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Journal Article

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Non-coding cis-element of Period2 is essential for maintaining organismal circadian behaviour and body temperature rhythmicity

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Non-coding cis-regulatory elements are essential determinants of development, but their exact impacts on behavior and physiology in adults remain elusive. Cis-element-based transcriptional regulation is believed to be crucial for generating circadian rhythms in behavior and physiology. However, genetic evidence supporting this model is based on mutations in the protein-coding sequences of clock genes. Here, we report generation of mutant mice carrying a mutation only at the E′-box cis-element in the promoter region of the core clock gene Per2. The Per2 E′-box mutation abolishes sustainable molecular clock oscillations and renders circadian locomotor activity and body temperature rhythms unstable. Without the E′-box, Per2 messenger RNA and protein expression remain at mid-to-high levels. Our work delineates the Per2 E′-box as a critical nodal element for keeping sustainable cell-autonomous circadian oscillation and reveals the extent of the impact of the non-coding cis-element in daily maintenance of animal locomotor activity and body temperature rhythmicity.
Evidences show that non-coding cis-regulatory elements are as important as protein-coding sequences for determining cell identity and morphological development\textsuperscript{1,3}. The degree of the effects of non-coding cis-regulatory elements on animal behavior and physiology after development, however, remains elusive. Circadian clocks generate \texttexttt{~24 h} rhythms in behavior and physiology, which allow organisms to anticipate and adjust to daily environmental changes. The rhythm generating mechanism of the circadian clock involves clock genes, which regulate their own transcription in a negative transcription-translation feedback loop\textsuperscript{9,10}. In this canonical feedback model, non-coding cis-elements are fundamental to both initiating and closing the loop\textsuperscript{7,9}. Genetic evidence supporting this model, however, is still exclusively based on the effects of mutations in the protein-coding sequences of the clock genes. Therefore, while it is tempting to speculate that the circadian clock-related non-coding cis-elements are operational for daily dynamic regulation of behavior and physiology, there is currently no direct evidence to corroborate this notion.

Notwithstanding the recent intriguing discoveries of circadian oscillations in peroxiredoxin superoxidation in transcriptionally incompetent anucleate erythrocytes\textsuperscript{10} and the expression of such cycles in the neurons in the suprachiasmatic nucleus (SCN)\textsuperscript{13}, the consensus conjecture regarding the mammalian clockwork supports the transcriptional feedback model. However, the literature addressing mutations or deletions in the protein-coding sequences of the clock genes cannot negate the possible contribution of post-translational (or non-genomic) clock mechanisms. Moreover, the extent of the impact of the cis-regulated transcriptional feedback cycle on establishing circadian rhythmicity in vivo remains the subject of considerable debate\textsuperscript{4,12}, due in large part to the lack of genetic research on non-coding cis-element mutations.

In the current study, we develop mutant mice carrying a mutation in a circadian cis-acting element of the core clock gene \texttexttt{Per2}. Although \texttt{Per2} was cloned as a secondary period gene in mammals, gene knockout studies revealed that \texttt{Per2} mutant mice displayed a loss of circadian rhythmicity, revealing its prominent role in the mammalian molecular clockwork\textsuperscript{13-15}. Moreover, familial advanced sleep phase syndrome in humans is attributed to a missense mutation in the \texttt{Per2} gene\textsuperscript{16}. The \texttt{Per2} E′-box sequence (5′–CAGGTT–3′) located near the putative transcription initiation site\textsuperscript{17} (\texttexttt{−20 to −15}) has been demonstrated to be the principal circadian cis-element that is sufficient to induce oscillating levels of reporter transcription via the mouse \texttt{Per2} minimal promoter\textsuperscript{17,18}. The importance of this particular cis-element is further implied by the high degree of DNA sequence conservation in its flanking region between humans and mice\textsuperscript{17} and by extremely enriched clock protein binding to this element\textsuperscript{19}. Publicly available genome-wide ChIP-seq data highlight the predominant peak of clock protein binding activity around this E′-box (see Supplementary Fig. 1).

