Circadian rhythms regulate cell proliferation and differentiation, but circadian control of tissue regeneration remains elusive at the molecular level. Here, we show that proper myoblast differentiation and muscle regeneration are regulated by the circadian master regulators Per1 and Per2. Depletion of Per1 or Per2 suppressed myoblast differentiation in vitro and muscle regeneration in vivo, demonstrating their nonredundant functions. Both Per1 and Per2 were required for the activation of Igf2, an autocrine promoter of myoblast differentiation, accompanied by Per-dependent recruitment of RNA polymerase II, dynamic histone modifications at the Igf2 promoter and enhancer, and the promoter–enhancer interaction. This circadian epigenetic priming created a preferred time window for initiating myoblast differentiation. Consistently, muscle regeneration was faster if initiated at night, when Per1, Per2, and Igf2 were highly expressed compared with morning. This study reveals the circadian timing as a significant factor for effective muscle cell differentiation and regeneration.

**Introduction**

Regulation of mammalian circadian rhythms is centered around the Clock–Bmal1 transcription factor complex (Gustafson and Parth, 2015; Hirano et al., 2016; Papazyan et al., 2016; Takahashi, 2017). The complex binds the E-box (5′-CANNTG-3′) in promoters and enhancers of thousands of genes to activate their transcription, including the Cry (Cry1 and Cry2) and Per (Per1–Per3) genes. Gradually accumulated Cry and Per in turn bind Clock–Bmal1 on DNA and repress its transcription activity, forming a negative feedback loop. Subsequent phosphorylation and ubiquitination of Cry and Per lead to their degradation, reactivating Clock–Bmal1. This oscillating activity of Clock–Bmal1 creates transcriptional circadian rhythms in >20% of the genes in the genome in at least one tissue in the body. In addition, Clock–Bmal1 activates retinoic acid receptor–related orphan receptor (ROR) proteins (RORα–RORγ) and reverse orientation c-erb proteins (Rev-erba and Rev-erbβ), which compete for the retinoic acid–related orphan receptor response element (RORE) in the Bmal1 promoter. Opposing activities of ROR as an activator and Rev-erb as a repressor of Clock–Bmal1 form the second circadian feedback loop. The feedback loops in the suprachiasmatic nucleus in the hypothalamus are entrained by the light signal transmitted from the retina as the primary external cue (zeitgeber). The suprachiasmatic nucleus clock plays a dominant role (central clock) in synchronizing the feedback loops in other tissues (peripheral clocks), including skeletal muscle. In addition, the peripheral clocks are also entrained by various physiological factors, such as body temperature, feeding time, and physical activity.

Circadian regulation is tightly integrated into the genetic program of mammalian cell differentiation as demonstrated by several studies (Harffmann et al., 2015; Lefta et al., 2011; Mayeuf-Louchart et al., 2015). First, >2,000 genes, including the master myogenic regulators Myoblast determination protein 1 (MyoD) and Myogenin (Myog), show circadian oscillation in abundance (Harffmann et al., 2015; McCarthy et al., 2007; Miller et al., 2007; Pizarro et al., 2013). Second, whereas Bmal1 promotes satellite cell proliferation and differentiation and is required for muscle regeneration (Chatterjee et al., 2013; Chatterjee et al., 2015), Rev-erbα acts as an inhibitor of these processes (Chatterjee et al., 2019). Third, Clock–Bmal1 binds the E-box in the core enhancer of MyoD in a circadian manner; MyoD then binds the Bmal1 enhancer and increases the amplitude of Bmal1 expression, forming a feed-forward loop in myogenesis (Andrews et al., 2010; Hodge et al., 2019). Finally, we previously showed that Cry2 promotes myoblast proliferation and fusion during differentiation in a circadian manner through stabilization of mRNAs encoding cyclin D1, a G1/S phase transition driver, and

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Tmemi76b, a transmembrane regulator for myogenic cell fusion (Lowe et al., 2018).

Mouse Per1 and Per2 share 73.4% sequence similarity at the amino acid level but are not functionally redundant. Per1−/− and Per2−/− mice are grossly normal and fertile; however, both knockout (KO) mice exhibit circadian periods up to 2 h shorter than WT mice and eventually become arrhythmic in constant darkness (Bae et al., 2001; Cermakian et al., 2001; Zheng et al., 2001; Zheng et al., 1999). In contrast, Per1−/−:Per2−/− mice become arrhythmic immediately after transfer to constant darkness, although they are morphologically normal and fertile. As for muscle phenotypes, Per2−/− mice show a 20% shorter running distance with a treadmill test compared with WT and Per1−/− mice, although the length, weight, contractility, and abundance of several contractile proteins in the tibialis anterior (TA) muscle are similar in the three genotypes (Bae et al., 2006). Currently, virtually nothing is known about whether and how Per genes contribute to myogenic differentiation and muscle regeneration.

The present study uncovered insulin-like growth factor 2 (Igf2) as a critical link between Per1/Per2 and myoblast differentiation. Igf2 is a necessary and well-characterized autocrine differentiation promoter of myoblasts that increases in secretion levels during differentiation (Duan et al., 2010; Florini et al., 1991; Yoshiko et al., 2002). Igf2 is also up-regulated upon muscle injury and enhances regeneration (Keller et al., 1999; Kirk et al., 2003; Levinovitz et al., 2002). Additionally, several single-nucleotide polymorphisms of the human IGFBP genes are associated with a loss of muscle strength following strenuous exercise (Baumert et al., 2016; Devaney et al., 2007). Whole-body Igf2-null mice display impaired growth at birth but subsequently grow normally (Baker et al., 1993). Igf2 binds the type 1 Igf1 receptor with the highest affinity among several receptors, resulting in its auto-phosphorylation and subsequent activation of the P38/KAKT pathway and the RAS/MAPK pathway (Siddle, 2011; Taniguchi et al., 2006). In particular, activation of p38/A [MAPK by phosphorylation is an essential downstream effector for the promotion of myoblast differentiation by Igf2 (Knight and Kothary, 2011; Segalés et al., 2016b). p38 achieves the pro-differentiation function by triggering cell cycle exit, activating myogenic transcription factors, and opening the chromatin of muscle gene promoters (see Gardner et al., 2015; Segalés et al., 2016a). Most Igf2 in blood and local tissues is bound by Igf-binding proteins (IGFBP1-IGFBP7), which up- or down-regulate Igf2 functions (Allard and Duan, 2018). For example, whereas IGFBP3 inhibits myoblast differentiation (Huang et al., 2016), IGFBP5 is induced during early myoblast differentiation and amplifies the auto-regulatory loop of Igf2 expression, resulting in promoted differentiation (Ren et al., 2008). The current work demonstrated the Per1/Per2-Igf2 axis as a novel pathway underlying myogenesis and uncovered the presence of circadian epigenetic priming at the Igf2 gene during myoblast differentiation.

**Results**

**Disrupted muscle regeneration and myoblast differentiation by Per depletion**

To study the roles of Per1 and Per2 in skeletal muscle regeneration, TA muscle in Per KO mice was injured by barium chloride injection, and its regeneration was assessed by a series of histological analyses. The mice were entrained at 12-h light (zeitgeber time [ZT] 0–ZT12) and 12-h dark cycles (ZT12–ZT24) as diurnal rhythms for 2 wk before experiments. TA muscle was damaged and harvested at ZT14; ZT14 (20:00) was selected to compare with the results of the antephase time point (ZT2, 8:00) later. Hematoxylin and eosin (H&E) staining on day 4.5 after injury demonstrated smaller myofibers with centrally located nuclei, an indication of newly formed myofibers, in Per1−/−, Per2−/−, and particularly Per1−/−:Per2−/− mice compared with WT mice (Fig. 1, A–C). This trend continued at least until day 14 and was also observed in uninjured myofibers (Fig. S1, A and B). In addition, myofibers expressing embryonic myosin heavy chain (eMHC), a marker for newly generated myofibers, were smaller in Per2−/− and Per1−/−:Per2−/− mice than those in WT and Per1−/− mice (Fig. 1, D and E). Furthermore, the kinetics of cell cycle exit of satellite cells in the KO mice were different from those in WT mice. During muscle regeneration, activated satellite cells re-enter the cell cycle (5-ethynyl-2′-deoxyuridine [EdU]/Myod1 population), followed by exit from the cell cycle (EdU/Myod1) before terminal differentiation. Comparison of the frequency of each population on day 4.5 showed an increased frequency of EdU/Myod1 cells in the single- and double-KO mice compared with WT mice (Fig. 1, F and G). This finding could suggest delayed cell cycle exit of the satellite cells in the KO mice, which translates into delayed muscle regeneration on day 4.5. Finally, the single- and double-KO mice contained more fibrosis on day 14 after the injury as demonstrated by Sirius red staining, suggesting more extensive damage or delayed regeneration in the KO mice (Fig. 1, H and I). Uninjured Per2−/− and Per1−/−:Per2−/− mice already contained more fibrosis than WT mice, although it was less severe than in day 14 mice, implying that natural turnover of myofibers was also disrupted in the KO mice. Thus, both Per1 and Per2 are necessary for the proper regeneration of TA muscle, and they can partially compensate each other, a reminiscence of the milder arrhythmic phenotype in the single-KO mice than in the double-KO mice.

