activity patterns (both highly circadian in nature) potentially influencing the *T-cap* circadian oscillation. In order to account for the presence of such confounding factors we decided to assay the circadian rhythmicity of *T-cap* in an *in vitro* environment null of external time-cues. For our analysis we choose to record *T-cap* promoter:reporter luciferase bioluminescence in
Figure 17. Representative western-blot (A) of T-CAP and γ-TUBULIN in wild-type gastrocnemius tissue from iMS-Bmal1+/+ and iMS-Bmal1−/− at CT 2.0 and 14.0. Quantification (B) of the T-CAP to γ-TUBULIN loading control. Wild-type liver tissue was used a negative control for the T-CAP antibody. ***P ≤ 0.001.
synchronized C2C12 myotubes over multiple circadian cycles. The T-cap promoter:reporter construct we utilized in our study consists of a 421 base-pair proximal-promoter region cloned upstream of Firefly luciferase gene (Appendix File 10). To detect T-cap bioluminescence we used a Lumicycle incubator (Actimetrix), as this is a powerful tool for measuring multiple parameters of circadian rhythmicity (period length, amplitude, acrophase), because it allows for the recording of bioluminescence for successive days with high temporal resolution.[221] We observed a robust circadian oscillation of T-cap:luciferase activity with an approximate 22 to 24 hour period length in C2C12 myotubes (Figure 18). Furthermore, the T-cap:luciferase circadian pattern is antiphasic to that of Bmal1:luciferase activity in C2C12 myotubes synchronized at the same time. The observation that the T-cap:Luciferase reporter displays a circadian oscillation in C2C12 myotubes indicates that the T-cap promoter contains the essential elements necessary for downstream regulation by the endogenous molecular-clock in the absence of external time-cues. The antiphasic manner in which T-cap:luciferase is expressed in vitro, in comparison to Bmal1:luciferase, is very typical of a CCG and further supports the direct regulation of T-cap by the BMAL1:CLOCK transcription factors.

3.3.4 BMAL1:CLOCK transactivate T-cap:luciferase activity

The significant decreases in T-cap expression in iMS-Bmal1−/− muscle tissue argues that T-cap is transcriptionally regulated down-stream of the clock, but does not discount the possibility that T-cap mRNA and protein are decreased due to an
Figure 18. Representative graph of baseline subtracted Bmal1:luciferase (black circles) and T-cap:luciferase (red circles) over days in culture. Error bars denote SEM. T-cap:luciferase-JTK_CYCLE: p-value 8.53E-9, period 22, relative phase CT1. Bmal1:luciferase-JTK_CYCLE: p-value 2.12E-8, period 22, relative phase CT13.
alternative mechanism. For instance, \textit{T-cap} expression could dampen in response to \textit{Bmal1} knockout via alternative mechanisms such as enhanced mRNA instability or protein turnover since we have observed the circadian oscillation of a number of genes that encode muscle specific E3-ubiquitin ligases. Therefore, we sought to determine if BMAL1:CLOCK transcriptionally activate \textit{T-cap} expression in skeletal muscle by performing a series of dual-reporter assays (Dual-Luciferase Reporter Assay System, Promega) in C2C12 muscle cells co-transfected with \textit{T-cap}:Luciferase (reporter), \textit{Renilla luciferase} (control-reporter), and expression plasmids for BMAL1 and CLOCK. The dual-reporter system is commonly used to accurately quantify transactivation of promoter-reporter constructs. Overexpression of the molecular-clock factors, BMAL1:CLOCK, appeared to drive a slight increase \textit{T-cap}:Luciferase activity (~2-fold) in C2C12 myotubes (Figure 19). A possible explanation for why BMAL1:CLOCK do not drive a robust increase in \textit{T-cap}:Luciferase activity, is that additional factor(s) are required to promote \textit{T-cap} expression in skeletal muscle. Interestingly, we observed no apparent increase in \textit{T-cap}:Luciferase activity when we over-expressed a mutant form of BMAL1 that contains an A91G point mutation rendering the protein incapable of binding DNA (Figure 19).