The present study was designed to investigate the role of this unique E′-box sequence of the \texttt{Per2} promoter (hereafter, \texttt{Per2} E′-box) as a potential nodal cis-element in the mammalian clockwork. By introducing a site-specific mutation at this element in vivo, we provide empirical evidence to show that the \texttt{Per2} E′-box is essential for maintaining cell-autonomous circadian oscillations. The cells without the \texttt{Per2} E′-box cannot maintain circadian molecular oscillations in culture conditions. At the organismal level, mice lacking the \texttt{Per2} promoter E′-box show destabilized locomotor activity and body temperature rhythms under altered light conditions, including constant light (LL) and experimental jet-lag conditions. Due to compensatory mechanisms in vivo, the mutant mice kept under constant dark (DD) conditions remain rhythmic but exhibit considerably shorter circadian periods than WT mice. Our data therefore define the degree of the impact of the deletion of the \texttt{Per2} E′-box on the organismal clock: The \texttt{Per2} E′-box is essential for the period determination of behavioral rhythms in DD conditions and for sustaining stable rhythms under LL and jet-lag conditions. These data underscore the roles of the non-coding cis-element in the regulation of daily behavior and physiology in adulthood.

**Results**

**Generation of \texttt{Per2} E′-box mutant mice.** We developed mutant mice harboring a targeted mutation in only the \texttt{Per2} E′-box. To do this, we used the \texttt{piggyBac} (PB) transposase tool for genome engineering because it allows for seamless removal of the \texttt{PB}-flanked marker cassette (\texttt{PB-Neo}) from the host genome after the mutation is introduced (Fig. 1a). Conventional methods that rely on the Cre/loxP or Flp/FRT system leave behind a single loxP- or FRT-derived ectopic sequence after marker excision. Such a footprint sequence could affect promoter architecture and/or enhancer communication of the target gene\textsuperscript{20}. The PB transposase (\texttt{PBase}/\texttt{PB} system circumvents this problem\textsuperscript{21} (see Fig. 1b–d). Southern blot analysis confirmed that the \texttt{PB}-neo-mycin cassette was deleted without re-integration into the host genome (Fig. 1c). The footprint-free disappearance of the marker cassette was further confirmed by DNA sequencing (Fig. 1d).

To reduce the confounding effects of a mixed genetic background, the mutant mice were backcrossed with \texttt{C57BL/6J} mice until microsatellite markers covering all individual chromosomes were congenic to the \texttt{C57BL/6J} strain (see Supplementary Fig. 2). The targeted mutation of the E′-box sequence was finally verified by DNA sequencing using homozygous mutant mice (Fig. 1d).

Chromatin immunoprecipitation (ChIP) assays using homozygous \texttt{Per2} mutant mice revealed that the \texttt{WT} (+/+), \texttt{Cry1}, \texttt{Cry2}, and \texttt{CLOCK} had consistently low binding levels to the mutated \texttt{Per2} promoter (Fig. 1e). In a control experiment with \texttt{WT} (+/+), the peak binding levels of \texttt{PER1}, \texttt{Cry1}, \texttt{Cry2}, and \texttt{CLOCK} to the \texttt{Per2} promoter were observed at \texttt{CT12}, \texttt{CT4}, \texttt{CT20}, and \texttt{CT8}, respectively, consistent with previous reports\textsuperscript{19,22}. These results confirmed that \texttt{Per2} E′-box function was abolished in the mutant mice.

**\texttt{Per2} E′-box is essential to maintain cellular circadian oscillations.** To investigate the role of the \texttt{Per2} E′-box in maintaining cellular oscillation, we generated primary fibroblast cultures from \texttt{WT} (\texttt{Per2}E′+/+) and mutant (\texttt{Per2}E′/−/−) mouse lung tissues and monitored circadian fluctuations in the \texttt{PER2} protein levels over 80 h (Fig. 2a). As expected, the endogenous \texttt{PER2} protein in synchronized \texttt{WT} cells displayed characteristic circadian oscillations in both abundance and electrophoretic mobility, which continued over multiple cycles under constant culture conditions\textsuperscript{23} (Fig. 2a).

