Circadian rhythms in the absence of the clock gene Bmal1

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Circadian (~24-hour) clocks have a fundamental role in regulating daily physiology. The transcription factor BMAL1 is a principal driver of a molecular clock in mammals. Bmal1 deletion abolishes 24-hour activity patterning, one measure of clock output. We determined whether Bmal1 function is necessary for daily molecular oscillations in skin fibroblasts and liver slices. Unexpectedly, in Bmal1 knockout mice, both tissues exhibited 24-hour oscillations of the transcriptome, proteome, and phosphoproteome over 2 to 3 days in the absence of any exogenous drivers such as daily light or temperature cycles. This demonstrates a competent 24-hour molecular pacemaker in Bmal1 knockouts. We suggest that such oscillations might be underpinned by transcriptional regulation by the recruitment of ETS family transcription factors, and nontranscriptionally by co-optimizing redox oscillations.

The primary regulator of circadian rhythmicity in mammals is thought to comprise transcriptional-translational feedback loops (TTFLs) that drive periodic expression of clock gene products (1, 2). In this scheme, BMAL1 (also known as MOP3 or ARNTL) is believed to serve as an indispensable component of the system (3), acting as a transcription factor that heterodimerizes with CLOCK (4) to activate circadian gene expression. Disruption of Bmal1 in mammals leads to a range of physiological abnormalities, including the abolition of circadian behavior (3, 5), aberrations in the sleep-wake cycle (6, 7), abnormal retinal function (8), neurodegeneration (9), and shorter life span (10). Deletion of Bmal1 disrupts robust oscillations of core clock components (11). However, Bmal1 may not be essential for all molecular oscillations beyond the canonical circadian circuit (8), particularly at the whole-genome or proteome scale, and TTFL models may not provide a comprehensive representation of all molecular circadian clocks (12–16).

We explored whether 24-hour transcriptional oscillations are possible in Bmal1−/− mice under physiological conditions. To do this, we analyzed a liver RNA-sequencing (RNA-Seq) dataset in which Bmal1−/− mice (conventional Bmal1 knockout [KO]) had been entrained to a standard 12-hour light:12-hour dark (LD) cycle for several days (17). Under these conditions, Bmal1−/− mice exhibit 24-hour locomotor activity rhythms (18), which are not observed under constant conditions (continuous environmental darkness) (3). We found that 8002 genes displayed 24-hour rhythms at a false discovery rate (FDR) < 0.05. These were detected by the RAIN (rhythmicity analysis incorporating nonparametric methods) algorithm, which detects both symmetric and nonsymmetric waveforms in time series data (19). This demonstrates that many liver transcripts are rhythmic under synchronized conditions (i.e., in an LD cycle).

In mammals, tissue clocks, such as those in the skin and liver, exist in a hierarchy and are synchronized by a central pacemaker residing in the suprachiasmatic nucleus (SCN) of the brain through a range of mechanisms including endocrine, autonomic, temperature, and feeding cues. This synchronization occurs such that organs assume relative phases to each other and the SCN, but also within each tissue so that individual cells are in phase with each other (20). To avoid the effects of such synchronization (which may convey a desynchronized signal to tissues in vivo) and test whether Bmal1−/− mice might retain an intrinsic time-keeping function, we assayed skin fibroblasts and liver tissues from these animals outside the body.

This approach enabled us to synchronize tissues and then allow them to free-run under constant conditions, whereby they might reveal endogenous rhythmicity. To synchronize liver tissues from Bmal1−/− and Bmal1+/− mice, we treated them with a 15-min pulse of the glucocorticoid hormone dexamethasone (DEX), a standard and potent synchronizer of the molecular circadian clockwork in peripheral tissues (21). Forty-eight hours after synchronization, we collected samples every 3 hours for 3 days (Fig. 1A) and subjected these to RNA-Seq to quantify gene expression. Similar to what we saw in mice under entrained LD conditions, a large number of transcripts [5790 with p value < 0.05] oscillated in Bmal1−/− liver slices under constant conditions (Fig. 1B and fig. S1, C and D).

Even after applying more stringent FDR, we identified 5098 transcripts at FDR < 0.1 or 3822 at FDR < 0.05 in Bmal1−/− liver tissue (Fig. 1B). With few exceptions, if a transcript oscillated in Bmal1−/− liver, it did not do so in Bmal1+/− tissue and vice versa—that is, the sets of oscillating transcripts were almost mutually exclusive (Fig. 1C), with slightly different phase distribution patterns (fig. S1E). We tested the overlap between these rhythmic transcripts and those that we quantified as rhythmic in vivo. At FDR < 0.05, there was a highly significant overlap (2034 genes, Fisher’s exact test; p < 0.0001) between the rhythmic genes identified in both the datasets (Fig. 1D). We also determined which genes were synchronized by DEX by performing a pulse-chase experiment (fig. S2A). We found that 15.5% (788 out of 5098) of rhythmically expressed genes in Bmal1−/− tissues responded to glucocorticoid synchronization (fig. S2, B to F).

