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MOLECULAR METABOLISM

The calcineurin-NFAT pathway controls activity-dependent circadian gene expression in slow skeletal muscle

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ABSTRACT

Objective: Physical activity and circadian rhythms are well-established determinants of human health and disease, but the relationship between muscle activity and the circadian regulation of muscle genes is a relatively new area of research. It is unknown whether muscle activity and muscle clock rhythms are coupled together, nor whether activity rhythms can drive circadian gene expression in skeletal muscle.
Methods: We compared the circadian transcriptomes of two mouse hindlimb muscles with vastly different circadian activity patterns, the continuously active slow soleus and the sporadically active fast tibialis anterior, in the presence or absence of a functional skeletal muscle clock (skeletal muscle-specific *Bmal1* KO). In addition, we compared the effect of denervation on muscle circadian gene expression.
Results: We found that different skeletal muscles exhibit major differences in their circadian transcriptomes, yet core clock gene oscillations were essentially identical in fast and slow muscles. Furthermore, denervation caused relatively minor changes in circadian expression of most core clock genes, yet major differences in expression level, phase and amplitude of many muscle circadian genes.
Conclusions: We report that activity controls the oscillation of around 15% of skeletal muscle circadian genes independently of the core muscle clock, and we have identified the Ca^{2+}-dependent calcineurin-NFAT pathway as an important mediator of activity-dependent circadian gene expression, showing that circadian locomotor activity rhythms drive circadian rhythms of NFAT nuclear translocation and target gene expression.

Keywords Skeletal muscle; Circadian; Activity; NFAT

1. INTRODUCTION

The mammalian circadian clock is an evolutionarily conserved, adaptive means of coordinating internal physiology in anticipation of environmental conditions [34], and is composed of a central pacemaker in the suprachiasmatic nucleus (SCN) of the hypothalamus and autonomous molecular oscillators in cells of peripheral tissues. Timing information regarding the daily light/dark cycle is transmitted directly from the retina to the SCN, which in turn synchronizes clocks in the periphery by regulating various metabolic and behavioral outputs, including locomotor activity, feeding behavior and body temperature [13].
At the cellular level, circadian time is maintained by a rhythm generating molecular clock composed of two interlocking regulatory feedback loops [17]. In the primary loop, the bHLH-PAS domain-containing transcription factors CLOCK and BMAL1 form heterodimers in the cytoplasm and translocate to the nucleus where they bind to E-box sequences of target genes, including *Period (Per)* and *Cryptochrome (Cry)* transcription factors. PER and CRY proteins form heterodimers in the cytoplasm and translocate to the nucleus, repressing activation by the CLOCK-BMAL1 complex. In a second regulatory loop, CLOCK-BMAL1 activation leads to transcription and translation of *Rev-Erbα*, which represses *Bmal1* transcription by inhibiting the ROR enhancer element. In addition, all tissues contain a large number of 24-hour oscillating genes, most of which are tissue-specific [31,36,49,59] and some of which are directly regulated by extrinsic circadian signals independent of this core molecular clock [25].
From a circadian perspective, skeletal muscle is unique among peripheral tissues, as muscle fibers contain local circadian clocks and are

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targets of blood-born circadian signals like other cell types. Yet muscle fibers also respond to rhythmic motor neuron activity, which is likewise controlled by the SCN pacemaker [39]. Motor activity has traditionally been used as a read-out of the circadian timing system, but it is unclear whether muscle activity and muscle clock gene rhythms are coupled, and whether activity affects the entrainment of the muscle clock. While feeding time has emerged as a dominant entrainment factor for most peripheral tissues studied to date [12,48], including skeletal muscle [5,55] time-restricted feeding also alters circadian locomotor activity [55] and body temperature rhythms [12]. Similarly, various exercise protocols, including voluntary and involuntary running, have been shown to influence entrainment of the core muscle clock [55], but likewise affect the central pacemaker [15,30] circadian hormone release [54] and body temperature rhythms [46]. As such, a clear understanding of the relationship between activity rhythms and muscle circadian genes remained elusive. Muscle activity reflects nerve activity, and a role for neural signals entraining the muscle clock has been suggested by parabiosis experiments between intact and SCN-lesioned mice, showing that non-neural signals are able to maintain the circadian rhythm of clock gene expression in liver but not in skeletal muscle [18]. However, other studies have pointed to the dissociation between clock gene expression in skeletal muscle and rhythmic locomotor activity, as transplantation of a functional SCN into SCN-lesioned Syrian hamsters failed to reinstate normal gene oscillation in skeletal muscle, despite restoring locomotor activity rhythms with the period of the donor [19]. Nakao and colleagues recently reported that sciatic nerve denervation disrupts circadian expression of some skeletal muscle clock genes without affecting circadian rhythms of body temperature or plasma corticosterone levels [35], but whether the effects on muscle clock genes were directly dependent upon the loss of nerve activity or an indirect result of muscle remodeling could not be determined.

To investigate the relative roles of intrinsic versus extrinsic circadian signals in the regulation of circadian muscle gene expression, we previously generated mice with muscle-specific inactivation of the *Bmal1* gene (*Bmal1 mKO*), thus disrupting local muscle clock function [14]. We have shown that these mice display an essentially normal phenotype [14], in contrast to global knockout of *Bmal1*, which leads to severe muscle atrophy, reduced body weight and shorter life span [23]. In agreement with results obtained from other tissue-specific *Bmal1* knockout mice [25], we found a significant proportion of cycling genes maintain their circadian oscillation in muscle cells even with a disrupted core oscillator, and thus must respond to extrinsic circadian signals. To determine whether circadian activity rhythms drive the oscillation of these clock-independent genes, we examined circadian gene expression in fast and slow muscles with vastly different circadian activity patterns, and compared them to contralateral denervated muscles with complete loss of activity. Importantly, these comparisons were made in the same animals, thus avoiding systemic effects that complicate the interpretation of studies on the role of exercise on circadian rhythms. We found the oscillation pattern of core clock genes is essentially identical in fast and slow muscles, despite major differences in circadian activity levels. Furthermore, core clock gene expression shows relatively minor changes after denervation, in contrast to the striking changes induced by time-restricted feeding.

Furthermore, we report that nerve activity controls the oscillation of around 15% of skeletal muscle circadian genes independently of the core muscle clock, and we have identified the Ca^{2+}-dependent calcineurin-NFAT pathway as an important mediator of activity-dependent circadian gene expression, showing that circadian locomotor activity rhythms drive circadian rhythms of NFAT nuclear translocation and target gene expression.