In contrast, \texttt{Per2}E′/−/− cells failed to maintain normal \texttt{PER2} protein oscillations (Fig. 2a). It is important to note that the mutant cells could potentially form the first surge of \texttt{PER2} expression after synchronization (4–12 h). However, following a subsequent increase (28 h), \texttt{PER2} expression in the mutant cells remained at mid-to-high levels and eventually lost apparent circadian variation after 60 h (see also Supplementary Fig. 3). Electrophoretic migration patterns also disappeared from the mutant cells (Fig. 2a). The relative intensities of the three major bands of the \texttt{PER2} protein remained unchanged in the \texttt{Per2}E′/−/− cells. The post-translational rhythms were thus affected either directly or indirectly by the mutation of the E′-box. We experimentally confirmed that the protein-coding sequences of \texttt{PER2} and known clock protein kinases and phosphatases were unaffected in our mutant mice. These results indicate that the E′-box is indispensable for maintaining normal \texttt{PER2} protein oscillations.
Fig. 1 Generation of mice carrying a targeted mutation at Per2 E-`box. a Genome modification strategy using the piggyBac transposon. The CACGTG Per2 E-`box was mutated to GCTAGT. Top line, genome architecture of the mouse targeted allele. Middle line, genome architecture of the F1 allele. Southern blot probes. Numbering shows the position relative to the putative transcription start site. a Targeted allele. b Southern blot and PCR analysis showing the insertion (+/PB-Neo) and excision (+/m and +/+m) of the piggyBac transposon. c a recombined PB-Neo fragment. d Genomic DNA sequences of WT (+/+) and Per2 E-`box mutant (+/m and m/m) mice, illustrating the precise mutation of the E-`box and seamless excision of the PB-Neo cassette from the TATAA site. Heterozygous (+/m) mouse liver sampled at 4 h intervals for 24 h on the first day in DD. Values are means ± s.e.m. of three technical replicates. Source data for (c) and (d) are provided as a Source Data file.

Fig. 2 Per2 E-`box is essential for maintaining cell-autonomous circadian oscillations. a Temporal profiles of PER2 protein expression in Per2 E-`box and Per2 E-`box fibroblasts. Representative immunoblots and normalized densitometry values (n = 3, mean ± s.e.m.) are shown. b mRNA profiling of clock genes in Per2 E-`box and Per2 E-`box fibroblasts (n = 2, for each data point). For Per2, both intron and exon RNA were analyzed. The data are presented as the mean ± variation. Source data are provided as a Source Data file.

In the Per2 E-`box/m/m cells, we observed that both Per2 mRNA and pre-mRNA (intronic RNA) levels remained constitutively high, with values exceeding those of the circadian peak in the WT cells at 72 h (Fig. 2b). Thus, Per2 transcription remains active even without this particular E-`box sequence in the promoter. Moreover, extensive mRNA profiling revealed that the effects of the mutation of the Per2 E-`box were not limited to Per2 transcription. The dysfunctional Per2 E-`box also abolished the circadian expression of other clock genes and output genes, including Per1, Per3, Cry1, Cry2, Bmal1, Ntr1d1, Dbp, and E4bp4 (Fig. 2b). These pervasive effects of the mutation provide evidence that the Per2 E-`box is a fundamental cis-element that maintains normal molecular clock oscillations.
**Per2 E'-box is essential to maintain molecular oscillations in the SCN.** The SCN in the hypothalamus is the primary regulator of daily rhythms of behavior and physiology in mammals. We next examined the effect of the Per2 E'-box mutation on the SCN clock. To study the molecular rhythms in the SCN, we used a Bmal1 promoter-driven luciferase reporter (Bmal1-Eluc) as it allowed the assessment of molecular rhythms that are not a simple reflection of the Per2 loop. In agreement with previous reports, organotypic SCN slices prepared from control mice (Per2E'+/+; Bmal1-Eluc) displayed persistent circadian rhythms of luminescence, which continued for over a week in culture (Fig. 3a, see also Supplementary Fig. 4). In contrast, all tested SCN slices from the Per2 E'-box mutant mice (Per2E'−/−; Bmal1-Eluc) displayed attenuated rhythms of luminescence, which were damped within 2–3 cycles (Fig. 3a). Fast Fourier transform (FFT) analysis of the de-trended waveforms of days 1.5–7.5 (Fig. 3b) confirmed the reduced rhythmicity in the Per2E'm/m SCN. This finding indicated the relevance of the Per2 E'-box in maintaining normal molecular circadian oscillations in the SCN. Nevertheless, detectable rhythms were noted in the attenuated Per2E'm/m SCN slices over the initial 2–3 cycles (Fig. 3a). These remaining rhythms were likely dependent on Per1, because SCN explants from Per2E'm/m, Per1 heterozygous null mice (Per2E'm/m, Per1E'/−; Bmal1-Eluc) exhibited even fewer persistent rhythms, which were damped within 2 cycles (Fig. 3a–c). Importantly, similar attenuation of Bmal1-Eluc rhythmicity by Per2E'm/m and further by Per1E'/− could be reproduced in cultures of lung (Fig. 3d–f) and adrenal explants (Supplementary Fig. 4).
confirmed the importance of the Per2 E’-box in keeping sustained molecular circadian oscillations not only for the SCN but also for the peripheral tissues.