To understand cell-autonomous effects of Per KO, primary myoblasts (activated satellite cells) were purified from hind limbs and induced to differentiate into myotubes with 5% horse serum (HS) in vitro. Per1−/−, Per2−/−, and Per1−/−:Per2−/− myoblasts displayed delayed activation of MHC, a marker for differentiation (Bader et al., 1982). This was quantified as decreased differentiation index (frequency of nuclei in MHC+ cells among total nuclei; Fig. 1 J and Fig. S1, C–F). These cells also demonstrated decreased fusion index (frequency of nuclei in MHC+ cells containing more than one nucleus among total nuclei) and increased frequency of EdU uptake compared with WT cells (Fig. 1, K and L; and Fig. S1, C–F). These results exhibited impaired cell-autonomous myoblast differentiation by Per KO, which could explain the delayed TA muscle regeneration, although niche effects could not be excluded.

To obtain a large number of cells for a mechanistic study, we examined whether the mouse myoblast cell line C2C12 could recapitulate the KO phenotypes of the primary myoblasts. The Per1 or Per2 gene was depleted by shRNA-mediated knockdown (KD) and CRISPR-Cas9–mediated KO (Fig. S2, A–C). We used KD
Figure 1. Regeneration of TA muscle in Per1−/−, Per2−/−, and Per1−/−:Per2−/− mice. (A) H&E staining of day 4.5 TA muscle sections. TA muscle was injured with barium chloride at ZT14 on day 0, and EdU was injected i.p. 96 h later for F and G. The muscle was harvested 12 h later at day 4.5. Bar, 100 µm. (B) Size
and KO because we could identify only one effective shRNA clone for each gene. These cells were used in bulk without cloning since differentiation-resistant cells would be selected by cloning. When the cells were induced to differentiate, MHC+ cells became shorter and sparser in the KD and KO cells than control cells on differentiation days 3 and 5 (Fig. 2 A). This finding was quantified as decreased differentiation index and fusion index in the KD and KO cells (Fig. 2, B and C). The KO cells also demonstrated slightly delayed cell cycle exit during differentiation (Fig. 2 D). Additionally, expression of differentiation-specific genes encoding Myog, muscle creatinine kinase (Ckm), myomaker (Mymk), and myosin heavy chain 3 (Myh3) was decreased by the KD and KO (Fig. 2 E). The similarity of the consequences of Per1 and Per2 depletions was further highlighted by several transcriptome datasets. The list included a heat map, a principal component analysis, Venn diagrams of differentially expressed genes (>2,000 genes were commonly up- or downregulated more than twofold compared with control cells), and scatterplots (R² > 0.97; Fig. 2, F and G; and Fig. S2, D–F). In addition, the gene ontology analysis demonstrated that many myogenesis-related genes were commonly down-regulated by Per1 KO and Per2 KO in undifferentiated cells and differentiating cells on days 3 and 5 (Fig. S2 G), further underscoring the requirement of both Per1 and Per2 for proper myogenesis.

Essential role of Igf2 in myoblast differentiation and muscle regeneration

The common phenotypes of Per1 and Per2 depletion led us to search for important myogenic genes that were commonly dysregulated by each KO. RNA sequencing (RNA-seq) revealed four muscle genes commonly down-regulated in Per1 KO and Per2 KO C2C12 cells in an undifferentiated state and during differentiation on days 3 and 5 (Fig. S3 A, highlighted in yellow). They included Cav3 (encoding the membrane protein caveolin 3), Crsp3 (the multifunctional protein cysteine and glycine-rich protein 3), Igf2, and Myo21 (a signaling protein). We decided to focus on Igf2 because of its significance in myogenesis as described earlier. Down-regulation of Igf2 in Per KD and KO cells was verified by quantitative PCR (qPCR; Fig. S3 B). To investigate the involvement of Igf2 in the Per depletion phenotypes, Igf2 was knocked down with two shRNAs in C2C12 cells (Fig. S3 C). MHC+ cells in the KD cells were sparser and shorter than control KD cells, consistent with the lower differentiation index and fusion index, as well as the decreased expression of differentiation-specific genes (Fig. S3 D; and Fig. S4, A–C). Cell cycle exit was also delayed by Igf2 KD during differentiation (Fig. S4 D). Thus, Igf2 depletion recapitulated the phenotypes of Per depletion. Additionally, Igf2 KD downregulated Cav3, Crsp3, and Myo21, indicating that these genes were under the control of Igf2 (Fig. 3 A).

In a complementary study, Per1 and Per2 were overexpressed with retrovirus in C2C12 cells (Fig. S4 E) to investigate the effects on Igf2 expression and differentiation. This study was done comparing control KD and Igf2 KD cells to understand whether the effects of Per overexpression were dependent on the presence of Igf2. In the control KD cells, overexpressed Per1 and Per2 raised the Igf2 mRNA level up to fourfold higher than that with empty vector on differentiation days 3 and 5 (Fig. 3 B, Igf2 in the Control KD columns). Because Per1 and Per2 regulate gene expression through binding to the Clock–Bmal1 complex, Clock and Bmal1 were also overexpressed with or without Per1 and Per2. However, the additional overexpression of Clock and Bmal1 did not affect the Igf2 level (Fig. 3 B, Igf2 with CBE, CBP1, and CBP2 in the Control KD columns). A similar trend was observed in the effects of overexpressed Per1 and Per2 on differentiation. Whereas single overexpression of Per1 or Per2 raised the expression levels of differentiation-specific genes, differentiation index, and fusion index, an addition of Clock and Bmal1 did not substantially alter the effects (Fig. 3, B–D). Importantly, all the effects by the overexpressed Per1 and Per2 were erased in Igf2 KD cells (Fig. 3, B–D, Igf2 KD1 columns), demonstrating that Igf2 was an essential downstream effector for the overexpressed Per1 and Per2 to promote myoblast differentiation. Thus, up- and downregulation of Per1 and Per2 demonstrated the Per1/Per2–Igf2 axis as a novel myogenic pathway.

We also investigated whether Igf2 promotes muscle regeneration in an autocrine manner in vivo using mice with satellite cell–specific conditional KO of Igf2 (Igf2 cKO). Satellite cell–specific cKO was particularly important for Igf2 because the soluble factor from other tissues could regulate muscle regeneration. Although whole-body KO of Igf2 impairs mouse growth, muscle-specific roles of Igf2 in vivo remained unclear. We disrupted only the paternal allele inherited Igf2 allele with Foxp7 promoter-driver CreERT2 because Igf2 is imprint during the paternal allele being preferentially expressed (Sasaki et al., 1992). After induction of CreERT2 with repeated tamoxifen (TMX) injection, TA muscle was injured with barium chloride, and muscle regeneration was compared with control mice that did not harbor the CreERT2 gene (Fig. 4 A). Depletion of the Igf2 mRNA in satellite cells was verified by qPCR (Fig. 4 B). H&E staining displayed that although Igf2 cKO only slightly decreased myofiber size in uninjured muscle compared with control mice, the smaller size was more prominent in regenerating muscle on day 7 (Fig. 4, C–E). The size of eMHC+ myofibers on day 4 was...
Figure 2. Differentiation of C2C12 cells after depletion of Per1 and Per2. (A) Immunofluorescence staining of C2C12 cells with MHC antibody during differentiation with 5% HS. Per1 and Per2 were depleted with shRNA (KD) and CRISPR-Cas9 (KO). Nontargeting sequences were used as a control for each. Bar,
also smaller in Igf2 cKO mice (Fig. 4, F and G). Although Igf2 cKO did not increase the frequency of EdU+/MyoD+ cells, unlike Per KO, it decreased the population of EdU+/MyoD+ cells, an indicator of delayed cell cycle exit in satellite cells by Igf2 cKO (Fig. 4, H and I). Furthermore, differentiation index and fusion index in primary myoblasts were smaller in Igf2 cKO mice than in control mice in vitro (Fig. 4, J and K; and Fig. S4 F), verifying the previous results with C2C12 cells. Collectively, these findings indicated the requirement of autocrine Igf2 in myoblast differentiation and muscle regeneration.