We then investigated skin fibroblasts (MSFs) from Bmal1−/− mice. Confluent (nondividing) MSFs were synchronized with a pulse of DEX and then sampled under constant conditions (fig. S1, B and D). We identified rhythmic transcripts in Bmal1−/− MSFs (Fig. 1E) with negligible overlap with the rhythmic transcripts identified in wild-type cells (Fig. 1F). Rhythmic transcripts had similar amplitude distributions in Bmal1−/− and Bmal1+/− liver tissues (fig. S3A). Moreover, the amplitude distributions observed in our study are comparable with those of earlier circadian transcriptome studies performed using wild-type mice (fig. S3B). As expected, the amplitude for the rhythmic transcripts was higher in liver tissues compared to fibroblasts in both the genotypes (fig. S3, A and C). Furthermore, period analysis in fibroblasts indicated a predominant period of 24 to 27 hours in both the genotypes, although in Bmal1−/− liver tissue, we saw a greater number of transcripts that oscillated with a longer period (26 to 27 hours) (fig. S3, D and E). There were a few transcripts with a short period (18 to 21 hours), and harmonics of circadian period (8- to 12-hour ultradian rhythms) were negligible in all the datasets (table S1). Together, these results demonstrate circadian oscillations of gene expression in DEX-synchronized liver and fibroblasts of Bmal1 knockout mice.

A crucial feature of circadian clocks is that their free-running period remains ~24 hours throughout a broad range of physiological temperatures (1). To determine whether transcriptomic oscillations are seen in the absence of...
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**Fig. 1. Rhythmic transcriptome of Bmal1−/− mouse liver tissues and skin fibroblasts.** (A) Schematic representation of the experimental strategy used in this study. Cells and tissues were cultured outside the body (ex vivo) and synchronized by a single DEX pulse to evaluate their rhythmicity under constant conditions after this treatment (constant darkness (DD); gray and black bars show subjective day and night, respectively) without any masking signal from the SCN. (B) Twenty-four-hour oscillating transcripts identified at different stringency levels (with RAIN) in wild-type and Bmal1−/− liver tissues. (C) Heatmap representation of the rhythmic transcripts (FDR < 0.1 in RAIN) in Bmal1+/+ and Bmal1−/− liver tissues. Corresponding abundance profiles for the rhythmic candidates identified in each genotype are displayed in the same order. (D) Venn diagram showing the overlap between the rhythmic genes (FDR < 0.05) identified in Bmal1-KO mouse in LD cycle (Gene Expression Omnibus accession: GSE70499) (17), and in Bmal1−/− liver tissues under constant conditions (DD) as obtained in our study. (E) Twenty-four-hour oscillating transcripts identified at different stringency levels (with RAIN) in wild-type and Bmal1−/− MSFs. (F) Heatmap representation of the rhythmic transcripts (FDR < 0.1 in RAIN) in Bmal1+/+ and Bmal1−/− MSFs. Corresponding abundance profiles for the rhythmic candidates identified in each genotype are displayed in the same order.

*Bmal1* exhibit this key characteristic of circadian rhythmicity, we synchronized fibroblasts by DEX treatment and maintained them at 27°, 32°, or 37°C for two complete 24-hour cycles (Fig. 2A). The temperature coefficient (Q10) for the rhythmic transcriptome was ~1 in both genotypes (Fig. 2B). This indicates that there is temperature compensation of genome-scale circadian oscillations in Bmal1+/+ cells (Fig. 2, C and D). Circadian clocks also can be “entrained” by external cues (J). We synchronized fibroblasts from both genotypes with a DEX pulse 12 hours apart and then sampled them in free-running conditions at the same external (solar) time (Fig. 2E). Several transcripts in wild-type or Bmal1−/− fibroblasts had oppositely phased rhythms when in free-run (Fig. 2, F and G). This means that they retained their initial phases (i.e., antiphasic). If driven by an exogenous cue during free-run, the rhythms would instead be in an identical phase, which they are not. Taken together, these findings demonstrate the presence of free-running, temperature-compensated, and entrainable (i.e., circadian) rhythms in the absence of the core clock gene *Bmal1*.