2. MATERIALS AND METHODS

2.1. Animals

All experiments were performed on 2–4 month old adult male mice, with standard chow (Mucedola, Settimo Milanese, Italy) and water provided ad libitum. Mice with skeletal muscle-specific inactivation of *Bmal1* (*Bmal1 mKO*) were generated as previously described [14]. In brief, a mouse line with floxed *Bmal1* gene [50] was crossed with mice carrying a Cre recombinase transgene under control of the *Mlc1f* promoter (Mlc1f-Cre), which is exclusively active in skeletal muscle [4]. Cre-negative littermates were used as wildtype controls (Ctrl). Experimental protocols were reviewed and approved by the local Animal Care Committee, University of Padova. Prior to all experiments, animals were acclimated for at least 4 weeks in an isolated temperature controlled room (22 °C) under a 12-h light–dark regimen, with lights on at *Zeitgeber* time 0 (ZT0; 6 am), lights off at ZT12 (6 pm). Tibialis anterior (TA) and soleus (SOL) muscles, and in some experiments also liver and heart, were collected immediately after cervical dislocation at 4-h intervals across the day/night cycle. Tissues were snap frozen in liquid nitrogen and stored at −80 °C until subsequent use. For time-restricted feeding, food access was restricted to the light phase (ZT0-12) for 14 days. For denervation experiments, mice were anesthetized by i.p. injection of a mixture of Zoletil 100® (a combination of Zolazapam and Tiletamine, 1:1, 10 mg/kg, Labotatoire Virbac) and Rompun® (Xilazine 2%, 0.06 ml/kg, Bayer) and a 1–2 mm segment of the sciatic nerve was removed high in the thigh. Muscles from denervated and sham-operated contralateral leg used as control were removed after 7 days.

2.2. RNA isolation and qPCR

Total RNA was isolated using TRIzol (Invitrogen) followed by cleanup with the RNeasy Mini Kit (Qiagen). RNA integrity was evaluated with the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and quantified with a NanoVue spectrophotometer (GE Healthcare Life Sciences, Baie d'Urfe, QC). Complementary DNA from each sample was generated from 0.8 μg of RNA reverse-transcribed with Invitrogen Superscript III reverse transcriptase. Primer sets were designed using Primer-BLAST (NCBI) and were validated prior to use by gradient PCR and gel analysis to test for optimal annealing temperature, reaction efficiency and specificity. Duplicates of sample cDNA were then amplified on the 7900HT Fast Real-Time PCR System (Applied Biosystems) using the Fast SYBR Green RT-PCR kit (Applied Biosystems). Specificity of gene amplification was confirmed by analyzing the dissociation curve with SDS 2.3 software (Applied Biosystems). Analysis was performed using the standard curve method and all data were normalized relative to *36B4* expression.

2.3. Gene expression profiling and analyses

Gene expression profiling for the denervation experiment was carried out using 250 ng of pooled RNA from 6 biological replicates for each time point hybridized to Mouse Genome 430 2.0 Arrays (Affymetrix). The gene expression data were normalized and summarized with RMA using custom CDF files (Version 14.1.0; EntrezG) to remap the probes on the arrays to the genome and transcriptome library data [11]. Differentially expressed genes were determined using a default R2 threshold of 0.6 and a cubic regression model in maSigPro (microarray Significant Profiles), a two-step regression-based method to identify genes with significant temporal expression changes and significant differences between experimental groups in time series microarray experiments [10]. Rhythmic genes that cycle with a 24-hour period were identified using a Benjamini–Hochberg Q-value < 0.2 in the

non-parametric algorithm JTK_Cycle [21]. 24-hour cycling genes identified with this cut-off all had adjusted $p < 0.01$ (Dataset S1). Over-representation analysis was performed using Gene Set Enrichment Analysis software (GSEA; http://www.broadinstitute.org/gsea/index.jsp) [51] and genesets from the TRANSFAC database. Genesets were defined as significantly enriched if the False Discovery Rate (FDR) was <0.2 and $p < 0.05$ when using Signal 2 Noise as metric and 10,000 permutations of geneset labels.

2.4. Plasmids and in vivo electroporation

The NFATc1-GFP construct, a gift from Rhonda Bassel-Duby, consists of a pEGFP–N1 plasmid (Clontech) containing NFATc1 linked to EGFP [9]. The NFATc3-GFP construct was a gift from Shoichiro Miyatake [3]. The histone 2B red fluorescent protein construct consists of histone 2B linked to RFP (H2B-RFP) and was a gift from Manuela Zaccolo. Transfection of plasmid DNA was performed as previously described [53]. Prior to surgery, mice were anesthetized by i.p. injection of a mixture of Zoletil 100® (a combination of Zolazapam and Tiletamine, 1:1, 10 mg/kg, Laboratoire Virbac) and Rompun® (Xilazine 2%, 0.06 ml/kg, Bayer). The soleus and tibialis anterior muscles were surgically exposed and isolated by a small hindlimb incision. Plasmid injection was followed by electroporation with stainless steel spatula electrodes connected to a ECM830 BTX porator (Genetronics, San Diego, CA) as described [32]. Transverse cryosections (10 μm) of muscles were used for detection and quantification of transfected NFAT-GFP fusion proteins. NFATc3-GFP labeling was enhanced with polyclonal rabbit anti-GFP (1:200, Molecular Probes) and Cy2-conjugated anti-rabbit IgG (1:150, Jackson Immunoresearch). Images for NFATc1-GFP, NFATc3-GFP and nuclear H2B-RFP were collected with a BioRad Laboratories confocal microscope (Radiance 2100 MP equipped with an argon laser for 488 nm excitation of GFP fluorescence, a Nikon 60x/1.4 Plan Apo objective, a 500 DCLPXR beamsplitter and HQ515/30 emission filter; all filters were from Chroma Technology Corp.) using Lasersharp 2000 software (BioRad). All images were analyzed using Image-J software (NIH image) and mean nuclear/cytoplasmic fluorescence was quantified based on the ratio of nuclear to cytoplasmic fluorescence for each muscle fiber measured (see Figure S3). NFAT-dependent transcriptional activity was monitored as previously described [32] by an NFAT reporter construct consisting of 9 tandem NFAT-binding sites from the interleukin 4 gene fused to a basal α-MyHC promoter and linked to firefly luciferase [9]. The NFAT reporter was co-injected with a Renilla luciferase expression vector (pRL-TK, Promega) to normalize for transfection efficiency and muscles were collected 7 days after cotransfection, at ZT0, 4, 8, 12, 16, and 20. Frozen muscles were minced with pestle and mortar in liquid nitrogen and resuspended in 2.5 μL/mg passive lysis buffer (Promega). After centrifugation at 12,000 g for 30 min, luciferase activity of supernatant was measured with a previously calibrated FD-20/20 Luminometer (Turner Designs, Sunnyvale, CA). Protein concentration of the supernatant was determined using the Bradford method with bovine serum albumin as standard for the calibration curve. Results are expressed as the mean ratio of firefly luciferase to Renilla luciferase ± SEM.

3. RESULTS

3.1. Circadian genes are differentially expressed in slow and fast skeletal muscles

To investigate the role of activity on skeletal muscle circadian gene expression, we first compared the circadian expression of core clock genes and some of their canonical targets in the slow, continuously active soleus (SOL) and the fast, sporadically active tibialis anterior (TA), two muscles with completely different fiber type and motor unit composition [45]. Slow soleus (SOL) is composed of around 90% type 1 slow and 2A fibers (Figure S1), whereas fast tibialis anterior (TA) and extensor digitorum longus (EDL) are composed of around 90% fast 2B and 2X fibers. The activity profiles of the corresponding motor units are not known in the mouse, but firing pattern analyses of rat motor units, based on continuous electromyographic recording for extended periods, indicate that slow motor units in rat soleus are active for more than 30% of the time over 24 h, whereas the predominant motor units in EDL, a fast muscle similar to TA, display sporadic short bursts of activity, with total time occupied by bursts being less than 0.5% of the time over 24 h for most units [20]. Consistent with this are continuous electromyographic analyses showing that the rat soleus is active mostly during the night [16].