Circadian expression of Igf2

Igf2 was likely to be expressed in a circadian manner as a target gene of Per1 and Per2. This possibility was tested by Western blotting with C2C12 cells harvested every 4 h after circadian synchronization with dexamethasone. The protein level of Bmal1 reached a peak at 44 h after synchronization, which was anti-phasic to the expression patterns of Per1 and Per2 in control cells (Fig. 5 A). Igf2 expression reached peaks at 32–36 h and 56 h, similar to the patterns of Per1 and Per2. Phosphorylation of p38 (p-p38) followed the expression pattern of Igf2 as its downstream effector. In contrast, Igf2 and p-p38 were severely down-regulated in Per KO cells. Igf2 was also expressed in TA muscle in a circadian manner, but the rhythms were largely lost in Per1−/− and Per2−/− mice (Fig. 5 B). These results demonstrate that Igf2 shows circadian expression and that its expression level is regulated by Per1 and Per2.

Next, the concentration of Igf2 in the culture supernatant of C2C12 cells was measured with ELISA. The concentration was ~0.025 ng/ml with undifferentiated cells and was increased sixfold during differentiation as previously reported (Fig. 5 C; Florini et al., 1991). Although Igf2 in the supernatant of Per KO cells was also increased, the level remained ~20% of the control level on day 5, consistent with PCR and Western blotting results. The Igf2 concentration with control cells also displayed oscillation that was similar to the Western blotting result (Fig. 5 D). The concentration represented the amount of accumulated Igf2 since 0 h, when dexamethasone was replaced with fresh culture medium. The result likely reflected the gain by secretion and the loss by degradation and attachment to the culture dish and cell surface. The oscillation became more evident when an increase or a decrease between two time points was plotted (Fig. 5 E).

We also examined whether exogenous Igf2 could rescue the disrupted differentiation of Per KO cells by adding Igf2 to the culture medium from day 0 onward. Differentiation index and fusion index on days 3 and 5 exhibited improved differentiation with 1 ng/ml Igf2, reaching 40–60% levels of those observed with control cells without exogenous Igf2 (Fig. S4 G). Activation of Ckm and Myog by Igf2 was more efficient; the mRNA levels became indistinguishable from those with control cells without exogenous Igf2 on day 3 for both genes and on day 5 for Myg (Fig. S4 H). Thus, exogenous Igf2 could substantially rescue the disrupted differentiation of Per KO cells, consistent with the notion of the Per1/Per2–Igf2 axis. Note that the concentration of effective Igf2 in the culture medium in these experiments was unknown due to the presence of IGFBPs.

Epigenetic regulation of the Igf2 expression by Per1 and Per2

To elucidate how Per1 and Per2 promoted Igf2 expression, epigenetic changes caused by Per KO were studied with chromatin immunoprecipitation (ChIP)–qPCR. An Igf2 enhancer containing two E-boxes is embedded within an intron of the Nctd gene located 105 kb downstream of the Igf2 promoter (Alzhanov and Rotwein, 2016; Alzhanov et al., 2010). Publicly available ChIP-seq data obtained with nonsynchronized myoblasts demonstrated binding peaks of Bmal1, its binding partner histone acetylase p300 (Papazyan et al., 2016; Takahashi, 2017), and an enhancer marker H3K27ac (acetylated lysine 27 in histone H3) at the Igf2 enhancer, whereas another enhancer marker H3K4me1 was broadly distributed (Fig. 5 F). Our ChIP-qPCR with synchronized control C2C12 cells demonstrated circadian binding of Bmal and p300 at the enhancer, and the temporal profile was antiphasic to those of Per1 and Per2 as anticipated (Fig. 5 G). The binding pattern of Bmal1 was not significantly altered by KO of Per1 or Per2. The abundance of H3K27ac also demonstrated an oscillatory pattern that was flattened in Per1 and Per2 KO cells. The level of H3K4me1 did not exhibit a clear circadian pattern in control or Per KO cells. The histone modification patterns were consistent with the general trend in that, whereas H3K27ac marks active enhancers, H3K4me1 is present in both active and inactive/poised enhancers (Creyghton et al., 2010; Rada-Iglesias et al., 2011). All these protein levels were substantially decreased outside the enhancer, except for the less prominent or no decrease of H3K4me1 (Fig. S5 A, regions R1 and R3), underscoring the enhancer specificity of our results.

Muscle cells primarily use promoter 3 among the three promoters of the Igf2 gene (Baral and Rotwein, 2019; Kou and Rotwein, 1993), which was verified by qPCR (Fig. S5, B and C). Downloaded ChIP-seq data with nonsynchronized myoblasts did not show a specific increase or decrease of Bmal1, RNA polymerase II, or histone markers for active genes (H3K4me3 and H3K9ac) at promoter 3 (Fig. 5 H, region R5). However, synchronized C2C12 cells again demonstrated a Per-dependent increase of these proteins at promoter 3 (Fig. 5 I) with weaker or no oscillation in the surrounding regions (Fig. S5 D for regions R4 and R6). Thus, both Per1 and Per2 are necessary for the circadian dynamics of multiple epigenetic markers characteristic of gene activation at the Igf2 enhancer and promoter 3.

To understand the functional significance of the circadian epigenetics, the temporal profile of nascent Igf2 mRNA was

Katoku-Kikyo et al.

Circadian regulation of myogenesis

Journal of Cell Biology

6 of 20

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Figure 3. Disrupted differentiation of C2C12 cells by Igf2 KD. (A) Relative expression levels of Cav3, Csrp3, and Myoz1 in Igf2 KD cells. (B) Relative expression levels of Igf2 and muscle genes during differentiation of C2C12 cells. The cells were transduced with empty vector (Emp Vec), Per1, Per2, CBE (Clock, Bmal1, and empty vector), CBP1 (Clock, Bmal1, and Per1), and CBP2 (Clock, Bmal1, and Per2) before differentiation. Igf2 KD cells and control cells were compared. (C and D) Differentiation index (C) and fusion index (D) of the cells used in B. Data are presented as mean ± SEM of biological triplicates. Each replicate includes n = 1,000–1,500 nuclei in C and D. *, P < 0.05; **, P < 0.01; and ***, P < 0.001 with Student’s t test compared with control KD cells in A and with empty vector cells in B–D.
Figure 4. Impaired muscle regeneration in satellite cell–specific Igf2 cKO mice. (A) Experimental scheme for satellite cell–specific Igf2 cKO by injection of TMX and muscle regeneration from barium chloride (BaCl2)–induced injury. (B) Relative expression level of Igf2 in isolated satellite cells comparing control.
Quantified with synchronized cells. A nuclear run-on assay demonstrated that nascent Igf2 mRNA was most abundant at time points when the Igf2 enhancer and promoter were enriched with active gene markers in control cells (Fig. 5 J, 36 h and 60 h). However, the Igf2 level remained low throughout the process with Per KO cells as expected. Therefore, the circadian transcriptional changes of Igf2 indeed reflected the epigenetic dynamics of the gene.

The interaction between the Igf2 enhancer and promoter 3 has been shown in differentiating myoblasts and skeletal muscle (Alzhanov et al., 2010; Yoon et al., 2007). Because chromatin loop formation is also regulated by circadian rhythms (Pacheco-Bernal et al., 2019; Yeung et al., 2018), we hypothesized that the Igf2 enhancer–promoter interaction would also demonstrate circadian oscillation. This possibility was examined with chromosome conformation capture (3C) by studying the interaction between promoter 3 (anchor point in 3C) and the enhancer at 24 and 36 h after synchronization, which corresponded to the nadir and the peak of the Igf2 level, respectively (Fig. 6 A). The interaction (cross-linking frequency) at 36 h was more than twice as high as it was at 24 h in control cells (Fig. 6 B). This pattern was preserved in Per KO cells, but the cross-linking efficiency became less than half compared with control cells (Fig. 6, B and C). Therefore, both Per1 and Per2 are required for the circadian dynamics of the promoter–enhancer interaction at the Igf2 gene, just like the epigenetic modifications.

Coupling of differentiation efficiency and the circadian timing of differentiation initiation

The above findings led us to a hypothesis that myoblasts could differentiate more efficiently if differentiation cues are provided at the circadian timing when Per1, Per2, and Igf2 are highly expressed compared with other time points (“primed”). This was evaluated with C2C12 cells that were induced to differentiate at different time points after synchronization. Indeed, the cells differentiated more efficiently when induced at 36 h and 60 h after synchronization compared with 24 h and 48 h, as demonstrated by higher differentiation index, fusion index, and the expression levels of differentiation-specific genes (Myh3, Myog, and Clm) and Igf2 after 48 h of differentiation (Fig. 6, D–H). This trend was in agreement with the epigenetic modifications of the Igf2 enhancer and promoter. Note that since the cells kept proliferation between 24 h and 60 h before the differentiation induction, the increased cell density per se could promote differentiation in the later phase. In this sense, the drop of the differentiation efficiency at 44–48 h in comparison with 36 h was more significant than the increased differentiation from 48 h to 60 h. Igf2 KD cells differentiated poorly, regardless of when differentiation was initiated (Fig. 5 S, E–G).