**Destabilized organisinal rhythms of animal lacking Per2 E’-box.** Finally, to assess the effects of the mutation at the behavioral level, C57BL/6J-backcrossed WT and Per2E^-/-^ mice were housed in a 12-h light:12-h dark (LD) cycle and then subjected to DD, LL, or experimental jet-lag conditions. Actograms of the animals in the respective conditions were acquired (see Supplementary Figs. 5–9). Interestingly, mutant mice kept in DD showed overt circadian locomotor activity rhythms, although with a significantly shortened free-running circadian period relative to WT mice (WT vs. Per2E^-/-^, 23.71 ± 0.02 h vs. 23.50 ± 0.02 h; see Supplementary Fig. 5). This contrast with the in vitro data suggests that a compensatory mechanism functions in vivo. We thus examined the circadian behavior of Per1^-/-^; Per2E^-/-^ double-deficient mice (see Supplementary Fig. 6). Although their period length was unstable and changed gradually over extended exposure to constant darkness, Per1^-/-^; Per2E^-/-^ mice displayed behavioral rhythms over 40 days. With analogy, *Drosophila* strains expressing *period* or *timeless* under a constitutive promoter were reported to show behavioral rhythms with an altered period length. These data suggest that organisms might have a compensatory mechanism to mitigate defective transcriptional feedback regulation.

Compared to the modest phenotype in DD, we observed unstable rhythmicity of the mutant mice in LL (Fig. 4a). When placed in LL for 3 weeks, WT subjects (*n* = 7) stayed rhythmic with a period longer than 24 h, which is consistent with data from previous reports. However, none of the Per2E^-/-^ mice (*n* = 9) remained rhythmic (Fig. 4a, see also Supplementary Fig. 7); all tested mutant mice displayed behaviors characterized by a gradual decrease in the power of rhythmicity (FFT spectrogram, Fig. 4a). Since light has a negative masking effect on locomotor activity, we measured core body temperature fluctuations and confirmed that the body temperature recapitulated the reduced rhythmicity of the mutant mice in LL conditions (Fig. 4b, Rayleigh vector length of WT vs. Per2E^-/-^; 0.64 ± 0.06 vs. 0.42 ± 0.02).

The Per2 E’-box mutant mice were also distinct under experimental jet-lag conditions (Fig. 4c, see also Supplementary Fig. 8). When the ambient LD cycle was advanced by 8 h, WT mice re-entrained progressively over 8 to 9 days. In contrast, the Per2E^-/-^ mice adapted to the new cycle within 2–3 days. The 50% phase-shift value (*PS50*) measured from activity onset, indicated a rapidity of 5.33 ± 0.28 days for WT mice and 1.26 ± 0.17 days for Per2E^-/-^ mice (Fig. 4c). Notably, the difference between the two genotypes was even pronounced when the animals were exposed to a single 8-h light advance, followed by DD conditions (Fig. 4d, see also Supplementary Fig. 9). Under this light regime, all tested mutant mice shifted forward by approximately 12 h, which is nearly 180° out of phase in a 24-h
cycle, relative to WT mice (Fig. 4d). The underlying mechanism of this extremely large shift is unknown. While this could be due to an indirect effect of the weak clock, a limit cycle oscillator model predicts that a reduced-amplitude pacemaker in the mutant mice (reduced radius of the limit cycle) could have this effect. We also noticed that after a light pulse exposure, the mutant mice show a slightly enhanced Per2 mRNA induction in the SCN (see Supplementary Fig. 10). This perhaps also partly contributes to the enhanced resetting of the mutant mice.