To identify the possible mechanisms that can drive or sustain molecular oscillations in the absence of the known core clock machinery, we tested whether BMAL2 (MOP9), which is a closely related paralog of BMAL1, could substitute for BMAL1 in its absence. However, two independent lines of evidence exclude BMAL2 as a driver of rhythms in the absence of BMAL1. First, deletion of *Bmal1* alone leads to loss of *Bmal1* and *Bmal2* function because BMAL2 is entirely regulated by *Bmal1* (18). Accordingly, we did not detect BMAL1 or BMAL2 (MOP9) by immunoblotting in Bmal1−/− liver tissue (fig. S4, A to D). Second, if BMAL2 was able to substitute for BMAL1’s function at the genomic scale, there should be a substantial overlap of downstream rhythmic genes in both Bmal1−/− and Bmal1+/+ MSFs and liver tissues. However, we did not observe this (see Fig. 1, C and F). Thus, we did not find evidence to suggest that BMAL2 (and by extension other related basic helix loop helix transcription factors) substitutes for BMAL1 function in Bmal1−/− tissue or cells.

What might, therefore, be the underlying molecular mechanism driving circadian rhythmicity in Bmal1−/− tissue? To establish this, we analyzed the promoter regions of rhythmically expressed transcripts (FDR < 0.1), focusing on the two principal phase peaks at subjective dawn and dusk (Fig. 3A). Unbiased motif analysis indicated enrichment [q value (Benjamini) < 0.05] of E26 transformation-specific (ETS) factors, for the dawn phase
rhythmic transcripts in both \textit{Bmal1}^{+/+} and \textit{Bmal1}^{−/−} mice. (Fig. 3A). We found rhythmic expression of multiple ETS transcription factors with comparable peak phases clustered around dawn (Fig. 3B). We observed rhythmic expression of 9 and 11 ETS transcription factors in \textit{Bmal1}^{−/−} and \textit{Bmal1}^{+/+} liver tissues, respectively (FDR < 0.1), with 5 overlapping candidates (fig. S6A and table S3).

Furthermore, we found rhythmic expression of ETS transcription factors in \textit{Bmal1} knockout mice in vivo under LD cycles (17) (Fig. 3C). ETS binding sites are enriched in rhythmic enhancer RNAs in wild-type mice (22).

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Image 1: Schematic representation of the experimental strategy used in temperature compensation analysis of the rhythmic transcriptome. MSFs (wild-type and \textit{Bmal1}^{−/−}) were synchronized by one DEX pulse and maintained at a constant temperature of 27°, 32° or 37°C for two complete circadian cycles and were subsequently sampled every 2 hours at these three different temperatures under constant conditions (DD) for RNA-Seq analysis. (B) Temperature independence of transcriptome-level circadian oscillations in wild-type and \textit{Bmal1}^{−/−} cells as temperature coefficient (Q10) for the rhythmic transcriptome was found to be almost 1 in both the genotypes. Data are represented as mean ± SD, n = number of cyclic genes (FDR < 0.1) in each condition (\textit{Bmal1}^{+/+} 27°C = 113, 32°C = 577, 37°C = 1340; \textit{Bmal1}^{−/−} 27°C = 1397, 32°C = 1169, 37°C = 692), period = 18 to 30 hours. (C) Abundance profiles of temperature-compensated rhythmic transcripts identified at all three different temperatures (FDR < 0.1). \textit{Bmal1}^{+/+} (n = 32) and \textit{Bmal1}^{−/−} (n = 140), period = 24 hours. Transcript abundances were calculated as FPKM (fragments per kilobase per million mapped reads) and represented on a log2 scale after z-score normalization. (D) Abundance profiles (log2-transformed FPKM) of six representative temperature-compensated rhythmic genes in \textit{Bmal1}^{−/−} cells. Samples from three biological replicates were pooled together for RNA-Seq analysis at each time point. (E) Schematic showing the experimental design for oppositely phased initial synchronization in circadian transcriptomics analysis. MSFs (wild-type and \textit{Bmal1}^{−/−}) were synchronized with single DEX pulses 12 hours apart and then sampled (3-hour resolution) in free-running conditions at the same external (solar) time. (F) Abundance profiles (log2 transformed FPKM) of representative clock genes in wild-type cells showing oppositely phased transcripts. (G) Oppositely phased abundance profiles (log2 transformed FPKM) of representative rhythmic genes in \textit{Bmal1}^{−/−} fibroblasts.
Fig. 3. Twenty-four-hour rhythmicity of ETS transcription factors and peroxiredoxin (PRDX) oxidation in Bmal1+/+ and Bmal1−/− tissues. (A) Top sequence motifs (q < 0.05) of the circadian transcriptional regulators for the dawn phase and dusk phase rhythmic transcripts (FDR < 0.1) identified in Bmal1+/+ and Bmal1−/− liver tissues. De novo sequence motif analysis was performed with +/−300–base pair DNA sequence from the master peak binding sites by using HOMER. (B) Frequency distribution of the phases showing rhythmic expression of multiple ETS transcription factors with comparable peak phases with the cyclic dawn phase transcripts. (C) Rhythmic expression (q < 0.05) of three ETS transcription factors in organotypic liver culture in constant conditions (DD, left) and in Bmal1−/−KO mice in a light-dark cycle (LD, right; three biological replicates from a single cycle are concatenated to enable comparison with ex vivo liver data) (17). Samples from three biological replicates were pooled together for RNA-Seq analysis at each time point. (D) RNAi-mediated knockdown of the ETS transcription factors that are rhythmic in Bmal1+/+ and/or Bmal1−/− mice induce alteration in clock period length (fig. S6). Data analyzed from BioGPS circadian layout database http://biogps.org/circadian/ #goto=welcome (24). **Indicates p < 0.0001, ***indicates 0.0001 < p < 0.001, and *indicates 0.001 < p < 0.05 (t test). (E) Twenty-four-hour oscillation (RAIN, p < 0.05) of peroxiredoxin oxidation [oxidized/hyperoxidized peroxiredoxin (PRDX-SO2/3)] in Bmal1−/− and wild-type liver tissues detected by immunoblotting. Quantification of the immunoblots was done by densitometry, and data are represented as mean ± SEM (n = 3). β-actin was used as a loading control to normalize PRDX-SO2/3 monomer bands. Immunoblots for PRDX-SO2/3 and β-actin are provided in fig. S7.
We also investigated the effects of RNA interference (RNAi)-mediated inhibition of the ETS transcription factors that were identified as rhythmic in Bmal1+/+ and Bmal1−/− mice using BioGPS, a portal allowing access to circadian time courses from small interfering RNA (siRNA) knockdown of almost all genes (24). Depletion of many ETS transcription factors induced alteration in circadian period length in U2OS cells (Fig. 3D and fig. S6B). Thus, a range of ETS proteins could contribute to transcriptional oscillations in cells devoid of Bmal1.