Since Per1, Per2, and Igf2 were also expressed in TA muscle in a circadian manner (Fig. 5 B), the timing of the injury could be an important factor for muscle regeneration. To assess this possibility, TA muscle was injured at ZT2 (low Per1, Per2, and Igf2; the early inactive phase of mice) and ZT14 (high Per1, Per2, and Igf2; the early active phase) to compare the regeneration efficiency. H&E staining clearly showed larger TA muscle in the ZT14 WT mice than in the ZT2 mice on day 4.5, but this difference was lost by day 14 (Fig. 7, A and B; and Fig. S5 H). There was no statistically significant difference between ZT2 and ZT14 injuries in the single- and double-KO mice of Per1 and Per2. The average diameter of eMHC+ myofibers was also longer in WT mice damaged at ZT14 than that in the ZT2 damage, and this difference was also lost in the single- and double-KO mice (Fig. 7, C and D). Moreover, the frequency of the EdU+/MyoD+ population was diminished in the ZT14 WT mice compared with ZT2 mice on days 4.5 and 5.5, suggesting early cell cycle exit (Fig. 7, E and F). Finally, fibrosis was also less abundant in the ZT14 WT mice than in the ZT2 mice on day 14; this difference was again erased in the single- and double-KO mice (Fig. 7, G and H). These results collectively indicate that circadian timing of injury affects the efficiency of TA muscle regeneration in a Per1- and Per2-dependent manner.

Discussion

The most novel message of the present work is that the efficiency of myoblast differentiation and muscle regeneration is dependent on the circadian timing when these events are triggered. As a mechanistic explanation obtained with the myoblast model, the Igf2 gene was primed toward activation in a circadian manner while the cells were still in the proliferation medium. Despite extensive studies of circadian regulation of cell proliferation and differentiation (Benitah and Welz, 2020; Dierickx et al., 2018; Paatela et al., 2019), studies focused on the mechanistic influence of circadian timing on tissue regeneration are quite limited. One of the few studies concerns fibroblast migration during skin wound healing (Hoyle et al., 2017). Fibroblast mobilization to a mouse skin incision site, an early and essential step in wound healing, was greater when the wound was inflicted at ZT13 than at ZT5. Additionally, when a skin explant was harvested and wounded at different time points, the number and volume of fibroblasts invading the wound area were higher in the explant harvested at ZT13 than at ZT5. Circadian regulation of actin polymerization, which controls...
Figure 5. Circadian oscillation of the expression and epigenetics of the Igf2 gene. (A) Western blotting with control and Per KO C2C12 cells harvested every 4 h after circadian synchronization. Histone H2B was used as a loading control. (B) Relative expression levels of Igf2, Per1, and Per2 in TA muscle.
measured by qPCR. The value of WT mice at ZT2 was defined as 1.0. n = 3 mice with technical triplicates for each. (C) Igf2 concentration in the C2C12 cell supernatant measured with ELISA. The culture medium was not replaced for 48 h before measurement. (D) Igf2 concentration in the C2C12 cell supernatant after circadian synchronization. Cells were treated with dexamethasone between −1 h and 0 h for synchronization. The culture medium was replaced with fresh growth medium at 0 h and was not changed until the harvest at the indicated time point. The concentration indicates the accumulated Igf2 in the medium. (E) The change of the Igf2 concentration in D was highlighted by displaying the change of the concentration between two time points. (F) ChIP-sequencing analyses of the Igf2 enhancer within the Ntc1 gene downloaded from the GEO database. See Materials and methods for the accession number of each dataset. R1–R3 indicate the regions amplified by PCR in G and in Fig. S5A. (G) ChIP-PCR analyses of the indicated proteins in control and Per KO C2C12 cells at region R2. Relative abundance compared with control IgG is shown. Peak values of control cells that are higher than those of Per KO and Per2 KO cells are highlighted with asterisks. (H) ChIP-sequencing analyses of the Igf2 promoters downloaded from the GEO database. See Materials and methods for the accession number of each dataset. R4–R6 indicate the regions amplified by PCR in I and in Fig. S5 D. Variants 1, 2, and 3 correspond to NM_010514, NM_001122736, and NM_001122737, respectively. Pr1–Pr3 indicate promoter 1 to promoter 3. Pol II, RNA polymerase II. (I) ChIP-PCR analyses of indicated proteins in control and Per KO C2C12 cells at the R5 region. (J) Nascent transcription analysis with a nuclear run-on assay comparing control and Per KO cells. Synchronized C2C12 cells were labeled with EU for 4 h before harvesting every 4 h, and EU* RNA was isolated with a kit, followed by RT-PCR of the indicated genes. The values were normalized against Clock mRNA, whose expression was not influenced by circadian rhythms. The value with control cells at 24 h after synchronization was defined as 1.0. Data are presented as mean ± SEM of biological triplicates. *, P < 0.05; **, P < 0.01; and ***, P < 0.001 with Student’s t test compared with control. †, 24 h rhythmicity with Cosinor (P < 0.05); ††, P < 0.01; and †††, P < 0.001.

migration and adhesion, is one of the mechanisms for the time-dependent difference. In a related phenomenon, circadian timing of physical exercise influences muscle strength and oxidative capacity (Wolf and Esser, 2019). For example, muscle atrophy in the mouse hind limb due to reduced gravity was prevented more effectively by intermittent weight bearing between ZT12 and ZT16 than between ZT20 and ZT0 (Aoyama et al., 2018). Although we linked circadian timing and the efficiency of muscle regeneration from an acute injury, it remains an open question whether chronic muscle damage, such as exercise-induced injuries, exhibit the same level of circadian responses. At the mechanistic level, we demonstrated that Igf2 is a required downstream effector of Per1/Per2 for myogenesis and that Igf2 is expressed in a circadian manner. However, we did not determine whether it was the overall expression level or rhythm of Per1/Per2 and Igf2 that is important for myogenesis and muscle regeneration. Experiments with KO cells and mice cannot distinguish the two possibilities. A recently reported artificial circadian clock in clock-deficient mice could provide a valuable model to directly address this question (D’Alessandro et al., 2015).

Our findings on the circadian timing–dependent differentiation and regeneration should be interpreted in a broader perspective of circadian metabolic regulation that defines the availability of energy and cellular building blocks (Aoyama and Shibata, 2017; Aoyama and Shibata, 2020; Panda, 2016). A circadian transcriptome analysis of muscle uncovered clustered expression of genes with a common metabolic function at specific circadian phases in the mouse under constant darkness with ad libitum feeding (Hodge et al., 2015). Specifically, the genes involved in carbohydrate catabolism (the early active/dark phase), carbohydrate storage (the mid-active/dark phase), lipogenesis (the end of the active/dark phase), and fatty-acid uptake and β-oxidation (the mid-inactive/light phase) reached peaks at distinct circadian phases as indicated in the parentheses. Metabolomic profiling of muscle also demonstrated neutral lipid storage and decreased lipid and protein catabolism in the late inactive phase (Dyar et al., 2018). Given the global circadian oscillation of the numerous metabolites essential for tissue turnover, circadian timing could create a preferred time window for an effective response to major tissue disruption and repair, although experimental evidence is lacking. The interaction between the Per1/Per2–Igf2 axis and the global metabolic oscillation awaits further studies. This is further supported by a recent study demonstrating regulation of the Per protein level by insulin and Igf1, which are regulated by feeding and share signaling pathways with Igf2, at the transcription and translation levels (Crosby et al., 2019).

Per1 and Per2 are generally considered as transcription repressors. In our study, however, >1,000 genes were commonly up-regulated by Per1 KO and Per2 KO cells, suggesting that Per1 and Per2 can act as direct or indirect gene activators in this context. Per-induced gene activation has been demonstrated with several genes involved in sodium channels in the kidney (Gumz et al., 2009; Richards et al., 2014; Stow et al., 2012). In addition, Bmal1 and Per1 are required for the circadian activation of prolactin in a rat mammatropo cell line (Bose and Boocoff, 2010). Moreover, Per2 activates Cry1 by removing the Clock–Bmal1–Cry1 repressor complex from the Cry1 promoter in an ectopic expression model (Chiou et al., 2016). This study also showed that genes with complex promoters can be repressed or de-repressed by Per, depending on the regulatory elements at the promoters. Furthermore, Per2 can activate its own promoter, which has been explained by the blocking of the binding of Cry to the Clock–Bmal1 complex (Akashi et al., 2014). In our study, although the Per proteins bind the Igf2 enhancer, it remains unknown whether they directly contribute to the transcription activation of Igf2. Because Per proteins bind thousands of loci along with Clock–Bmal1, Igf2 activation could be an indirect activity of Pers. Identification of the binding proteins of Per1 and Per2 would be an important next step to further clarify how Per1 and Per2 activate Igf2 during myoblast differentiation.