Discussion

Non-coding cis-regulatory elements are known to play a critical role in development, but their precise effects on daily behavior and physiology in adulthood remain elusive. The present study was designed to provide genetic evidence for the roles of the circadian cis-element in vivo. Despite the presence of compensatory mechanisms in vivo, our work shows that the Per2 E-box is essential for maintaining optimal locomotor activity rhythms under LD conditions and enabling the phase of the clock to resist abrupt shifts in LD cycling. In general, adapting to new LD cycles is rarely instantaneous and requires repeated 24 h cycles. However, the Per2 E-box mutant mice adapted to a new phase immediately. These data elucidate the impact of the deletion of the non-coding cis-element in daily maintenance of behavioral activity in adulthood.

In addition, our cell culture data reinforce the concept that circadian clock phenotypes are more drastic at the cellular level than the organismic level. Per2 E-box mutation leads to unsustainable gene expression rhythms in organotypic SCN slices and cultured peripheral tissues and fibroblasts. These observations substantiate the general conjecture that cis-regulatory element-based gene transcription is essential for sustaining cellular clock oscillations.

Not surprisingly, the overall behavioral phenotypes of the Per2 E-box mutant mice reveal that the Per2 E-box is not an absolute requirement for behavioral rhythm generation. Under DD conditions, its deletion affects only the circadian period (Supplementary Figs. 5 and 6), a phenotype analogous to that observed in transgenic Drosophila strains expressing period or timeless via a constitutive promoter. At the gene expression level, Per2 expression in the liver and SCN in the DD-kept mutant mice in vivo was still rhythmic, albeit with an increased baseline (see Supplementary Fig. 11). These observations suggest that organisms have a compensatory mechanism to mitigate defective transcriptional feedback regulation. Correspondingly, previous studies demonstrate that behavioral rhythms do not necessarily reflect cellular clock phenotypes. Superior circadian performance of behavioral rhythmicity has been observed in several clock gene mutant mice, compared to tissue cultures obtained from the same animal strains. In vivo multicellular and/or inter-organ systemic circuitry might compensate for poor core clock function within individual cells. In this regard, it is worth noting that systemic extracellular signals are known to affect the Per2 promoter via non-E-box cis-regulatory elements, such as Ca2+/cAMP response element (CRE) and glucoorticoid response element (GRE). Extracellular circadian feedback pathways through these non-E-box elements might contribute to the compensation mechanisms in vivo.

Our work differs from transgenic studies in which non-native promoters are used. Transgenic studies and rescue experiments can be affected by shorter promoter regulatory sequences and position effects. In comparison, native promoters are characterized by endogenous enhancer elements and normal chromatin structure, allowing for the preservation of regulation by epigenetic factors, which are known to be crucial for controlling transcription. Under these near-native conditions, we noticed that the deletion of the Per2 E-box leads to accumulation of Per2 mRNA and protein in cultured fibroblasts. Constitutively un-suppressed transcription of Per2 likely underlies the constant accumulation of Per2 protein and resultant compromised gene expression rhythms in the mutant cells.

Cis-element provides a place where both active and repressive transcription complexes are recruited. The net effect of its absence thus seems context-dependent. The question of this context specificity is still unresolved in the field of transcription and chromatin remodeling. Particularly, in our case, the mechanism(s) that allows the continued transcription of Per2 without relying on the E-box is still unknown. It is possible that native chromatin structure of the Per2 might permit its transcription. It is also conceivable that other circadian cis-elements on the Per2 promoter, such as D-box and CRE, might contribute to the basal transcription of Per2. In this regard, the continued expression of Per2 might reflect a confounding effect of being chronically deficient in the functional E-box. Given that Per2 is under regulation of interlocked feedback cycles, retaining Per2 transcription might be a homeostatic consequence of a nonfunctional clock in the mutant cells. A complete understanding of circadian regulation of Per2 in vivo under native conditions remains a challenge for future study.

Genome-wide association studies recently identified many non-coding variants that account for human chronotypes. Given the physiologic relevance of the Per2 cis-element, a targeted point mutation strategy would facilitate hypothesis-driven approaches to understand the extent of the impact of the non-coding elements on the daily physiology and pathophysiology of the organism.