To identify circadian genes in SOL and TA muscles we used the algorithm JTK_CYCLE [21] and found 1359 (SOL) and 684 (TA) 24-h cycling genes (Figure 1A). Applying False Discovery Rate (FDR) cut-offs of varying stringencies had little impact on the relative abundance of 24-hour cycling genes found in SOL and TA, and we consistently found around 2–3 times more cycling genes in SOL than TA (Figure S4). Interestingly, peak expression of these 24-hour cycling genes displayed a bimodal distribution, with larger clusters of genes peaking around the light–dark and dark–light transitions in both SOL and TA, coinciding with rest/activity and fasting/feeding phase transitions (Figure 1B). Comparing circadian genes between muscles, we further identified a subset of 334 cycling genes common to both SOL and TA (Figure 1C; Dataset S1). In contrast to major differences in the number of circadian genes between SOL and TA, circadian expression of the core clock genes was essentially the same, as verified by qPCR (Figure 1D), with only a few core clock-associated genes showing significant differences between SOL and TA. Specifically, *Dbp* amplitude was higher in SOL, whereas expression levels of *Rora* and *Dec1* were higher in TA.

On the other hand, the majority of 24-hour cycling transcripts were specific to each muscle (Figure 1C; Dataset S1), with 75% and 51% of circadian genes cycling only in SOL or TA, respectively. These differences underscore the specificity of circadian regulation between different muscles, and likely reflect known differences in activity levels, metabolism and functional properties. Selected SOL- and TA-specific circadian genes are shown in Figure 1E and F, while a comprehensive list of muscle type-specific circadian genes can be found in Dataset S1.

3.2. Skeletal muscles show Bmal1-dependent and -independent circadian genes

To identify muscle circadian genes regulated by the core muscle clock, we compared the list of 24-hour cycling genes found in SOL and TA muscles of wildtype mice (Figure 2A) with the lists of genes differentially regulated genes in SOL and TA muscles of their muscle-specific *Bmal1* KO littermates [14]. Accordingly, we identified a subset of 132 *skeletal muscle clock-dependent circadian genes* common to both SOL and TA (Dataset S2), again including most of the known core clock and clock-dependent genes, in addition to novel muscle circadian genes (see Figure 2B for selected genes; see Dataset S2 for the complete list).

These newly identified muscle clock-dependent circadian genes code for a range of structural proteins, receptor subunits, signaling molecules, metabolic enzymes and transcription factors, and many already have a well-defined physiological relevance, including *Adrb2*, encoding the beta2-adrenergic receptor, *Acvr1b*, encoding the activin receptor