An alternative mechanism that could generate molecular rhythms in Bmal1−/− is a non-transcriptional, biochemical oscillation (25). Oxidation-reduction state of peroxiredoxin (PRDX) proteins exhibit self-sustained oscillation in the absence of any TTFL mechanisms (12, 13, 26). Moreover, siRNA knockdown of PRDX proteins affects circadian rhythms in nucleated U2OS cells (12) (table S4). Consequently, we next determined whether similar oscillations of PRDX oxidation might be seen in DEX-synchronized Bmal1−/− liver. Lysates were immunoblotted by using an antiserum specific to overoxidized peroxiredoxin (PRDX-SO2/3) to monitor the redox status of PRDX. Statistically significant (RAIN, p < 0.05) cycling of PRDX-SO2/3 abundance was detected with a period ~24 hours in both Bmal1−/− and Bmal1+/+ liver tissues (Fig. 3E and fig. S7). We investigated the possible interactions among ETS transcription factors, PRDX proteins, and core clock components using the Search Tool for the Retrieval of Interacting Genes or Proteins (STRING) database. There were multiple interactions among ETS transcription factors, PRDX proteins, and clock components mediated through Trp53 and Sirt1 (fig. S8), which are important regulators of circadian clock gene expression (27–29).

Next, we investigated whether such non-canonical rhythmicity is extended to the proteome and phosphoproteome levels in Bmal1−/− mice. Circadian proteome and phosphoproteome have been reported in wild-type mice (30–32), but not in Bmal1−/− tissues. After synchronization with DEX, as described above, samples were harvested over two circadian cycles, labeled with 10-plex tandem mass tags...
demonstrated that although...MSFs, with comparable peak phases of protein
abundance of proteins and phosphosites was
represented, particularly the mitochondrion and
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SUPPLEMENTARY MATERIALS

science.sciencemag.org/content/367/6479/800/suppl/DC1

Figs. S1 to S13

Tables S1 to S4

References (30–57)

View/request a protocol for this paper from Bio-protocol.
Redundancy in circadian clocks?

The transcription factor BMAL1 is a core component of the mammalian circadian clock; without it, circadian behaviors are abolished. However, Ray et al. found that in animals lacking BMAL1, peripheral tissues synchronized with a brief pulse of the glucocorticoid hormone dexamethasone appear to retain a 24-hour pacemaker that sustains rhythmic gene expression, protein abundance, and protein phosphorylation in excised liver cells and fibroblasts (see the Perspective by Brown and Sato). These oscillations persisted in the absence of cues from changes in light or temperature. The results raise intriguing questions about the possible nature of the oscillator that maintains the observed rhythms. Science, this issue p. 800; see also p. 740.