Methods

Generation of the Per2 E-box mutant mice. The CAGCTT E-box sequence located in the Per2 promoter (−20 to −15; +1, the putative transcription start site) was targeted. Note that numbering of the relative position of the E-box sequence will change according to the definition of the transcription initiation site. We employed piggyBac (PB) transposon-based gene engineering system , because conventional methods relying on Cre/loxP or E/FLP systems leave behind a single loxP or FRT-derived sequence after excision, which causes the creation of a chimeric gene (e.g., dual ectopic sequences) that might affect promoter architecture and/or enhancer communication of target gene. The targeting vector to generate PB mutant allele of Per2 was constructed by using a Red/ET recombination system (Gene Bridges). The bacterial artificial chromosome (BAC) containing Per2 was obtained from BACPAC Resources at the Children’s Hospital and Research Center at Oakland (RP23-343F13). With a Red/ET cloning method (Gene Bridges), a 10-kb genomic region of Per2 (−5899 to +4413; +1, the putative transcription start site) that corresponds to the region of 6.5 kb upstream (long arm) and 3.5 kb downstream (short arm) of the Per2 E-box (−20/−15) was obtained by fusion PCR, and this was further modified by inserting a PB terminal repeat sequence (5’T-3’T)-flanked neomycin cassette (PB-NEO) into the TTAa quadruplet sequence of Per2 (+600/+603). With a second-round Red/ET cloning, this fragment was recombined into the 10-kb Per2 BAC/pDTA vector. The resulting Per2 targeting vector was verified by DNA sequencing. Gene targeting was carried out with TT2 embryonic stem cell. Germline transmission was verified by PCR and Southern blotting. The Per2 E-box mutant mouse was determined by DNA sequencing and/or TaqMan qPCR with the following probes: WT, 5′-FAM-TAG TGG AAA AGC TGA CGG M-C-G-M-3′; and mutant, 3′-VIC-TAG TGG AAA CTA GCA CCG TGG-5′. The use of different reporter dyes with separate emission wavelength maxima (FAM and VIC) enabled concurrent detection of the two alleles in a single PCR with a common primer set for Per2; forward, 5′-GGG GCC GCT AGT CCC AGT-3′; reverse, 5′-AGG TGG CAC TCC GAC TAA T-3′. The established mutant mice were backcrossed into the C57BL/6J background using a marker-assisted breeding (i.e., speed congenic) approach. Where specified, Per2+/E-box mice were intercrossed with mice carrying a Bmal1-Eluc reporter and/or Per1-deficient mice of C57BL/6J background (The Jackson Laboratory, 10491).
All animals were conducted in compliance with ethical regulations in Kyoto University and performed under protocols approved by the Animal Care and Experimentation Committee of Kyoto University and Institutional Animal Care and Use Committee of RIKEN Kobe Branch.

**ChIP.** ChIP assays were performed with technical replicates. We repeated chromatin/DNA sequencing with equal amounts of aliquots from the same liver nuclear sample. In brief, whole liver sample was homogenized with ice-cold PBS (4 ml/l) and cross-linked in two steps using first 2 ml dSSUcinimidyl glutarate for 20 min then 1% methanol-free ultrapure formaldehyde for 5 min at room temperature. Glutaraldehyde for 5 min in 125 mM final concentrace of CV 34S doglycine homogenates (~5 ml/l) were mixed with ice-cold 2.3 mM sucrose buffer (10 ml/l) containing 125 mM glycin, 10 mM HEPES pH 7.6, 15 mM KCl, 2 mM EDTA, 0.15 mM spermin, 0.5 mM spermidine, 0.5 mM DTT, and 0.5 mM PMSF. 

Two amounts of a 1:5 molar solution of 1.85 M sucrose, on a Beckman SW28 rotor. The resultant nuclei pellets were stored at ~80 °C until use. The nuclei were resuspended in 1.5 ml per of IBF buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM PMSF, protease inhibitor cocktail) and divided equally into three aliquots, which were each separately sonicated around 15 s for 80 times at 4 °C using a Biorevpet UCW-2001M apparatus (Torazo Denki, Japan). For each reaction, 10 μg fragmented chromat was resuspended in 500 ml of IBF buffer, pre-cleaved with protein A-agarose, and incubated overnight at 4 °C with the following antibody: for PER1, 1 μg of anti-mPER1 rabbit antiserum (Millpore, #AB2201), for CR1, 1 μg of affinity-purified anti-mCR1 guinea pig polyclonal antibody, and 1 μg of affinity-purified anti-mCR2 guinea pig polyclonal antibody, and for CLOCK, 1 μg of anti-mCLOCK mouse monoclonal antibody. The nucleoimmune complex, 40 ml of Protein A/G-Plus-agarose beads (Santa Cruz) were added and further incubated at 4 °C for 1 h. The beads were washed and resuspended in 0.1% Triton X-100 in ice-cold lysis buffer. 