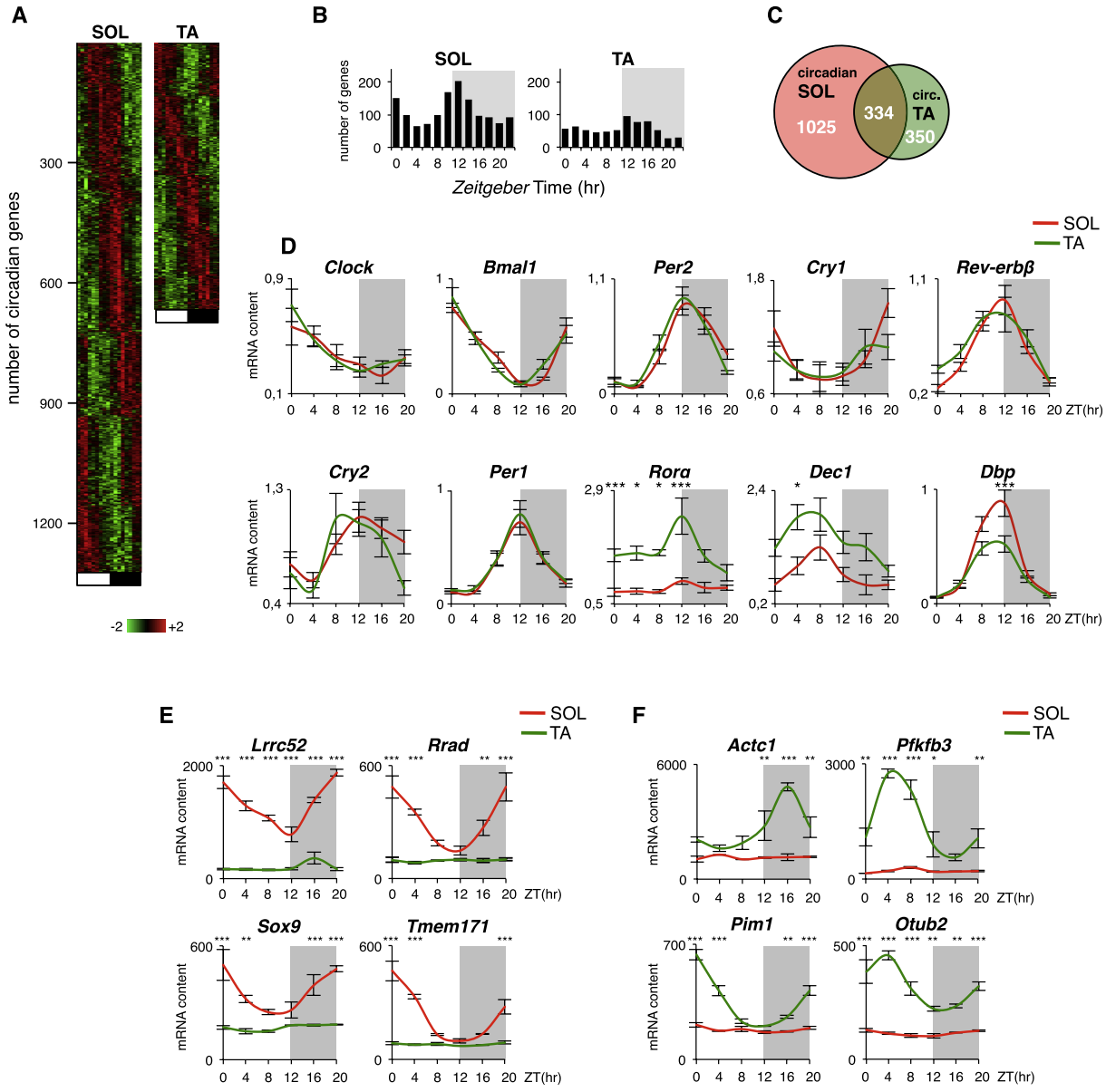

Figure 1: Circadian genes in different skeletal muscles. **A**. Phase map of circadian genes in slow/oxidative soleus (SOL) and fast/glycolytic tibialis anterior (TA) skeletal muscles identified by JTK_Cycle. Muscles (n = 3/group/time point) were collected at 4 h intervals over 24 h, throughout the light (white box) and dark phase (black box). **B**. Temporal distribution of circadian genes according to peak expression. Note bimodal distribution, with increased numbers of genes peaking around the transitions from light/resting/fasting and dark/active/feeding phases, particularly in the highly active SOL. **C**. Comparison of circadian genes between SOL and TA. **D**. Diurnal expression profiles of core clock genes and clock-associated genes in SOL and TA muscles determined by qPCR and expressed relative to *36b4* (arbitrary units; mean ± SEM; n = 6/time point; *$p = 0.05$, ***$p = 0.001$, 2-way ANOVA with Bonferroni correction). **E**–**F**. Diurnal expression profiles of selected SOL-specific **(E)** and TA-specific **(F)** circadian genes determined by microarray and plotted as mean absolute expression levels (n = 3/time point; mean ± SEM *$p = 0.05$, **$p = 0.01$, ***$p = 0.001$, 2-way ANOVA with Bonferroni correction). See Dataset S1 for complete list.

1b (also called Alk4), a component of the myostatin receptor complex, and *Glul*, coding for the enzyme responsible for glutamine synthesis. Other less well-known clock-dependent muscle circadian genes include *Mylk4*, coding for a highly expressed myosin light chain kinase of unknown physiological relevance.

Despite significant changes in circadian expression of several hundred genes due to muscle clock disruption [14], we found the majority of circadian genes in both SOL and TA were not significantly altered (Figure 2A), suggesting they are regulated by extrinsic circadian signals, and not by the core skeletal muscle clock. Accordingly, around 70% of SOL circadian genes (942 of 1359) and about 60% of TA circadian genes (414 of 684) remained unchanged in mKO muscles according to differential expression analysis using maSigPro. We found 121 of these *clock-independent circadian genes* common to both SOL and TA, including important insulin-responsive metabolic regulators (Figure 2C) such as Forkhead box protein O1 (*Foxo1*), encoding a transcription factor involved in muscle mass regulation, and G(0)/G(1) switch gene 2 (*G0s2)* which is induced by insulin in human skeletal muscle [37] and codes for an inhibitor of adipose triglyceride lipase (ATGL) thus blocking lipid droplet degradation [57].

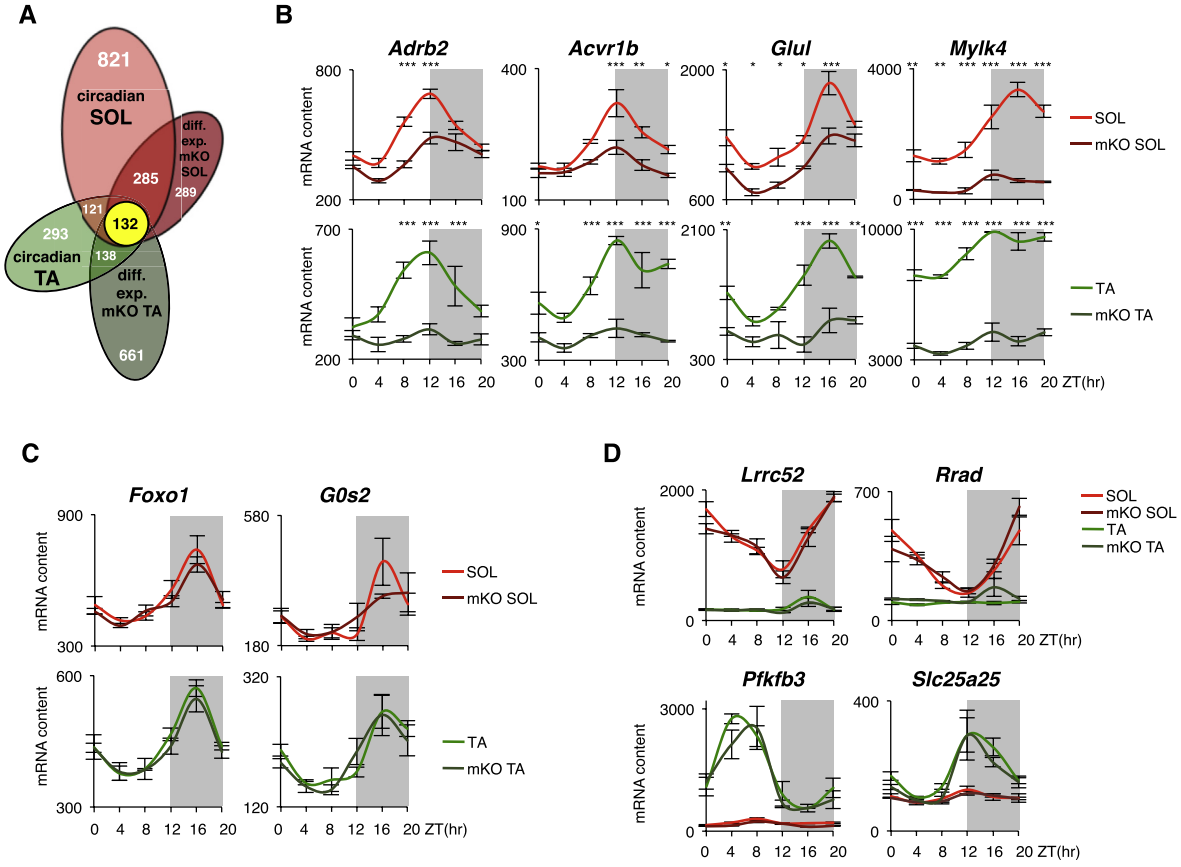

Figure 2: Clock-dependent and clock-independent circadian genes. **A**. Comparative analysis of circadian genes identified in wildtype SOL and TA muscles with genes differentially expressed in SOL and TA from littermates with muscle-specific *Bmal1* knockout. **B**. Diurnal expression profiles in SOL and TA of selected clock-dependent circadian genes determined by microarray and plotted as mean absolute expression levels (n = 3/time point; mean ± SEM $*p = 0.05$, $**p = 0.01$, $***p = 0.001$, 2-way ANOVA with Bonferroni correction). **C**–**D**. Diurnal expression profiles of selected common **(C)** and SOL- or TA-specific **(D)** clock-independent circadian genes determined by microarray and plotted as mean absolute expression levels (n = 3/time point; mean ± SEM). See Dataset S2 for the complete list.

In addition, a surprisingly large proportion of muscle circadian genes remained specific to each muscle type, with 821 *clock-independent circadian genes* specific to SOL, and 293 specific to TA. Selected examples of these SOL- and TA-specific *clock-independent circadian genes* are shown in Figure 2D, while the comprehensive list can be found in Dataset S2.