3.3.5 \textbf{MYOD1} works in a synergistic fashion with BMAL1:CLOCK to drive \textit{T-cap} expression

Zhang \textit{et al.} have previously shown that the \textit{T-cap} promoter is responsive to the muscle specific transcription factor MYOD1 in NIH-3T3 fibroblasts.[99] Additionally, this group suggests that MYOD1 plays a role in promoting \textit{T-cap} expression during
Figure 19. Dual-luciferase results for T-cap:luciferase reporter co-transfected with expression plasmids for BMAL1 and CLOCK, or BMAL1-R91A mutant and CLOCK normalized to the empty vector control. n=3/sample.
development as they demonstrated MYOD1 binding to the T-cap promoter during embryogenesis.\cite{99} We observed a 5-fold induction of T-cap:luciferase activity with transient transfection of MYOD1 in C2C12 myotubes compared to the empty vector pGL3 control (Figure 20). The MYOD1 protein contains multiple domains that function to promote gene expression in several ways.\cite{222} MYOD1 contains a transactivating domain (TAD) involved in protein:protein interactions that recruit transcriptional machinery and other transcription factors to myogenic promoter regions.\cite{223, 224} MYOD1 also contains two chromatin remodeling domains that recruit histone acetylases to promote euchromatin formation to allow additional transcription factors to more readily access the DNA.\cite{225} To determine the functional importance of MYOD1’s TAD in regulating T-cap expression we repeated the dual-luciferase assay with a MYOD1 mutant expression plasmid, Myod1-3\Delta56, that lacks the TAD.\cite{224} We observed no enhancement of T-cap:luciferase activity with overexpression of Myod1-3\Delta56, and luciferase activity appeared to be dampened below that of the control levels (Figure 20). These results suggest that the MYOD1 mutant may be acting in a dominate-negative fashion, where it may be out-competing endogenous MYOD1 for the T-cap promoter.

To test whether MYOD1 is required for BMAL1:CLOCK mediated transactivation of T-cap we measured T-cap:luciferase activity in C2C12 myotubes co-transfected with MYOD1 and BMAL1:CLOCK. Interestingly, over-expression of MYOD1 with BMAL1:CLOCK resulted in a \~20 fold induction of T-cap:luciferase activity (Figure 21), which is approximately 10 fold higher than BMAL1:CLOCK over-expression alone. These data indicate that MYOD1 and the molecular-clock factors work in a
Figure 20. Dual-luciferase results for T-cap: luciferase reporter co-transfected with expression plasmids for MYOD1 and MYOD1-3Δ56 mutant normalized to the empty vector control. n=3/sample ***P ≤ 0.001 compared to empty vector control.
Figure 21. Dual-luciferase results for T-cap: luciferase reporter co-transfected with expression plasmids for MYOD1+BMAL1+CLOCK, MYOD1+BMAL1-R91A mutant+CLOCK, and MYOD1-3Δ56 mutant+BMAL1+CLOCK normalized to the empty vector control. n=3/sample ***P ≤ 0.001 compared to empty vector control.
synergistic fashion to promote $T$-cap expression, as the induction of $T$-cap:luciferase activity is much greater than one would expect if the interaction was additive. The ability of BMAL1:CLOCK to transactivate $T$-cap was abolished with over-expression of the mutant form of MYOD1 (Figure 21), suggesting that MYOD1 function is required for the molecular-clock to promote $T$-cap expression in skeletal muscle. A number of possibilities exist that would help explain as to why $T$-cap oscillates in heart tissue in the absence of MYOD1. It is possible that BMAL1:CLOCK interact with a heart-specific co-factor that is required for the expression of $T$-cap in the heart such as the heart-specific bHLH transcription factors Hand1 and Hand2. Another possibility is that skeletal muscle possesses a negative-regulator that suppresses the ability of BMAL1:CLOCK to transactivate $T$-cap expression in the absence of MYOD1.