This study revealed circadian regulation of myoblast differentiation and muscle regeneration and demonstrated epigenetic regulation of the Igf2 gene by Per1 and Per2 as one of the underlying mechanisms using a myoblast differentiation model. Future genome-wide epigenetic analysis of histone modifications and chromatin interactions would further uncover other unexpected underpinnings for the time of the day-dependent regeneration of muscle and other tissues.
Figure 6. Circadian regulation of the Igf2 gene and C2C12 cell differentiation. (A) Locations of the primers used in the 3C experiments and BamHI sites in relation to the Igf2 promoter 3 and enhancer. The primer shown in red was used in combination with one of the primers shown in black in 3C, and the results

Katoku-Kikyo et al. Circadian regulation of myogenesis

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Muscle was injected with 50 µl 1.2% BaCl₂ in 0.9% NaCl at experiments. ZT0 corresponds to 6:00 and ZT12 to 18:00. TA

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Igf2 Association recommendations.

Panel of Euthanasia of the American Veterinary Medical

extracted within 1 wk. Control mice that contained the WT allele were also injected with TMX.

All methods aligned with the Institutional Animal Care and Use Committee of the University of Minnesota (1902-36737A and 1903-36906A). Per1+/−/ mice (B6.129-Per1tm1Drw/J; stock no. 010491) and Per2+/−/ mice (B6.129-Per2tm1Drw/J; stock no. 010492) were purchased from The Jackson Laboratory. Per1−/−, Per2−/−, Per1−/−:Per2−/−, and WT mice were obtained by breeding and identified by genotyping according to The Jackson Laboratory protocols. The same number of male and female mice at the age of 8–10 wk old were mixed in each group. The number of mice used is described in each figure legend. Mice were entrained at 12-h light and 12-h dark cycles (6:00–18:00 light and 18:00–6:00 dark) for 2 wk before experiments. ZT0 corresponds to 6:00 and ZT12 to 18:00. TA muscle was injected with 50 µl 1.2% BaCl₂ in 0.9% NaCl at ZT2 or ZT14 to induce muscle injury and subsequent regeneration. Mice were euthanized on day 4.5, 7, or 14 after injury, and the TA muscle was extracted at ZT2 or ZT14. In addition, uninjured TA muscle was isolated every 4 h for qPCR. Mice were monitored by the Research Animal Resources staff of the University of Minnesota in specific pathogen–free housing. Mice were given standard chow and access to drinking water without restrictions. Mice were euthanized via CO₂ inhalation. All methods aligned with the Panel of Euthanasia of the American Veterinary Medical Association recommendations.

Igf2 cKO mice

Pax7CreERT2 mice [B6.Cg-Pax7tm1(cre/ERT2)Gaka/J; stock no. 017763] and IGF2LoxP/LoxP mice (Igf2tm1.LThor/J; stock no. 032493) were purchased from The Jackson Laboratory (Modi et al., 2015; Murphy et al., 2011). Igf2 is imprinted with preferential expression of the paternal allele (Sasaki et al., 1992). Superscript P and M indicate paternal and maternal inheritance of the Igf2 allele in the IGF2 M-LoxP/P-LoxP and IGF2 M-LoxP/P-LoxP male mice were bred with Pax7CreERT2 female mice to generate Pax7−/−:IGF2M−/P−LoxP (control) and Pax7+/−:CreERT2:IGF2M+/P−LoxP (cKO) mice. Genotyping to detect the mutant alleles was performed by PCR using the primers shown in Table S1. Cre recombination was induced with TMX (TS648; MilliporeSigma) at 75 µg/g body weight four times between 6 and 7 wk of age. TA muscle was injured with BaCl₂ at 7 wk of age, and the muscle was extracted within 1 wk. Control mice that contained the WT Pax7 allele were also injected with TMX.

Preparation of primary myoblasts

Mouse muscle mononuclear cells were prepared from hind limbs of 8–10-wk-old male mice as previously described (Asakura et al., 2002; Motohashi et al., 2014). More specifically, muscles were minced and digested with 0.2% collagenase type 2 ( Worthington Biochemical; CLS-2) in DMEM to dissociate muscle cells. Satellite cells were purified with LD columns (Miltenyi Biotec; 130-042-901) by negative selection with antibodies against CD31-PE, CD45-PE, and Sca1-PE, followed by anti-PE MicroBeads (Miltenyi Biotec; 130-048-801). See Table S2 for the details of the antibodies. Positive selection was subsequently applied with an antibody against biotin-conjugated integrin α7-biotin and anti-biotin MicroBeads (Miltenyi Biotec; 130-090-485), followed by MS columns (Miltenyi Biotec; 130-042-201). Isolated satellite cells were cultured on dishes coated with 0.01% rat tail collagen (BD Biosciences; 354236) in myoblast growth medium (Ham’s F-10 medium with 20% FBS, 10 ng/ml basic FGF [Thermo Fisher Scientific; PHG0263], 100 U/ml penicillin, and 100 mg/ml streptomycin) at 37°C with 5% CO₂. Low-passage satellite cell–derived primary myoblasts (typically fewer than eight passages) were used for differentiation and immunostaining. DMEM with 5% HS, penicillin, and streptomycin was used for myogenic differentiation.

Culture of C2C12 cells

Female mouse myoblast C2C12 cells (American Type Culture Collection; CRL-1772) were maintained in the growth medium (10% FBS in DMEM) in a 37°C and 5% CO₂ incubator. Cells were authenticated by immunofluorescence staining of MyoD. Differentiation was induced on day 0, when cells were at 90% confluence, by rinsing with PBS twice and adding the differentiation medium (5% HS in DMEM). The medium was changed every 2 d thereafter. Concentration of endogenous Igf2 was measured with a mouse Igf2 ELISA kit (Thermo Fisher Scientific; EMIGF2). Because of the low Igf2 concentration, 300 µl culture supernatant was added seven times for 3 h each (2.1 ml in total). The effect of exogenous Igf2 was tested with recombinant human IGF2 (FeproTech; 100-12). To synchronize circadian rhythms, C2C12 cells were seeded on day −1, and 0.2 µM dexamethasone was added at −1 h on day 0. Cells were washed with PBS twice, and fresh 10% FBS in DMEM was added at 0 h. Cells were harvested for PCR or fixed for immunofluorescence staining every 4 h at the indicated time points.
Figure 7. Differential regeneration efficiency of TA muscle depending on the circadian timing of the injury. (A) Size distribution of H&E-stained myofibers containing centrally located nuclei on day 4.5. TA muscle was injured with barium chloride at ZT2 or ZT14 on day 0 and harvested on day 4.5. The
minimal Feret’s diameter of each myofiber was calculated. n = 8 mice with 4 males and 4 females in each group in A and B. (B) Average of the minimal Feret’s diameters of myofibers with centrally located nuclei on day 4.5. 2 and 14 at the end of each genotype indicate the injury time at ZT2 and ZT14, respectively. (C) Immunofluorescence staining of WT TA muscle injured at ZT2 and ZT14 with antibodies against eMHC and laminin on day 4.5. DNA was counterstained with DAPI. Bar, 100 µm. (D) Average of the minimal Feret’s diameters of the eMHC+ areas on day 4.5. n = 4 mice. (E) Immunofluorescence staining of WT TA muscle sections with the MyoD antibody and an Edu kit. TA muscle was injured with barium chloride at ZT2 or ZT14 on day 0, and Edu was injected i.p. 96 or 120 h later. The muscle was harvested 12 h later at day 4.5 or 5.5. Bar, 25 µm. (F) Frequency of positive cells for Edu uptake and MyoD staining in TA muscle sections shown in E. n = 4 mice. (G) Sirius red staining of WT TA muscle on day 14 after injury. Bar, 200 µm. (H) The area percentage of fibrosis indicated by positive Sirius red staining on days 7 and 14. Data are presented as mean ± SEM. *, P < 0.05; **, P < 0.01; and ***, P < 0.001 with Student’s t test. The values at ZT14 in Fig. 1 were reused in these figures.

**H&E staining**

Cryosections of the TA muscle were fixed with 2% PFA for 10 min. The sections were then treated as follows: deionized water for 1 min, Harris Modified Hematoxylin (Thermo Fisher Scientific; SH26-500D) for 2 min, tap water for 1 min, deionized water for 1 min, Eosin-Y (Thermo Fisher Scientific; 22-220-104) for 5 min, 95% ethanol for 30 s, 100% ethanol for 2 min twice, and xylen for 10 min twice. The sections were mounted with Permount (Thermo Fisher Scientific; SP15-100). Images were captured with cellSens Entry 1.11 software (Olympus) and a DP26 camera (Olympus) attached to an IX73 microscope with a UPlan FL N 10×/0.30 Ph1 lens (Olympus). The minimal Feret’s diameter of each myofiber was quantified with Fiji (National Institutes of Health).

**Sirius red staining**

The Sirius red solution was composed of 1% Direct Red 80 (MilliporeSigma; 365548) in 1.3% picric acid. TA sections were fixed with acetone, prechilled at −20°C, for 10 min. The sections were then washed in deionized water for 1 min, stained with Sirius red for 15 min, and rinsed with 0.5% acetic acid for 1 min. The sections were subsequently washed with 100% ethanol for 2 min and twice with xylene for 10 min. Finally, the sections were mounted with Permount for taking images as described for H&E staining. Quantification of Sirius red fibrosis was done using the entire section area with Fiji (Shimizu-Motohashi et al., 2015).