3.3. A subset of muscle circadian genes is strictly activity-dependent

To identify activity-dependent circadian genes in SOL and TA we used a loss-of-function approach by investigating muscle circadian gene expression in the absence of motor nerve activity. We compared global 24-hour gene expression of innervated SOL and TA muscles with denervated contralateral SOL and TA muscles after unilateral sciatic nerve transection. Muscle denervation is known to cause numerous changes in functional properties, metabolic demands and gene expression [29], including increased protein degradation and muscle atrophy [44]. Accordingly, microarray analysis revealed a massive reorganization of gene expression in denervated muscles, with 1629 genes in SOL and 1979 genes in TA significantly altered over 24 h according to maSigPro analysis (Figure 3A; Dataset S3). These included 179 and 118 circadian genes in SOL and TA, respectively (Figure 3B; Dataset S3). However, despite major muscle remodeling, the complete absence of nerve activity played only a minor role regulating core clock gene expression, with most core clock genes showing only modest changes in phase, amplitude and expression level in response to denervation (Figure 3C), in agreement with a recent report by Nakao and colleagues [35]. For example, while expression phase for *Per1* was identical in innervated and denervated muscles, amplitude was decreased around 30% by denervation in SOL and TA. On the other hand, denervation increased amplitude of *Cry2* around 30% in SOL and TA, whereas *Rora* amplitude was increased 2-fold in denervated SOL. Expression phase was slightly, but significantly altered after denervation for *Bmal1*, *Clock*, *Cry1* and *Cry2*, as well as *Dbp*, which all showed a phase advance of around 4 h in denervated muscles when considering peak and trough times. These relatively minor changes in core clock gene expression induced by denervation are in striking contrast to the drastic changes induced by daytime-restricted feeding, a major extrinsic circadian entrainment factor. In agreement with previous results in gastrocnemius [5] we observed that the expression phase of the core muscle clock genes *Bmal1*, *Per1* and *Per2* are shifted around 12 h by daytime-restricted feeding in both fast TA and slow SOL skeletal muscles, similar to liver and heart (Figure S2).

On the other hand, denervation caused more dramatic alterations in absolute expression level, phase and amplitude of many clock-dependent and-independent muscle circadian genes (Figure 3B). As shown in Figure 3D, these include the clock-independent, SOL-specific

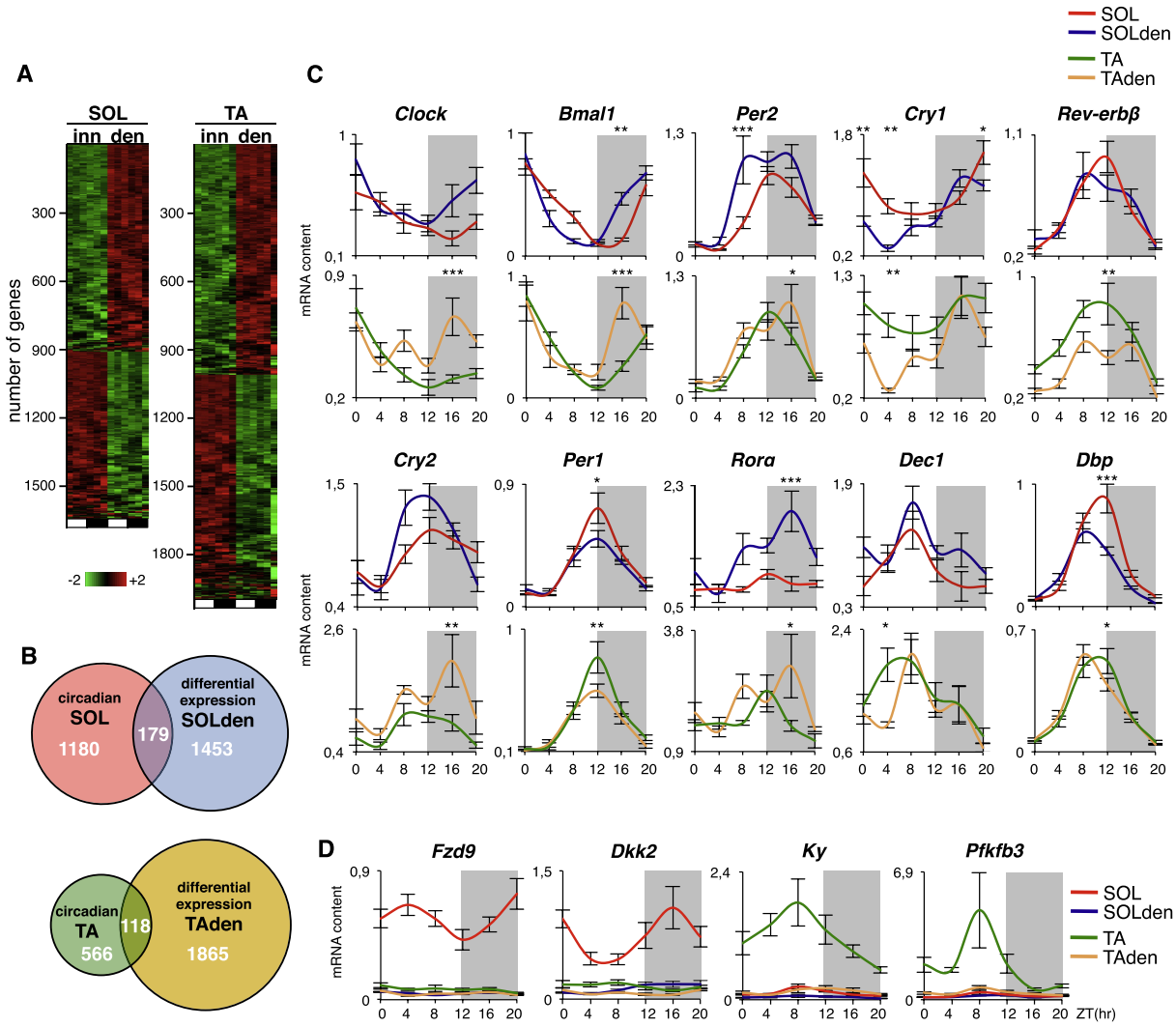

Figure 3: Activity-dependent circadian genes. **A**. Phase map of differentially expressed genes in control SOL and TA muscles and denervated contralateral muscles over 24-h identified by maSigPro. Muscles (n = 6/group/time point) were collected at 4-h intervals over 24 h, with RNA pooled and hybridized to Affymetrix arrays as described in the methods. **B**. Comparative analysis of circadian genes identified in wildtype SOL and TA muscles with genes differentially expressed in response to denervation. **C**. Diurnal expression profiles of core clock genes and clock-associated genes in innervated and contralateral denervated SOL and TA muscles determined by qPCR and expressed relative to *36b4* (arbitrary units; mean ± SEM; n = 6/group/time point; $*p = 0.05$, $**p = 0.01$, $***p = 0.001$, 2-way ANOVA with Bonferroni correction). **D**. Diurnal expression profiles of selected activity-dependent genes in innervated and contralateral denervated SOL and TA muscles determined by qPCR and expressed relative to *36b4* (arbitrary units; mean ± SEM; n = 6/group/time point).

circadian gene *Frizzled9* (*Fzd9*), coding for a 7-transmembrane domain Wnt receptor. Interestingly, *Fzd9* was expressed antiphase to the Wnt inhibitor Dickkopf 2 (*Dkk2*), and expression levels for *Fzd9* and *Dkk2* were both severely reduced by denervation, with *Fzd9* expression reduced 92% in denervated SOL, and Dkk2 expression reduced 85%. Similar changes were observed for circadian genes predominantly expressed in TA, such as the clock-dependent circadian gene *Kyphoscoliosis peptidase (Ky)*, coding for a titin- and filamin C-binding cytoskeletal protein, which showed 88% reduced expression and 30% reduced amplitude in denervated TA, and the clock-independent circadian gene *Pfkfb3,* which codes for a homodimeric bifunctional enzyme that catalyzes the synthesis and degradation of fructose 2,6-bisphosphate, and which showed 80% reduced expression and 70% reduced amplitude in denervated TA.