3.3.5.1 MYOD1 enhances the amplitude of the $T$-cap:luciferase oscillation

To further explore the role MYOD1 plays in regulating the circadian expression of $T$-cap we assayed $T$-cap activity (lumicycle) for approximately 4.5 days while over-expressing wild-type or mutant MYOD1. In addition to enhancing $T$-cap expression, MYOD1 over-expression promoted a $\sim$10-fold increase in the amplitude of the $T$-cap:Luciferase rhythm (Figure 22A). The increases in the amplitude of the rhythm seemed to have little effect on period length or phase. We observed a marked decrease in the amplitude of the $T$-cap:Luciferase rhythm with over-expression of MYOD1-3Δ56(Figure 22B). Interestingly, the circadian pattern of the $T$-cap-
Figure 22. Representative graph of baseline subtracted $T$-cap:luciferase reporter values with co-transfection of MYOD1 (A) and MYOD1-3Δ56 (B). Error bars denote SEM. Red circles indicate the co-transfected values. The black circles represents $T$-cap:luciferase values with no co-transfection. $T$-cap:luciferase-JTK_CYCLE: p-value 2.07E-11, period 20, relative phase CT 5.0. $T$-cap:luciferase+MYOD1-JTK_CYCLE: p-value 2.87E-8, period 22, relative phase CT 3.0. $T$-cap:luciferase+MYOD1-3Δ56-JTK_CYCLE: p-value 8.09E-11, relative phase CT 2.0.
luciferase rhythm appeared to be unaltered with over-expression of the MYOD1-3Δ56 construct, suggesting that MYOD1 may only regulate the transactivation and amplitude of the T-cap oscillation while having minimal control on the actually timing of the rhythm. MYOD1 protein expression peaks in the mid-inactive phase (~CT 6.0), which is also approximately when BMAL1:CLOCK reaches peak transcriptional activity.[66] These data suggest that BMAL1:CLOCK and MYOD1 may transactivate genes during the inactive-phase to promote CCG expression to peak during the early active-phase. Further temporal binding and transcriptional studies are required to determine if MYOD1 and BMAL1:CLOCK target the same promoter regions, and whether they bind at similar times through the day.

3.3.6 BMAL1 and MYOD1 bind to the tandem E-box element in the T-cap promoter

Two E-box elements have previously been identified within the T-cap promoter.[99] Zhang et al. demonstrated MYOD1 binding on the proximal E-box element during embryogenesis, while Podobed et al. have shown BMAL1 binding to the distal tandem E-box element in heart tissue.[96] We performed a ChIP assay to determine if both BMAL1 and MYOD1 bind to the T-cap promoter in C2C12 myotubes. Compared to IgG control, we observed an 8 and 3-fold enrichment of BMAL1 and MYOD1 binding on the distal portion of the T-cap promoter where the tandem E-box element is located, respectively (Figure 23A, B). While we did not detect any BMAL1 binding on the proximal portion of the T-cap promoter, we did see a small
Figure 23. Representative ChIP real-time PCR products (A) for the region of the T-cap promoter containing the tandem-E-Box element. Real-time PCR results for IgG, BMAL1, MYOD1, and serum as percent of the Input values for binding to the tandem E-Box element (B), the Myogenin promoter (C), and the Period-2 promoter. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.
amount of MYOD1 binding (data not shown). As positive controls for our antibodies we observed binding of MYOD1 on the Myogenin promoter, and BMAL1 binding on the Period-2 promoter (Figure 23C, D).