**RNA extraction and RT-qPCR.** Total RNA was extracted with RNeasy kit (Qiagen) and converted to cDNA with SuperScript VILO cDNA Synthesis kit (Invitrogen). qPCR was run on a BioMark HD System (Fluidigm) with a 48.48 Fluidigm Chip (Fluidigm). TaqMan probe and primer sets were as follows: for Bmal1, probe, FAM-TGG CAA TCC CTG ACG CAC TGG GC-3'; reverse primer, 5'-GGC TCT AGA CCA GGA CAC TTA TCA-3'; for Per2, probe, FAM-AGC CCA GCA GAG TGG CCC GC-TAMRA, 5'-AGA CCA GTT GGC GTG GAC TCT GAC-3' (Millipore, #AB2201); for CRY1, 1 probe, FAM-TGG CAA TCC CTG ACG CAC TGG GC-3'; reverse primer, 5'-GGC TCT AGA CCA GGA CAC TTA TCA-3'; for Per2, probe, FAM-AGC CCA GCA GAG TGG CCC GC-TAMRA, 5'-AGA CCA GTT GGC GTG GAC TCT GAC-3' (Millipore, #AB2201); for CRY1, 1 probe, FAM-TGG CAA TCC CTG ACG CAC TGG GC-3'; reverse primer, 5'-GGC TCT AGA CCA GGA CAC TTA TCA-3'; for Per2, probe, FAM-AGC CCA GCA GAG TGG CCC GC-TAMRA, 5'-AGA CCA GTT GGC GTG GAC TCT GAC-3' (Millipore, #AB2201); for CRY1, 1 probe, FAM-TGG CAA TCC CTG ACG CAC TGG GC-3'; reverse primer, 5'-GGC TCT AGA CCA GGA CAC TTA TCA-3'; for Per2, probe, FAM-AGC CCA GCA GAG TGG CCC GC-TAMRA, 5'-AGA CCA GTT GGC GTG GAC TCT GAC-3' (Millipore, #AB2201); for CRY1, 1 probe, FAM-TGG CAA TCC CTG ACG CAC TGG GC-3'; reverse primer, 5'-GGC TCT AGA CCA GGA CAC TTA TCA-3'; for Per2, probe, FAM-AGC CCA GCA GAG TGG CCC GC-TAMRA, 5'-AGA CCA GTT GGC GTG GAC TCT GAC-3' (Millipore, #AB2201); for CRY1, 1 probe, FAM-TGG CAA TCC CTG ACG CAC TGG GC-3'; reverse primer, 5'-GGC TCT AGA CCA GGA CAC TTA TCA-3'; for Per2, probe, FAM-AGC CCA GCA GAG TGG CCC GC-TAMRA, 5'-AGA CCA GTT GGC GTG GAC TCT GAC-3' (Millipore, #AB2201); for CRY1, 1 probe, FAM-TGG CAA TCC CTG ACG CAC TGG GC-3'; reverse primer, 5'-GGC TCT AGA CCA GGA CAC TTA TCA-3'; for Per2, probe, FAM-AGC CCA GCA GAG TGG CCC GC-TAMRA, 5'-AGA CCA GTT GGC GTG GAC TCT GAC-3' (Millipore, #AB2201); for CRY1, 1 probe, FAM-TGG CAA TCC CTG ACG CAC TGG GC-3'; reverse primer, 5'-GGC TCT AGA CCA GGA CAC TTA TCA-3'; for Per2, probe, FAM-AGC CCA GCA GAG TGG CCC GC-TAMRA, 5'-AGA CCA GTT GGC GTG GAC TCT GAC-3' (Millipore, #AB2201); for CRY1, 1 probe, FAM-TGG CAA TCC CTG ACG CAC TGG GC-3'; reverse primer, 5'-GGC TCT AGA CCA GGA CAC TTA TCA-3'; for Per2, probe, FAM-AGC CCA GCA GAG TGG CCC GC-TAMRA, 5'-AGA CCA GTT GGC GTG GAC TCT GAC-3' (Millipore, #AB2201); for CRY1, 1 probe, FAM-TGG CAA TCC CTG ACG CAC TGG GC-3'; reverse primer, 5'-GGC TCT AGA CCA GGA CAC TTA TCA-3'; for Per2, probe, FAM-AGC CCA GCA GAG TGG CCC GC-TAMRA, 5'-AGA CCA GTT GGC GTG GAC TCT GAC-3' (Millipore, #AB2201); for CRY1, 1 probe, FAM-TGG CAA TCC CTG ACG CAC TGG GC-3'; reverse primer, 5'-GGC TCT AGA CCA GGA CAC TTA TCA-3'.