3.4. Activity-dependent circadian pathways in the highly active soleus

To gain biological insight into the specificity of circadian gene expression potentially regulated by muscle activity, we focused our attention on the highly active soleus. We performed pathway enrichment analysis of the 1025 SOL-specific circadian genes using the computational software Enrichr [8] and genesets from the BioCarta database. We found 15 significantly enriched pathways which, in essence, are all pathways involving either p38 mitogen-activated protein kinase (MAPK) and activation of downstream transcription factors AP1 and/or CREB, or calcium-dependent pathways involving calcineurin and activation of NFAT transcription factors (Figure 4A). Both p38 MAPK and calcineurin are nerve activity-dependent regulators of muscle gene expression, and play important roles in skeletal

A

BioCarta pathway	*p*-value
EGFR SMRTE	0.002
TSP1	0.004
EGF	0.004
BCR	0.004
Calcineurin	0.015
CREB	0.015
PDGF	0.015
TOLL	0.018
P38 MAPK	0.026
TPO	0.029
NTHI	0.033
HCMV	0.034
Cardiac EGF	0.046
CCR5	0.046
Keratinocyte	0.046

B

TRANSFAC Geneset	*p*-val
TGANTCA_V$AP1_C	0.007
TTGTTT_V$FOXO4_01	0.009
RYTTCCTG_V$ETS2_B	0.011
CTTTGT_V$LEF1_Q2	0.012
TGGAAA_V$NFAT_Q4_01	0.016
CTTTGA_V$LEF1_Q2	0.023

Figure 4: Activity-dependent circadian pathways identified by enrichment analysis. A. BioCarta pathway enrichment analysis performed on wildtype SOL-specific circadian genes. All significant pathways involve p38 MAPK and activation of downstream transcription factors AP1 and/or CREB, or calcineurin and activation of NFAT transcription factors. **B**. Activity-dependent transcriptional mediators identified by Gene Set Enrichment Analysis using the TRANSFAC database.

muscle adaptation in response to exercise [1] and maintenance of the slow fiber phenotype [47].

To better define transcriptional mediators of activity-dependent circadian gene expression, we performed Gene Set Enrichment Analysis (GSEA [51]), interrogating genesets from the TRANSFAC database with SOL-specific circadian genes found to be differentially expressed after denervation. The TRANSFAC geneset collection calculates enrichment scores for groups of genes that share specific transcription factor binding motifs within promoter regions, +/−2 kb from their transcription start sites. Only 5 transcription factors were significantly associated with changes in activity-dependent circadian gene expression in SOL (Figure 4B): AP1, Foxo4, Ets-2, NFAT, and LEF1. NFAT and AP1 are particularly interesting candidates, as they are known to interact and cooperate at promoters or enhancers to activate or repress target genes in a context-dependent manner, despite being activated by distinct upstream signaling pathways [27].

3.5. NFAT transcription factors regulate activity-dependent circadian gene regulation

To better define how activity-dependent signaling pathways regulate circadian gene expression, we focused our attention on calcineurin-NFAT. We previously showed that NFATc1, a member of the NFAT family of transcription factors, is a slow-type nerve activity sensor in vivo [32,53]. In fast skeletal muscles like TA, NFATc1 is normally phosphorylated and found localized in the cytoplasm, but slow-type electrical stimulation causes calcineurin-dependent NFATc1 dephosphorylation and nuclear translocation, as revealed by the nuclear/cytoplasmic distribution of an NFATc1-GFP fusion protein [26,53]. Conversely, NFATc1-GFP displays a greater nuclear localization in SOL, but nuclear export is rapidly induced by denervation [53].

To monitor 24-hour calcineurin-NFAT signaling in vivo, we transfected mouse SOL and TA muscles with plasmids coding for NFATc1-GFP or NFATc3-GFP fusion proteins, and measured NFAT nuclear/cytoplasmic localization over 24 h. In SOL muscles, as shown in Figure 5A and B, NFATc1 and NFATc3 were both predominantly cytoplasmic in the middle of the light/resting phase (ZT4), yet displayed a robust nuclear localization during the dark/active phase (ZT16). Quantification of nuclear to cytoplasmic GFP fluorescence (Figure S3) confirmed 24-hour nucleocytoplasmic shuttling, with accumulation of both NFATc1 and NFATc3 in myonuclei of the soleus starting around the beginning of the dark phase (ZT12), with a clear peak at ZT16 (Figure 5A&B). This 24-hour NFAT nuclear translocation was absent in TA muscles from the same mice, with maximum nuclear localization in the TA remaining around the basal levels detected during the light/resting phase in SOL. To investigate whether 24-hr rhythms of NFAT nuclear translocation are nerve activity-dependent, we performed unilateral transection of the sciatic nerve and collected NFATc1- or NFATc3-GFP transfected soleus muscles 12 h later, at the previously established peak (ZT16) or trough (ZT4) time points for NFAT nuclear localization. As shown in Figure 5C, the normal peak of nuclear NFATc1 and NFATc3 accumulation seen during the dark phase (ZT16) in the innervated SOL (inn) is abolished in the contralateral denervated SOL (den).

To assess whether the circadian nuclear accumulation of NFATc1 and NFATc3 correlates with transcriptional activation of NFAT targets, we transfected SOL and TA muscles with an NFAT reporter consisting of a concatemer of 9 tandem NFAT sites linked to luciferase [7]. We observed a 24-hour variation in luciferase activity that closely followed the cycle of nuclear NFAT accumulation, but with a 4—8 h delay. A reliable endogenous calcineurin-NFAT reporter is *Rcan1.4* expression, since it codes for the *Rcan1* isoform selectively activated by calcineurin-NFAT [42] due to a dense cluster of 15 consensus NFAT binding sites within the promoter [56]. As shown in Figure 5E, *Rcan1.4 is* a SOL-specific and clock-independent circadian gene in skeletal muscle (left graph). Furthermore, endogenous *Rcan1.4* transcripts display a robust activity-dependent circadian oscillation specifically in innervated SOL muscles, (Figure 5E, right graph) with increased expression starting at around ZT16, the peak of NFAT nuclear localization (Figure 5A and B), and with peak expression between ZT20-ZT0, coinciding with the peak of the NFAT-luciferase reporter (Figure. 5D). Importantly, denervation caused a 70% reduction in mean 24-hour *Rcan1.4* expression and 30% reduced amplitude (Figure 5E, right graph). These changes are likely due to the lack of activity *per se,* rather than secondary changes in gene expression accompanying denervation, as we detected 90% reduced *Rcan1.4* expression at ZT0 already 12hr after performing denervation (Figure 5F).