**Immunofluorescence staining of TA sections**

Sections were fixed with 2% PFA for 10 min, followed by permeabilization with 0.2% Triton X-100 in PBS for 5 min. The sections were treated with two blocking reagents: 3% Mouse-on-Mouse Blocking Reagent (Vector Laboratories; MBK-2213) in PBS for 1 h and 10% BSA in PBS for 30 min. Primary antibodies against eMHC and laminin diluted in 10% BSA in PBS were applied overnight, followed by washing twice with 0.01% Triton X-100 in PBS. The secondary antibodies Alexa Fluor 488 donkey anti-mouse IgG and Alexa Fluor 594 donkey anti-rat IgG diluted in 10% BSA in PBS were used for 2 h. DNA was counterstained with DAPI (MilliporeSigma; 10236276001). Sections were mounted using fluorescent mounting medium (Dako; S302380-2). To label proliferating myoblasts, mice were injected i.p. with EdU at 50 µg/g body weight on day 4 and euthanized 12 h later on day 4.5 after injury. EdU was detected using the Click-IT Edu Alexa Fluor 488 Imaging Kit (Thermo Fisher Scientific; C10337) followed by staining with anti-MyoD antibody, Alexa Fluor 594 donkey anti-rabbit IgG antibody, and DAPI (Verma et al., 2018). Fluorescence images were captured using MetaMorph Basic software (Molecular Devices) and an ORCA-flash4.0 LT camera (Hamamatsu) attached to an IX73 microscope (Olympus) with an LUCPlan FL N 20×/0.45 Ph1 lens. Images were processed with Photoshop and Illustrator CS6 (Adobe). The minimal Feret’s diameter of each myofiber was quantified with Fiji (National Institutes of Health).

**Gene KO in C2C12 cells**

On day 1, 293FT cells (Thermo Fisher Scientific; R70007) were seeded in DMEM with 10% FBS at 3 × 105 cells/well in a 12-well plate. On day 2, cells were transfected with 0.5 µg pLKO.1 lentivirus vector encoding an shRNA sequence shown in Table S3, along with 0.2 µg each of pCMV–vesicular stomatitis virus G (Addgene; 8454), pRSV-Rev (Addgene; 12253), and pMDLG/pPRE (Addgene; 12251) using 2.75 µl Lipofectamine 2000 (Thermo Fisher Scientific; 11668019). The culture medium was replaced with fresh DMEM with 10% FBS 5 h after transfection. On day 4, C2C12 cells were seeded at 105 cells/well in 12-well plates. On day 5, the culture supernatant of the transfected 293FT cells was applied to a 0.45-µm syringe filter and added to C2C12 cells with 0.8 µg/ml polybrene (MilliporeSigma; H9268). The culture medium was replaced with fresh DMEM with 10% FBS on day 6. Virus-integrated cells were selected with 1 µg/ml puromycin dihydrochloride (MB Biomedicals; 100552) between days 7 and 14. Selected cells were expanded and frozen in liquid nitrogen.

**Gene KOs in C2C12 cells**

On day 1, C2C12 cells were seeded in DMEM with 10% FBS at 1.8 × 105 cells/well in 12-well plates. Cells were transfected with 0.5 µg single-guide RNA (sgRNA; Synthego) against Peri and Per2 (Table S3), 0.5 µg CleanCap Cas9 mRNA (TrLink; L-7206), 0.2 µg CleanCap mCherry mRNA (TrLink; L-7203), and 1 µl TransIT-mRNA transfection kit (Mirus Bio) at a density of 105 cells/well in 12-well plates. On day 2, cells were subcultured on day 3 to expand and freeze them. A small aliquot of cells was used for Sanger sequencing of genomic DNA to verify the KO efficiency.

**Overexpression of Clock, Bmal1, Per1, and Per2**

Mouse Clock, Bmal1, Per1, and Per2 cDNAs were inserted into the pMXs-IP vector (Kitamura et al., 2003). PLAT-E cells (Morita et al., 2000) were seeded at a density of 2.5 × 105 cells/well in a 12-well plate with 10% FBS on day 1. On day 2, cells were transfected with 750 ng of the pMXs-IP vectors with 2.3 µl FuGENE 6 (Promega; E2691). The culture medium was replaced with fresh DMEM with 10% FBS, and C2C12 cells were seeded at
Circadian regulation of myogenesis

Whole-cell extracts obtained from $2 \times 10^5$ cells with an NE-PER Western blotting nuclei in each replicate. The differentiation index, fusion index, and EdU uptake were analyzed with biological triplicates, including 80–130 nuclei in each replicate.

C2C12 cells were similarly stained with primary antibody against MHC, secondary antibody Alexa Fluor 488 goat anti-mouse IgG, and 5 µg/ml Hoechst 33342 (MilliporeSigma; B2261). The differentiation index, fusion index, and EdU uptake were analyzed with biological triplicates, including 1,000–1,500 nuclei in each replicate.

**Immunofluorescence staining of cells**

Primary myoblasts were fixed with 2% PFA for 10 min and blocked with 1% BSA in PBS for 30 min. Permeabilized cells were stained with antibodies against MHC and MyoD and then incubated with secondary antibody Alexa Fluor 488 anti-mouse IgG and Alexa Fluor 594 anti-rabbit IgG. DNA was counterstained with DAPI. To measure proliferating primary myoblasts, cells were pulsed with 1 µg/ml of EdU 3 h before harvest. EdU was detected using a Click-it EdU Alexa Fluor 488 Imaging Kit (Thermo Fisher Scientific; CI0337) followed by staining with anti-MyoD antibody, Alexa Fluor 594 donkey anti-rabbit IgG antibody, and DAPI (Verma et al., 2018). Fluorescence images were captured using MetaMorph Basic software (Molecular Devices) with an LUCPlan FL N 20×/0.45 Ph1 lens (Olympus) and an ORCA-flash4.0 LT camera (Hamamatsu) attached to an IX73P2F microscope (Olympus). The images were processed with Adobe Photoshop and Illustrator CS6. The differentiation index was defined as a percentage of nuclei (DAPI-stained structure) existing within MHC+ cells. The fusion index is a percentage of nuclei that were located in MHC+ cells containing more than one nucleus. The differentiation index, fusion index, and EdU uptake were analyzed with biological triplicates, including 80–130 nuclei in each replicate.

C2C12 cells were similarly stained with primary antibody against MHC, secondary antibody Alexa Fluor 488 goat anti-mouse IgG, and 5 µg/ml Hoechst 33342 (MilliporeSigma; B2261). The differentiation index, fusion index, and EdU uptake were analyzed with biological triplicates, including 1,000–1,500 nuclei in each replicate.

**Quantitative RT-PCR**

RNA was extracted from cells using a Quick RNA MicroPrep (Zymo Research; R1051) or RNeasy Plus Mini (Qiagen; 74136) kit, depending on cell number. RNA quantity and purity were assessed using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific). cDNA was synthesized with ProtoScript II reverse transcription (New England Biolabs; M0368L). qPCR was performed with the primers listed in Table S1 and GoTag qPCR Master Mix (Promega; A6002) in a Mastercycler RealPlex² thermocycler (Eppendorf). PCR conditions were as follows: initial denaturation at 95°C for 10 min, 40 cycles of 95°C for 15 s, 30 s at the specific annealing temperature for each set of primers, and 72°C for 30 s and a melting curve step to check the specificity of the reaction. mRNA expression levels were analyzed by normalizing expression values to Gapdh expression. Mean ± SEM of biological triplicates with technical triplicates each were calculated.

Nascent mRNA was measured with a Click-it Nascent RNA Capture kit (Thermo Fisher Scientific; CI0365). C2C12 cells were incubated with 0.1 mM 5-ethyluridine (EU) for 4 h, followed by the isolation of EU-labeled mRNAs with magnetic beads for cDNA synthesis and qPCR.