3.3.6.1 The tandem E-box in the T-cap promoter is required for BMAL1:CLOCK and MYOD1 transactivation and circadian expression pattern

Due to the observed binding of both BMAL1 and MYOD1 within the region of distal promoter region containing the tandem E-box element, we aimed to identify whether the tandem E-box element is required for BMAL1:CLOCK/MYOD1 mediated transactivation and/or the circadian rhythmicity of T-cap. To this end, we mutated the 5’-E-box of the tandem E-Box element of the T-cap:luciferase reporter by site-directed mutagenesis (CAGCAG to GTGCAG), and performed a series of dual-reporter and lumicycle assays. The mutated T-cap reporter (denoted as T-mut) was unresponsive to transactivation by over-expression of BMAL1:CLOCK, MYOD1, and BMAL1:CLOCK+MYOD1 compared to empty vector controls (Figure 24). The T-mut reporter also displayed abnormal temporal expression patterns, and the MYOD1 mediate enhancement of the amplitude observed for the wild-type T-cap:luciferase reporter was abolished (Figure 25A, B). Along with the ChIP studies, these data indicate that BMAL1:CLOCK and MYOD1 interact with the tandem E-box element within the T-cap promoter, and this element is required for the circadian expression pattern of T-cap.
Figure 24. Dual-luciferase results for the mutated T-cap:luciferase reporter co-transfected with expression plasmids for MYOD1, BMAL1+CLOCK, and MYOD1+BMAL1+CLOCK normalized to the empty vector control. n=2/sample.
Figure 25. Representative graph of baseline subtracted *T-cap*:luciferase (black) and *Tmut*:luciferase (red) reporter values (A). *T-mut*:luciferase reporter values co-transfected with MYOD1 (red) or no co-transfection (black) (B). Error bars denote SEM. *T-cap*:luciferase-JTK_CYCLE: p-value 2.94E-16, period 20, relative phase CT12. *T-mut*:luciferase-JTK_CYCLE: p-value 1, period n.a., relative phase n.a.
3.3.7 **MYOD1 and BMAL1 do not form a protein:protein interaction in C2C12 myotubes**

The synergistic interaction of MYOD1 and BMAL1:CLOCK, and the observed increases in the circadian amplitude of *T-cap*:luciferase with overexpression of MYOD1 suggests the potential for these factors to physically interact. To test this potential interaction we performed a series of co-immunoprecipitation assays. We first over-expressed either MYOD1 or BMAL1 fusion proteins contacting a hemaglutanin tag on their C-terminal end (MYOD1-HA, BMAL1-HA). We then pulled down these proteins using an antibody against hemaglutanin (IgG was used as the control pull-down). Western-blotting for either BMAL1 (bound to MYOD1-HA) or MYOD1 (bound to BMAL1-HA) did not produce sufficient bands, and we therefore do argue that these factors do not directly dimerize (Figure 26).

3.3.8 **Conclusion**

Here we report the identification and validation of *T-cap* as a direct clock-controlled gene in skeletal muscle. The temporal expression pattern of the T-CAP protein suggests that it is most-highly expressed during the active phase of the circadian cycle. At this time, the potential functional consequences of rhythmic T-CAP expression are unknown, but the highly conserved E-box elements within its promoter and the complex nature in which it is transcriptionally regulated suggests that this is more than a mere vestigial mechanism in skeletal muscle. The novelty of this study exists not so much in the identification of *T-cap* as a CCG, but in the observations that a muscle-specific transcription factor, MYOD1, can work in a synergistic nature with the molecular-clock to drive the expression of a muscle-
Figure 26. Representative MYOD-HA and IgG CoIP (A) western-blottting for BMAL1 and BMAL1-HA and IgG CoIP (B) western-blottting for MYOD1.
specific gene. The fact that *T-cap* is robustly circadian in heart-tissue, which does not express MYOD1, suggests the existence of an unknown heart-specific transcription factor. Additional studies are required to fully elucidate the molecular mechanisms in which these co-factors work together, but it is clear, at-least in our hands, that MYOD1 and the molecular-clock factors BMAL1:CLOCK do not bind in a protein:protein interaction.