4. DISCUSSION

The first clock gene, later identified as *period* (*per*), was discovered in *Drosophila* by studying mutations that altered free-running circadian locomotor activity rhythms [24]. Activity has since been widely used as a behavioral readout of circadian timing in both flies and mammals, but the relationship between rhythmic activity and circadian gene rhythms in skeletal muscle, and whether changes in activity can directly affect the intrinsic muscle clock, remain largely unknown. Clock gene mutations often disrupt rhythmic locomotor activity, and the SCN clock is both necessary and sufficient to drive circadian activity rhythms without participation of other circadian clocks [22,52]. On the other

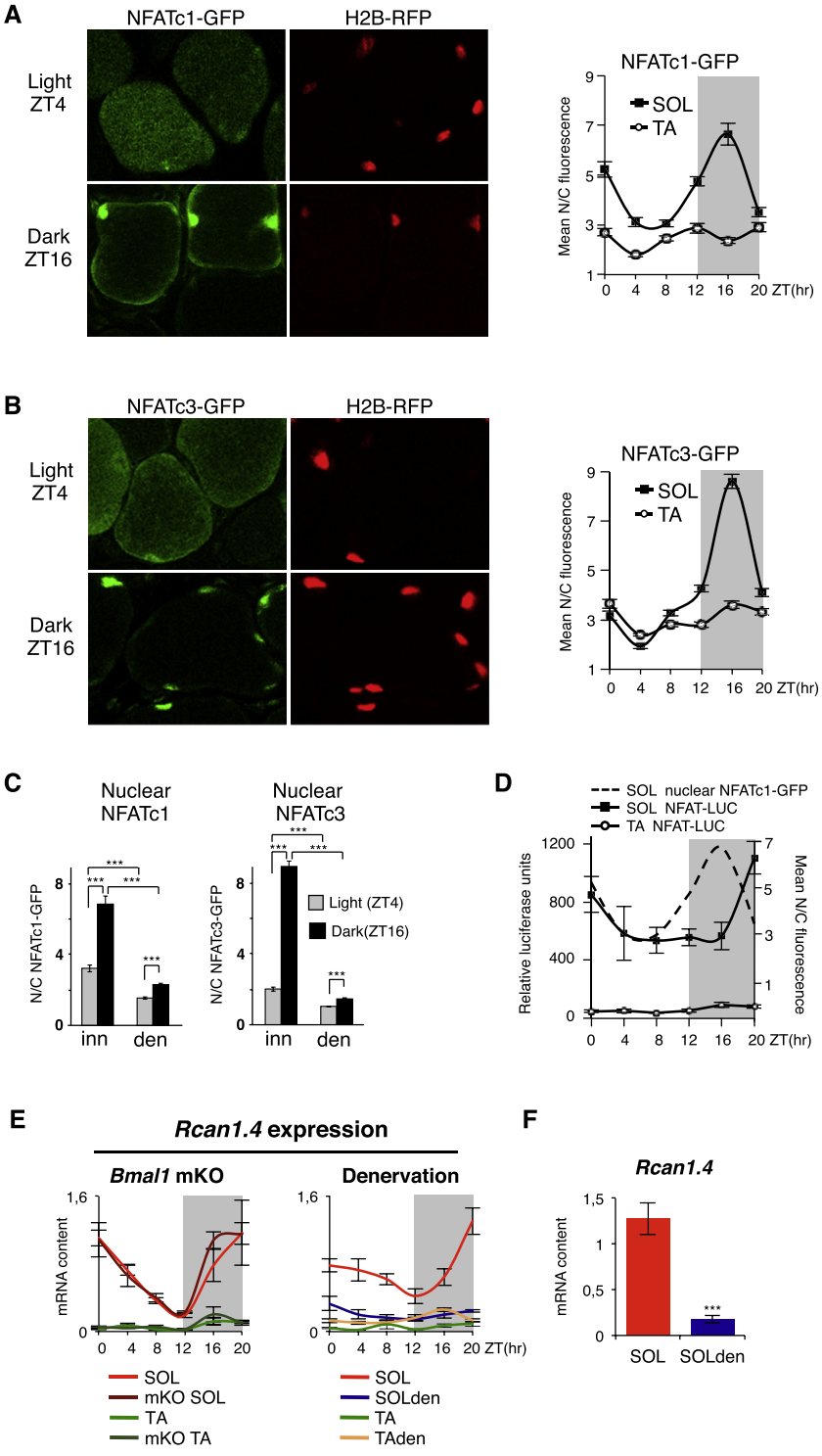

Figure 5: Activity-dependent circadian rhythms of NFAT nucleocytoplasmic shuttling and transcriptional activity. **A**–**B**. Nucleocytoplasmic localization of NFATc1- and NFATc3-GFP in transverse sections of transfected SOL muscles during the light (ZT4) and dark phases (ZT16). Transfected myofibers were identified by co-transfection with a constitutively nuclear Histone 2B-RFP (H2B-RFP) construct. Right panels show quantification of circadian nucleocytoplasmic shuttling of NFATc1- and NFATc3-GFP in transfected SOL and TA muscles (mean nuclear/cytoplasmic fluorescence ± SEM; n > 100 transfected fibers per time point; see Figure S3 for procedure). **C**. Diurnal rhythm of NFAT nuclear translocation is nerve activity-dependent: unilateral transection of the sciatic nerve 12 h prior to tissue collection reduced the previously observed peak of nuclear localization at night (ZT16) to mere basal levels observed during the day (ZT4). Nuclear localization was quantified as the ratio of mean nuclear/cytoplasmic fluorescence ± SEM; n = 4 muscles/group/time point, with n > 100 transfected fibers analyzed for each group/time point (***$p < 0.001$ Student's t-test). **D**. Diurnal NFAT transcriptional activity in SOL and TA muscles transfected with an NFAT-dependent luciferase reporter. Nuclear localization of NFATc1-GFP (dashed line) has been superimposed to demonstrate its correlation with NFAT reporter activity. Note the ~8 h delay between NFAT nuclear localization and reporter activity in SOL (mean ± SEM; n = 5 per time point). **E**. Diurnal expression profiles of endogenous NFAT sensor *Rcan1.4* in SOL and TA muscles from control and *Bmal1 mKO* littermates (left panel), and denervated (den) muscles compared to contralateral innervated muscles

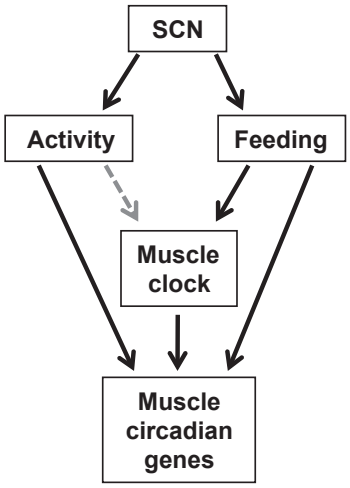

Figure 6: Intrinsic and extrinsic signals regulate skeletal muscle circadian gene expression. The hypothalamic suprachiasmatic nucleus (SCN) controls circadian rhythms of locomotor activity and feeding, which in turn drive the circadian rhythm of the muscle clock and of all other muscle oscillating genes, many of which are also regulated by the clock. Additional extrinsic factors related to activity and feeding rhythms, such as temperature, hormone release or autonomic innervation are not indicated.

hand, changes in activity have been shown to exert feedback control over the central pacemaker.