**ChIP-PCR**

2 million C2C12 cells were treated with 1% PFA for 10 min and then with 125 mM glycerol, followed by washing with PBS twice. Chromatin was prepared by treatment with 300 µl cell lysis buffer (50 mM Hepes, pH 7.8, 85 mM NaCl, 0.5% NP-40, and Complete Mini Protease Inhibitor Cocktail [MilliporeSigma; 11836153001]) for 15 min on ice with vortexing for 15 s every 5 min. After centrifugation at 1,500 × g for 5 min at 4°C, the pellet was resuspended in 50 µl nuclear lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS, and Complete Mini Protease Inhibitor Cocktail) and incubated for 5 min on ice. Chromatin was sheared by sonication with a Bioruptor 300 (Diagenode) with 30 cycles of 30 s on and 30 s off with the high power setting at 4°C. After centrifugation at 15,000 × g for 15 min at 4°C, supernatant was incubated with 2 µg antibody, 2 µl Dynabeads Protein G (Thermo Fisher Scientific; 10004D), and 400 µl dilution buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, and Complete Mini Protease Inhibitor Cocktail) for 16 h at 4°C with rotation at 20 rpm. The beads were sequentially washed with 500 µl each of dilution buffer, LiCl buffer (20 mM Tris-HCl, pH 8.0, 250 mM LiCl, 2 mM EDTA, 0.1% SDS, and 1% Triton X-100) twice, and 10 mM Tris-HCl with 1 mM EDTA, rotating for 5 min at 4°C each. The beads were resuspended in 100 µl elution solution (0.1 M NaHCO₃, 1% SDS, and 200 µg/ml proteinase K) and incubated for 2 h at 65°C.

The p-p38 antibody was used as follows to avoid the sequestration of the antibody by casein in the milk. The membrane was blocked with 5% nonfat dry milk in TBST (50 mM Tris-HCl, pH 7.5, 0.1% Tween 20) for 1 h at 25°C, followed by washing with TBST for 5 min three times. The p-p38 antibody was diluted in 5% BSA in TBST, and three subsequent washes were done with TBST. Goat anti-rabbit IgG–HRP was diluted in 5% milk in TBST, and six subsequent washes were done with TBST.

**Western blotting**

Whole-cell extracts obtained from 2 × 10⁵ cells with an NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Fisher Scientific; 78833) were loaded into a 12% SDS-PAGE gel. After completion of electrophoresis, the proteins were transferred to an Immobilon P membrane (EMD Millipore; IPVH00010) at 25°C overnight. The next day, the membrane was blocked with 5% nonfat dry milk (Bio-Rad Laboratories; 180171A) in PBT (0.2% Tween 20 in PBS) for 1 h at 25°C. Proteins were then labeled with the primary antibody of interest diluted in 5% milk in PBT at 25°C for 1 h. After washing with PBT for 5 min three times, the membranes were incubated with the secondary antibodies goat anti-rabbit IgG–HRP or goat anti-mouse IgG–HRP, both diluted at 1:1,000 in 5% milk in PBT for 1 h at 25°C. After washing the membrane with PBT six times, the chemiluminescent signal was detected with a SuperSignal West Dura kit (Thermo Fisher Scientific; 34075) and x-ray films.

**Circadian regulation of myogenesis**

Katoku-Kiyogo et al.

Journal of Cell Biology

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with rotation to reverse cross-linking. Proteinase K was inactivated by heating for 10 min at 95°C. DNA was purified with a ChIP DNA Clean & Concentrator (Zymo Research; D5205) and applied for qPCR as described above. Results of biological triplicates with technical duplicates each were presented as ratios for comparison with the results with normal mouse IgG as a control.

The ChIP-seq data GSE25308 (RNA polymerase II, H3K4me1, H3K4me3, and H3K9ac), GSE97257 (H3K27ac and p300), and GSE108650 (Bmal1) were downloaded from the University of California Santa Cruz Mouse Genome Browser mm9. The quality of all downloaded data were evaluated using FastQC version 0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and the adapter sequence was trimmed using Trimmomatic version 0.33 (http://www.usadellab.org/cms/index.php?page=trimmomatic; Bolger et al., 2014). The filtered high-quality reads were then mapped to a reference genome (GRCm38/mm10) using HISAT2 version 2.0.2 (https://ccb.jhu.edu/software/hisat2/index.shtml; Kim et al., 2015). Resulting BAM files with MACS version 2.2.5 (https://github.com/macs3-project/MACS) were used to generate peaks (Zhang et al., 2008).

### 3C analysis

3C was performed combining two published protocols (Hagèze et al., 2007; Naumova et al., 2012). To make a bacterial artificial chromosome (BAC) control library, a mouse 110-kb BAC clone encoding NtcI and Iγf2 was purchased from Thermo Fisher Scientific (RPC123.C), and the DNA was prepared with a ZR BAC DNA Miniprep kit (Zymo Research; D4048) as a control. 10 μg of DNA was digested with BamHI for 16 h, followed by phenol-chloroform extraction and ethanol precipitation twice. DNA fragments were ligated with T4 ligase for 16 h at 16°C. DNA was purified with phenol-chloroform extraction and ethanol precipitation twice and used in qPCR.

C2C12 cells were treated with 1% PFA and then with 125 mM glycine as described in the ChIP-PCR method. 10 million cells were treated with lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, and 0.2% NP-40) for 10 min on ice. After centrifugation, the pellet was washed once with the digestion buffer for BamHI and incubated in a series of buffers for 1 h at 37°C each: BamHI buffer, BamHI buffer with 0.3% SDS, and BamHI buffer with 0.3% SDS and 2% Triton X-100. Finally, the cells were incubated with 400 U BamHI for 16 h at 37°C with rotation. BamHI was inactivated with 1.6% SDS and incubation at 65°C for 30 min. The cell suspension was diluted 10-fold with lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 0.2% NP-4, and 1% Triton X-100) and incubated for 1 h at 37°C with rotation. After the addition of 100 U T4 DNA ligase, the reaction mix was incubated for 4 h at 16°C, followed by incubation for 1 h at 25°C. Finally, the reaction mix was incubated for 16 h at 65°C in the presence of 300 μg proteinase K, followed by phenol-chloroform extraction and ethanol precipitation twice as a 3C library.

PCR data were analyzed as detailed by Naumova et al. (2012). Briefly, PCR was performed with 10 ng BAC control library and 3C library as templates using the 3C-0 kb primer in combination with each of 3C-76 kb through 3C-112 kb primers. The primer pair 3C-Clock1 and 3C-Clock2 at the Clock gene, which showed a consistent amplification throughout the circadian rhythms, was used as an internal control. PCR conditions were as follows: initial denaturation at 95°C for 5 min, 35 cycles of 95°C for 30 s, 70°C for 30 s, and 72°C for 20 s and an extension at 72°C for 5 min. PCR products were resolved with a 2% agarose gel and stained with ethidium bromide. The images were captured with a Gel Logic 212 Pro system (CareStream Molecular Imaging), and the band intensity was quantified with Fiji. Biological triplicates were analyzed for each group.

### RNA-seq

Total RNA was prepared from KO C2C12 cells before differentiation (day 0) and on days 3 and 5 during differentiation. RNA concentration and RNA integrity number were measured with an Agilent BioAnalyzer 2100. Samples with RNA integrity number >8 were used to create sequencing libraries at the University of Minnesota Genomics Center. 1 μg of total RNA was used to create each sequencing library using a TruSeq RNA Sample Preparation Kit (Illumina; RS-122-2001). Briefly, poly-adenylated RNA was first purified using oligo(dT)-coated magnetic beads. RNA was then fragmented and reverse transcribed into cDNA. The cDNA was further fragmented, blunt ended, and ligated to barcoded adapters and amplified. The final library size distribution was validated with capillary electrophoresis and quantified with a Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific; P11496) and qPCR. Indexed libraries were pooled and size selected to 320 bp ± 5% with a LabChip XT (PerkinElmer). Libraries were loaded onto a single-read flow cell and amplified on a cBot (Illumina) before sequencing using a NextSeq High kit (Illumina). The RNA-seq data have been deposited to Gene Expression Omnibus (GEO) under accession no. GSE150785.

### Bioinformatics analysis

On average, 58.78 million reads (51.23 million to 64.24 million) were generated per library. The demultiplexed FASTQ files were analyzed using a customized pipeline (gopher-pipelines; https://bitbucket.org/jgarbe/gopher-pipelines/overview) developed and maintained by the Minnesota Supercomputing Institute. Briefly, FastQC version 0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to check the sequencing quality of the FASTQ files. Then adapters and low-quality reads were trimmed using Trimmomatic version 0.33 (http://www.usadellab.org/cms/index.php?page=trimmomatic; Bolger et al., 2014). An additional quality check with FastQC was performed on the post-trimming sequences to ensure successful adapter and quality trimming. The remaining sequences were then aligned to the GRCh38/mm10 reference genome using HISAT2 version 2.0.2 (https://daehwankimlab.github.io/hisat2/), and transcript abundance was counted using subread version 1.4.6 (http://subread.sourceforge.net/; Kim et al., 2015; Liao et al., 2014). Differential gene expression analysis was performed with R version 3.6.2, edgeR (https://bioconductor.org/packages/release/bioc/html/edgeR.html; Robinson et al., 2010). Gene ontology analysis of differentially expressed genes was performed by functionally annotating the genes and performing an overrepresentation enrichment test using PANTHER.
Circadian regulation of myogenesis (http://pantherdb.org/; Mi et al., 2013). Heat maps were generated using the log-transformed counts with pheatmap version 1.0.12 (https://cran.r-project.org/web/packages/pheatmap/index.html) packages. Hierarchical clustering was performed using the average linkage clustering method with the correlation coefficient as a similarity metric.