One possible mechanism in which muscle-specific genes are targeted for circadian oscillation is that MYOD1 and/or BMAL1:CLOCK work as pioneering factors that recruit chromatin remodeling enzymes to expose binding elements within promoter regions. Previous studies have shown the ability of BMAL1:CLOCK to recruit enzymes to the DNA to promote the rhythmic opening of the chromatin structure, and thus promote the rhythmic binding of adjacent transcription-factors.[226] The demonstration that viral vectors undergo epigenetic changes and incorporate nucleosomes in a similar fashion to genomic DNA promotes the likelihood of the ‘pioneering-factor’ hypothesis in the explanation of our results utilizing the pGL3-basic luciferase reporter.[227, 228] Multiple findings in our current study support the possibility that BMAL1:CLOCK are acting as pioneering factors to regulate the rhythmic binding and/or activation of MYOD1 to promote the circadian oscillation of muscle-specific genes. Most notably, the finding that over-expression of wild-type MYOD1 enhances the amplitude of the *T-cap* circadian rhythm. In theory, use of a viral-vector to over-express MYOD1 should result in a non-oscillating steady state of MYOD1 protein at all circadian time-points, and therefore the oscillation of *T-cap*:luciferase activity must be due to the circadian oscillation in BMAL1:CLOCK
activity. This is further supported by the \textit{T-cap} expression iMS-\textit{Bmal1}−/− muscle tissue that displays a very low and non-oscillatory expression pattern. Additionally, the dual-reporter assays in C2C12 muscle cells, and the fact that these transcription factors bind at the same DNA motif, both highly suggest that \textit{MYOD1} and BMAL1:CLOCK work as co-activators, possibly in a larger transcriptional complex.

4 Chapter 4: Future Directions

4.1 Circadian Regulation of Substrate Metabolism in Skeletal Muscle

The idea that metabolic tissues regulate the timing of anabolic and catabolic metabolism is not novel, nor is the notion that the molecular-clock regulates many metabolic processes throughout the body. The novelty of our study lies in our approach to identify the acrophase (or peak expression) of metabolic genes, and our observations that genes encoding rate-limiting metabolic enzymes are functionally clustered in time. To our knowledge we are the first to utilize JTK\_CYCLE’s ability to accurately identify the acrophase of circadian genes in skeletal muscle, which has allowed us to make predictions about how the molecular-clock temporally regulates substrate metabolism. From our results we would predict that skeletal muscle is primed for lipid-oxidation in the early active period, carbohydrate utilization in the mid-active period, and glycogen and lipid storage toward the end of the active phase. Since our predictions are based solely on transcript level data we require a multitude of additional studies to determine if the circadian metabolic genes we have identified are rhythmic at the protein-level and whether this translates into varying rates of substrate utilization/storage throughout the day. One global
approach would be to perform a time-course metabolomics study in wild-type mice to assay substrate levels and metabolic rates throughout the day. Analysis of the gene changes in the iMS-Bmal1−/− skeletal muscle has provided clues as to what metabolic genes are downstream of the molecular clock. We predict that BMAL1:CLOCK target genes involved in catabolic processes to be expressed during the early active period, while we predict REV-ERB’s to target genes involved in anabolic processes to be repressed during the active period. A time-course ChIP-Seq analysis of wild-type skeletal muscle probing for both BMAL1 and REV-ERBα would help to elucidate target genes, as well as a temporal landscape of binding.