While exercise has recently been shown to regulate core clock gene expression in skeletal muscle [55], exercise also produces a variety of relevant systemic physiological responses, including hormonal changes and increased body temperature, thus affecting many different tissues, including the central pacemaker. As such, it has been difficult to discern whether activity plays a direct role regulating the muscle clock, or whether some correlate of activity is responsible for the observed changes. A few studies have addressed a direct effect of muscle activity on the core skeletal muscle clock by ruling out systemic effects of exercise. Acute one-leg exercise in humans was found to modulate the expression of some core clock genes in the exercised but not in the contralateral leg [58]. However, only two time points were examined and so it could not be determined whether exercise causes a phase advance or a phase delay in core clock gene expression, and the eccentric exercise protocol used in this experiment is known to lead to muscle damage and inflammation [28]. More recently, Nakao and colleagues monitored circadian gene expression in the fast gastrocnemius muscle at 7 and 28 days after unilateral sciatic nerve transection [35]. In agreement with our results, they reported various gene-specific alterations in core clock gene expression in response to denervation.

Comparing muscles with different activity levels, we show that core clock gene expression is essentially identical in two muscles with completely different circadian activity patterns: the continuously active slow SOL and the sporadically active fast TA. Furthermore, complete loss of muscle activity induced by denervation does not abrogate the diurnal cycling of core clock genes, and, although it causes some significant changes to their circadian expression pattern, the effect is much less striking than the complete phase shift induced by restricting food access to the light phase [5,55]. Indeed, in light of the fact that core clock genes are only slightly affected by denervation in the midst of such a massive global reorganization of gene expression (Figure 3A), our data suggest that activity plays only a minor, if any, direct role in regulating the muscle clock. While *Per1* expression maintained an identical phase, and showed only reduced amplitude after denervation, *Cry2* showed increased amplitude. Since both are CLOCK/BMAL1 targets, they must receive additional activity-dependent inputs. Many of the observed changes due to denervation, including the modest phase advance of *Bmal1*, *Clock*, *Cry1*, *Cry2*, and *Dbp*, will require further investigation to clarify whether they are indeed a result of altered activity-dependent signaling to clock genes, or whether they are secondary effects of denervation.

An important finding of this study is the demonstration that muscle circadian genes differ greatly among different skeletal muscles, with the order of magnitude of these differences similar to what is found when comparing circadian transcriptomes among different organs [59]. Our results thus underline the complexity of circadian regulation among different muscle types, and the importance of taking fiber composition, metabolism and activity levels into account when conducting circadian experiments. While most skeletal muscles of mice are composed of 90% or more type 2 fast muscle fibers, human muscles are composed of 40% or greater type I slow fibers. Our study therefore provides a novel perspective relevant for human physiology, as previous skeletal muscle circadian transcriptome studies have focused predominantly on fast muscles like gastrocnemius [2,5,31,35]. We also identify a set of muscle clock-dependent circadian genes common to different skeletal muscles, giving insight into the general regulation of skeletal muscle physiology and function by the intrinsic muscle clock.

Another major result of this study is the identification of circadian muscle genes that are dependent on activity and independent of the core oscillator. To better define the role of activity, we examined the effect of denervation, which allowed us to compare normally active and paralyzed muscles from the same animals. Even though denervation completely blocks muscle activity, there are some limitations to the denervation model that must be considered. For example, blood flow is reduced in denervated muscles, and so availability of circulating factors may be impaired in denervated muscle. In addition, denervation causes skeletal muscle remodeling, with downregulation of genes encoding contractile and mitochondrial proteins and up-regulation of genes involved in the proteasome and lysosome pathways of protein degradation [38]. Thus, as pointed out by Nakao and colleagues [35], it is possible that some of the observed changes are an indirect consequence of these transcriptional changes rather than direct loss of nerve activity. However, the finding that nerve section has an acute effect on the expression of circadian genes that are not dependent on the core muscle clock, like *Rcan1.4*, supports a direct role of nerve-dependent muscle activity on circadian gene expression in skeletal muscle. Finally, section of the sciatic nerve, which completely blocks motor nerve activity, could also alter sympathetic innervation of leg muscles; however, a large proportion of sympathetic nerves that innervate mouse hindlimb muscles are contained in the femoral and obturator nerves, and not exclusively within the sciatic nerve [33].

(right panel) determined by qPCR relative to *36b4* expression (mean ± SEM; n = 3/time point in left panel, n = 6/time point in right panel). Note *Rcan1.4* expression is higher in SOL than in TA, peaks during the dark/active phase (shaded area) and is markedly reduced by denervation. **F**. Acute unilateral transection of the sciatic nerve 12 h prior to tissue collection similarly reduced peak *Rcan1.4* expression seen in contralateral SOL at ZT0, as determined by qPCR relative to *36b4* (mean ± SEM; n = 5; ***$p < 0.001$ Student's t-test).

How can activity drive the oscillation of skeletal muscle circadian genes? Unbiased enrichment analyses of SOL-specific and activity-dependent circadian genes indicate the Ca^{2+}-dependent calcineurin-NFAT pathway is one important mediator of activity-dependent circadian gene expression in slow muscles. Upon dephosphorylation by the phosphatase calcineurin, NFAT proteins translocate to the nucleus and activate or repress target genes [40,41]. We report here that NFAT responds to circadian activity rhythms, as shown by increased nuclear import of NFATc1 and NFATc3 during the active/dark phase, followed by increased transcriptional activity of an NFAT-dependent reporter. Similar circadian changes in calcineurin-NFAT activity were recently described in the heart [43]. Accordingly, a well established NFAT target, *Rcan1* (DSCR1/MCIP1) is a 24-hour oscillating gene in liver and heart [6,49], and *Rcan1.4* transcripts have been shown to display circadian oscillations in mouse heart similar to what we observed in mouse SOL, with peak expression around ZT0 and a trough around ZT12, immediately preceding the peak and trough of RCAN1.4 protein levels [43]. We show that circadian oscillation of *Rcan1.4* is abolished by denervation, yet unaffected by disruption of the core muscle clock (Figure 5E).

In summary, our work gives regulatory context to the hundreds of genes cycling day and night in different skeletal muscles by identifying specific signaling pathways driving their oscillation. We show how various extrinsic and intrinsic circadian factors, including activity and the core muscle clock, can be integrated to organize circadian gene expression, and ultimately skeletal muscle physiology and function. Our work highlights the coordinated control of muscle circadian genes by both activity and feeding-dependent mechanisms, either acting directly on gene expression, or indirectly through the core muscle clock (Figure 6).

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2015.09.004.

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