**Principal component analysis**
Principal component analysis was performed to investigate the clustering of the datasets. The transformed and normalized gene expression values were used, and principal components were computed with R version 3.6.2 [prcomp(); https://www.rdocumentation.org/packages/utils/versions/3.6.2/topics/promp] The first and second largest variance components in the data (principal components 1 and 2) were visualized as a scatterplot using R version 3.6.2, ggplot2 version 3.2.1 (https://cran.r-project.org/web/packages/ggplot2/index.html).

**Scatterplot analysis**
Regression analysis was used to evaluate correlations between the two groups. Normalized expression values of each group were transformed with natural logarithms for better linear fitting and plotted against each other. The $R^2$ value was calculated and is shown on each regression line. All plots were produced in R version 3.6.2, ggplot2 version 3.2.1.

**Statistical analysis**
One-sided Student’s t tests were used in the analysis of statistical significance of the differences in the differentiation index, the fusion index, EdU uptake, quantitative RT-PCR, and ChIP-PCR data. The mean + or ± SEM obtained from biological triplicates is shown in each graph unless stated otherwise. 24-h rhythmicity was detected with Cosinor at DiscoRhythm (https://mcarlucci.shinyapps.io/discorhythm/; Carlucci et al., 2020).

**Online supplemental material**
Fig. S1 shows the regeneration of TA muscle and myoblast differentiation in Per KO mice. Fig. S2 shows the results of RNA-seq analysis of Per KO C2C12 cells. Fig. S3 shows the results of RNA-seq analysis of Per KO C2C12 cells and differentiation of Igf2 KD C2C12 cells. Fig. S4 shows the Per1/Per2-Igf2 axis and myoblast differentiation. Fig. S5 shows the Per1/Per2-Igf2 axis and circadian timing-dependent myogenesis and muscle regeneration. Table S1 displays the sequences of PCR primers. Table S2 lists antibodies used. Table S3 lists the sequences of shRNA and sgRNA.

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Figure S1. Regeneration of TA muscle and myoblast differentiation comparing Per1<sup>+/−</sup>, Per2<sup>+/−</sup>, and Per1<sup>−/−</sup>:Per2<sup>−/−</sup> mice. (A) Average of the minimal Feret’s diameters of myofibers with centrally located nuclei on day 14. TA muscle was injured with barium chloride at ZT14 on day 0 and harvested 14 d later. n = 8 mice with 4 males and 4 females in each group in A and B. (B) Average of the minimal Feret’s diameters of myofibers in uninjured mice. (C–F) EdU uptake and immunofluorescence staining of undifferentiated (C) and differentiating primary myoblasts on day 1 (D), day 2 (E), and day 3 (F) with antibodies against MHC and MyoD. DNA was counterstained with DAPI. Bars, 100 µm. *, P < 0.05 and ***, P < 0.001 with Student’s t test compared with WT mice. Data are presented as mean ± SEM.
Figure S2. RNA-seq analysis of Per KO C2C12 cells. (A) Relative expression levels of Per1 and Per2 mRNAs in C2C12 cells after KD of each gene. The expression level with control scrambled shRNA was defined as 1.0 for each gene. ***, P < 0.001 with Student’s t test. Data are presented as mean ± SEM of biological triplicates.

(B) Indel frequency in Per1 KO and Per2 KO C2C12 cells analyzed with TIDE software (https://tide.nki.nl/).

(C) Western blotting demonstrating down-regulation of Per1 and Per2 in KD and KO cells. Histone H2B was used as a loading control (Cont).

(D) Venn diagrams displaying the number of genes whose expression levels were >200% or <50% of those of control KO cells.

(E) The number of genes that were commonly up-regulated (> Cont × 2) or down-regulated (< Cont × 0.5) more than twofold in Per1 KO and Per2 KO cells compared with control KO cells.

(F) Scatterplots comparing control, Per1 KO, and Per2 KO C2C12 cells.

(G) Gene ontology (GO) terms relevant to muscle differentiation that were enriched in the genes commonly down-regulated in Per1 KO and Per2 KO cells compared with control KO cells. FC, fold change.
Figure S3. RNA-seq analysis of Per KO cells and differentiation of Igf2 KD cells. (A) List of genes belonging to the gene ontology terms shown in Fig. S2 G. Four genes commonly down-regulated in Per1 KO and Per2 KO cells in an undifferentiated state and during differentiation are highlighted in yellow. n = 1 in A and B.

(B) Relative expression level of Igf2 mRNA determined by qPCR in C2C12 cells after depletion of Per1 and Per2. The value obtained with control scrambled shRNA on day 0 (before differentiation) was defined as 1.0. (C) Relative expression level of Igf2 mRNA after KD with two shRNAs. The expression level with control shRNA was defined as 1.0. **, P < 0.01 and ***, P < 0.001 with Student’s t test compared with control cells (Cont). Data are presented as mean ± SEM of biological triplicates. FC, fold change.
Figure S4. Per1/Per2–Igf2 axis and myoblast differentiation. (A and B) Differentiation index (A) and fusion index (B) during differentiation of Igf2 KD cells. (C) Relative expression levels of five muscle genes during differentiation of Igf2 KD cells. The value obtained with day 0 control (Cont) KD cells was defined as 1.0. (D) Temporal profile of the frequency of EdU+ nuclei in Igf2 KD cells during differentiation. (E) Western blotting of Clock, Bmal1, Per1, and Per2 after retrovirus-mediated transduction of these genes in C2C12 cells. Empty vector (Emp Vec) was used as a control. Cells transduced with Igf2 shRNA clone 1 (Igf2 KD1) and those with control shRNA were compared. Histone H2B was used as a loading control. (F) EdU uptake and immunofluorescence staining of undifferentiated and differentiating primary myoblasts with antibodies against MHC and MyoD. DNA was counterstained with DAPI. Bar, 100 µm. (G) Differentiation index (top) and fusion index (bottom) of control and Per KO C2C12 cells cultured with exogenous Igf2 at different concentrations for 3 and 5 d. Igf2 was not added to the control cells. (H) Relative expression levels of Ckm (top) and Myog (bottom) of control and Per KO C2C12 cells treated with exogenous Igf2 for 3 and 5 d. The expression level of the control cells before differentiation was defined as 1.0. Data are presented as mean ± SEM of biological triplicates. Each replicate includes n = 1,000–1,500 nuclei in A and B. *, P < 0.05; **, P < 0.01; and ***, P < 0.001 with Student’s t test compared with control. NS, P ≥ 0.05. Asterisks were only added to the values with 1 ng/ml Igf2 in G and H.
Figure S5. **Per1/Per2–Igf2 axis and circadian timing–dependent myogenesis and muscle regeneration.** (A) ChIP-PCR analyses of indicated proteins in control and Per KO C2C12 cells at regions R1 and R3 shown in Fig. 5 F. Relative abundance compared with control IgG is shown. Peak time points when the control cell values were higher than those of Per1 KO and Per2 KO cells are highlighted with asterisks. (B) Locations of the PCR primers specific to two variants and common to all three variants of Igf2. (C) qPCR results of the Igf2 variant mRNAs in control (Cont) and Per KO cells. The PCR products obtained with the common primers largely represented the expression levels of variant 3 because the levels of variants 1 and 2 were by far lower than the level of variant 3. (D) ChIP-PCR analyses of the indicated proteins in control and Per KO C2C12 cells at regions R4 and R6 in Fig. 5 H. Relative abundance compared with control IgG is shown. Peak time points when the control cell values were higher than those of Per1 KO and Per2 KO cells are highlighted with asterisks. Pol II, RNA polymerase II. (E–G) Analyses of differentiation index (E), fusion index (F), and relative expression levels of differentiation-specific genes (G) with C2C12 cells that were induced to differentiate at the indicated post-synchronization time points. Control and Igf2 KO cells prepared with two shRNA clones were compared. (H) Average Feret’s diameters of myofibers with centrally located nuclei on day 14. TA muscle was injured with barium chloride at ZT2 or ZT14 on day 0 and harvested 14 d later. Mean ± SEM of 8 mice, including 4 males and 4 females in each group, is shown. 2 and 14 at the end of each genotype indicate the injury time at ZT2 and ZT14, respectively. Data are presented as mean ± SEM of biological triplicates. Each replicate includes n = 1,000–1,500 nuclei in E and F. *, P < 0.05; **, P < 0.01; and ***, P < 0.001 with Student’s t test compared with control values in A and D, control common primers in B, and different time points in control cells in E–G. *, 24 h rhythmicity with Cosinor (P < 0.05); **, P < 0.01; and ***, P < 0.001.

Katoku-Kikyo et al. Journal of Cell Biology

Circadian regulation of myogenesis

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Provided online are three tables. Table S1 displays the sequences of PCR primers. Table S2 lists antibodies used. Table S3 lists the sequences of shRNA and sgRNA.