4.2 Muscle Specific Regulation of Clock-Controlled Genes

The observation that the molecular-clock directly targets the z-line protein T-cap suggests a novel mechanism whereby the maintenance of myofibrillar structure, and thus muscle function, are temporally regulated. The protein expression profile of T-CAP suggests that this molecule reaches peak levels during the mid- to late-active phase. One possible explanation for the functional importance of the temporal expression profile of T-CAP is to add or subtract stability to the myofibrils in relation to the amount of stress the muscle will experience. In effect, having high levels of T-CAP at the z-line could provide more stability and rigidity to the myofibrils during the active period when the demand on skeletal muscle is high. Since skeletal muscle is in a state of partial paralysis during sleep this period coincides with a decreased demand for the muscle. During the inactive period low T-CAP levels could result in a partial relaxation of the myofibrils. It is possible that
repair mechanisms are under temporal regulation and the inactive phase may be an optimal time to repair/reorganize the structural components within muscle. The observations that Murf1 mRNA is highly circadian and peaks just prior to the inactive period further supports the possibility that sarcomeric proteins are targeted for proteasomal degradation during the inactive period. As myofibrillar proteins turnover, the presence of a partially relaxed structure may facilitate the incorporation of newly translated proteins and sarcomeric units. T-cap has also been shown to interact with multiple t-tubule proteins to direct the localization of the neuromuscular junction to the z-line. Therefore, the functional importance of the T-CAP oscillation in muscle may also be to promote a time of day sensitivity for excitation-contraction coupling as well as calcium induced intracellular signaling and mechanotransduction.

We have provided evidence that the molecular-clock factors BMAL1:CLOCK work in a synergistic fashion with the myogenic regulatory factor MYOD1 to promote the circadian expression of the muscle-specific gene T-cap. The findings in this study provoke numerous questions regarding the interaction of MYOD1 with BMAL1:CLOCK. Firstly, is this interaction specific to T-cap or do MYOD1 and BMAL1:CLOCK target the majority of muscle-specific circadian genes? Our lab is actively pursuing these questions by performing a time-course ChIP-Seq analysis for BMAL1 and MYOD1. These experiments will allow us to determine whether BMAL1:CLOCK and MYOD1 target the same circadian genes, and will also allow us to quantify the extent of binding over time of day. This may provide clues as to how these factors are working. For instance we may be able to predict if BMAL1:CLOCK
or MYOD1 work as pioneering factors by determining which factors bind to the promoter regions first. We have demonstrated that the tandem-E-Box element within the proximal promoter of T-cap appears to be the primary binding site for BMAL1:CLOCK and MYOD1, and that it is absolutely essential for the circadian expression of T-cap. Using the ChIP-Seq data as a guide we could perform a bioinformatics study in which we examine the promoters of muscle-specific genes to determine if the tandem-E-Box elements are essential for BMAL1:CLOCK and MYOD1 cooperatively. We provided evidence that MYOD1 function is required for the ability of BMAL1:CLOCK to drive T-cap expression (mutant MYOD1 assays). Additional studies are required to determine if other myogenic regulatory factors, such as MYOGENIN, can be substituted for MYOD1 and still promote the circadian oscillation of T-cap.
Chapter 5: Dissertation summary