**Cell culture and immunoblotting.** WT and Per2emm primary fibroblasts were isolated from the lung tissue of adult male mice according to a protocol established by Sekolog et al. Cells were used for time course assay were between passages 4 and 6. Dispersed cells were uniformly plated in 24-well plates at a density of 1×10⁶ cells per well and cultured for 2 days prior to synchronization, for which cells were treated with dexamethasone (DEX, final concentration, 200 nM) for 3 h, followed by medium refreshment at Time0. Cells were harvested every 4 h in either TRizol reagent (Invitrogen) for RNA analysis or 2× Laemmli buffer for Western blot analysis. Immunoblotting was performed using an affinity-purified anti-mPER2 rabbit polyclonal antibody (final concentration, 2 μg/ml) and β-Actin antibody (A3441, Sigma, 1:1000).

**Body temperature recording and data analysis.** Precalibrated temperature data loggers (Thermochron iButtons, DS1921H, Maxim) were surgically implanted to the peritoneal cavity of mice under general anaesthesia. After a week of recovery in LD, mice were transferred into LL. The iButtons were programmed for collecting temperature data every 20 min. For data analysis, the temperature mean of the entire data series during days 7 to 21 in LL was calculated, and the data points above the mean were displayed in double-plotted format using CLOCKLAB software. For clock analysis, the data were detrended by subtracting a 24 h running average, and periods of top 20% temperature were deployed in Rayleigh format using Oriana software (Kovacs Computer Services, UK). CT12 was extrapolated from the onsets of locomotor activity during days 3–6. Phaselock distribution was assessed by mean vector length (Oriana 4).

**Real-time bioluminescence recording and data analysis.** Mice were intercrossed to produce homozygous mutant (Per2emm /emm) and WT (Per2emm /emm) progenies using in vitro fertilization. We used adult male mice (8–10 week old). The animals were housed individually in light-tight, ventilated cages under indicated lighting conditions with ad libitum access to food and water. Locomotor activity was recorded via passive infrared sensors (PIR, FA-055SF, Omron) with 1 min-resolution and analyzed with CLOCKLAB software (Actimetrics). A fourth-order Blackman-Harris window was applied before the power spectrum calculation. The spectrum was normalized to an integral of one by dividing each of its elements by the sum of all elements. Circadian rhythm was defined as relative spectral power density at the peak in the circadian range (20–30 h). The peak values represent the power within a frequency band of 0.009. Speed of behavioral re-entrainment was evaluated using 50% phase-shift value (PS50). To determine PS50, sigmoidal dose-response curve with variable slope, Y = Bottom + (Top - Bottom)/(1 + (log P50 - X)²) HillSlope, was fitted to the onset time points using GraphPad Prism software.


PLase knock-in mice and Bmal1-Eluc mice, respectively. This work was supported by the Core Research for Evolutional Science and Technology, Japan Science and Technology Agency (JPMJCR14W3), and the Ministry of Education, Culture, Sports, Science and Technology of Japan (15H05933, 17H01524, and 18H04015), and AMED Project for Elucidating and Controlling Mechanisms of Ageing and Longevity (JP17gm5010002, JP18gm5010002).

Author contributions
M.D. and H.O. conceived the project and designed the research; M.D., H.S. and Y.A. contributed equally as first authors who performed experiments in collaboration with I.M., H.H., Y.T., J-M.F., Y.Y., H.K., N.K., K.Y., C.L., M.A., K.S. and H.O.; M.D. and H.O. drafted the manuscript, supported by H.S., Y.A. and J-M.F.

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