Through the use of high-resolution time-course microarray data we were able to identify a significantly large number of genes expressed in a circadian manner in skeletal muscle. Utilizing the circadian statistical algorithm, JTK_CYCLE, we were able to accurately identify the acrophase of metabolic genes, which resulted in the observations that catabolic and anabolic metabolic processes are temporally segregated in skeletal muscle. Our data suggests that skeletal muscle prefers lipid oxidation in the inactive and early active-phase (morning) prior to that of carbohydrate breakdown. In addition to rate-limiting enzymes we also identified multiple genes that encode for activators/repressors of substrate metabolism (Pdk4, Pdp1, Nrip1, etc). In this manner the molecular-clock controls the temporal expression of genes that act as molecular switches, so metabolic processes are always followed by a break to the system. These mechanisms help to promote the precise temporal control of substrate preference or the capacity of the muscle to store over use, or vice-versa. The observation that there is a switch toward a more oxidative state in iMS-Bmal1-/- skeletal muscle with a decrease in genes involved in carbohydrate metabolism strongly suggests that the molecular-clock is vitally important in controlling the timing of carbohydrate metabolism. Since skeletal muscle is the main site for post-prandial glucose uptake, understanding how the molecular clock regulates carbohydrate uptake, storage, and breakdown can be a potential new focus for understanding diseases such as diabetes, obesity, and metabolic syndrome.
It is becoming increasingly evident that the mechanisms in which the molecular-clock regulates the precise timing is not only highly-complex, but diverse over different cell types. Herein we provide evidence that the molecular-clock and the myogenic regulatory factor, MYOD1, work in a synergistic fashion to promote the expression of a muscle-specific gene. These factors not only target the same promoter element, but seem to work as co-factors that require each other’s presence to promote circadian rhythmicity. Future experiments are needed to determine if the molecular-clock works with other myogenic regulatory factors, and whether these factors changes throughout the development and maturation of skeletal muscle. We propose that the molecular clock may be interacting with many different tissue-specific factors to promote circadian output in a tissue dependent manner. Identifying such factors may result in the identification of drug-able targets for entraining the molecular-clock or promoting molecular-clock output.
Appendix File 1. Normalized gene expression traces of circadian lipid metabolic genes. Normalized expression traces of the circadian metabolic genes from the high-resolution skeletal muscle time-course transcriptome (data were downloaded from NCBI GEO datasets-GSE54652). Grey bars indicated the active period and white bars indicate the inactive period. Note that mice were in constant darkness during the time-course collection. Red lines indicate the acrophase (time of peak expression) calculated by JTK_CY-CLE algorithm. A 6° polynomial was fit to the data to highlight the temporal expression pattern (black line).
Appendix File 2. Normalized gene expression traces of circadian lipid metabolic genes. Normalized expression traces of the circadian metabolic genes from the high-resolution skeletal muscle time-course transcriptome (data were downloaded from NCBI GEO datasets-GSE54652). Grey bars indicated the active period and white bars indicate the inactive period. Note that mice were in constant darkness during the time-course collection. Red lines indicate the acrophase (time of peak expression) calculated by JTK_CYCLE algorithm. A 6° polynomial was fit to the data to highlight the temporal expression pattern (black line).
Appendix File 3. Normalized gene expression traces of circadian glycolytic metabolic genes. Normalized expression traces of the circadian metabolic genes from the high-resolution skeletal muscle time-course transcriptome (data were downloaded from NCBI GEO datasets-GSE54652). Grey bars indicated the active period and white bars indicate the inactive period. Note that mice were in constant darkness during the time-course collection. Red lines indicate the acrophase (time of peak expression) calculated by JTK_CYCLE algorithm. A 6° polynomial was fit to the data to highlight the temporal expression pattern (black line).
Appendix File 4. Normalized gene expression traces of circadian glycolytic metabolic genes. Normalized expression traces of the circadian metabolic genes from the high-resolution skeletal muscle time-course transcriptome (data were downloaded from NCBI GEO datasets-GSE54652). Grey bars indicated the active period and white bars indicate the inactive period. Note that mice were in constant darkness during the time-course collection. Red lines indicate the acrophase (time of peak expression) calculated by JTK_CYCLE algorithm. A 6° polynomial was fit to the data to highlight the temporal expression pattern (black line).
Appendix File 5. Temporal gene expression traces of circadian metabolic genes. Gene expression traces for circadian metabolic genes from the Mouse ST 1.0 Affymetrix gene array for gastrocnemius tissue collected at circadian times 18 to 38. iMS-\textit{Bmal}1+/+ control values are indicated as black diamonds and iMS-\textit{Bmal}1−/− are indicated as red squares. Grey bars indicated the active period, and white bars indicate the inactive period. Note that mice were in constant darkness during the time-course collection.
Appendix File 6. Real-time PCR results for circadian metabolic genes. Real-time PCR results of time-course expression values for Fabp3, Pnpla3, Hk2, and Pdp1 in the iMS-Bmal1+/+ (black) and iMS-Bmal1−/− (red). Paired t test of Fabp3 (P value = 0.02), Pnpla3 (P value = 0.4), Hk2 (P value = 0.001), and Pdp1 (P value = 0.15).
Appendix File 7. Temporal gene expression traces of the core molecular-clock genes. Gene expression traces are from the Mouse ST 1.0 Affymetrix gene array for gastrocnemius tissue collected at circadian times 18 to 38. iMS-Bmal1+/+ control values are indicated as black diamonds and iMS-Bmal1 −/− are indicated as red squares. Grey bars indicated the active period, and white bars indicate the inactive period. Note that mice were in constant darkness during the time-course collection.
Appendix File 8. Temporal expression pattern of $T$-cap and $Murf1$ from the Hogenesch skeletal muscle circadian transcriptome. The circles represent the expression values of $T$-cap at each circadian time-point. The dotted line is a 6-degree polynomial fitted to the expression data. Grey bars indicated the active period, and white bars indicate the inactive period. Note that mice were in constant darkness during the time-course collection.
Appendix File 9. Western-blot of iMS-Bmal1+/+ and iMS-Bmal1-/- gastrocnemius tissue for T-CAP and gamma-Tubulin control at CT2 (top) and CT14 (bottom). First lane of the left is the protein ladder, top band is 50KDa and the bottom band is 20KDa.
**Appendix File 10.** *T-cap* proximal promoter sequence. The letters inside the parenthesis indicate the nucleotides following site directed mutagenesis. The tandem E-box element is highlighted red, proximal E-box element is highlighted green:

5' -

GAGGGCATCAGTTCCTGCTTTCTCTTTTTACAGACAAAGCTGCTGTCTTCTCCCTGAGC
AAGGGGGAGACAGAAAGAAGCAGTGGCCAAGGTGGC(G)A(T)C(G)CAGCTGCCCATG
TGCCTGTCCGAGGTTGTTTGTGTCAGCATGCCAGACACCCACAGAGGTGCTACTCTGAGAAC
CCAGGTCCCAACAGAGCATCTTGAGGCTTTGAAAGCTGACCTCTGACCTGGGAGCAAC
CAGAATCTGGGTACCCCAACCCCCGGCCGGACTCAAGCCCCATCAACACAGTGATCT
TGCTCTGCTTTATAGCATCTGACGCGAGGGGCTGAAATAGCCCTGGAGAAGGGGG
AGAGGGGAAGAGGGGCTATTTAAAGGGCTCTGGAGAGGAGCAGACATAGCAGAGGGAG
CAATCAGAAAATCAGATCTGCGATCTAAGGATGCTTGGCATTTCCGGTACTGTGTGAAAGC
CACCATGGAAGACGGCCAAAAACATAAAAGGAGGACCGGGCAGCATTCTAGCCTGGAAAGA
TGGGACCCGCTGGAGAACTGCATAAGAGGCTATGAGATACCTGCCCTGGTCTGGAAC
AATTTGCTTTTACAGATGCACTATCGAGGTTGAGCAGACATCCTACGCTGAGTACTCTGAAAT
GTCGTTTGTTGGCAGAAGCTATGAAACGATATGGGCTGAATACAAATCA-3'
References


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5. **Hodge, B.A.,** Zhang, X., Arnold, N., Esser, K.A. MYOD1 and the molecular-clock work in a synergistic fashion to promote the circadian expression of *Titin-cap* in skeletal muscle. (In preparation)

**ABSTRACTS:**


**PRESENTATIONS:**


INVITED PRESENTATIONS:

1. **Hodge, B.** “Keeping time in skeletal muscle” Biochemistry and Molecular Biology Research Seminar, Centre College, Danville, Kentucky, April 13th, 2015.

RESEARCH INTERESTS:

- Exercise induced mechanotransduction and downstream signaling events in muscle.
- Regulation of signaling mechanisms underlying muscle hypertrophy/atrophy.
- Molecular mechanisms of genetic skeletal muscle diseases.
- Circadian Rhythms: Tissue-specific regulation of clock-controlled genes and how disruptions in peripheral tissue clocks affect systemic biological processes.
- Bioinformatics approaches to understanding circadian genomics.

ACTIVITIES:

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- Big Brothers Big Sisters of the Bluegrass, Volunteer Fall 2010-2011.
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