Doctoral Dissertation (Censored)

博士論文（要約）

Posttranslational modifications regulating the stability of clock proteins PER2 and DBP

（時計タンパク質 PER2 及び DBP の
安定性を制御する翻訳後修飾）

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Abstract

The molecular architecture of circadian clockwork is based on a transcriptional/translational feedback loop. While many of previous studies focused on the transcriptional regulations in the clockwork, the regulation of protein stability also plays a critical role in the circadian clockwork as in other physiological processes including cell cycle, signal transduction and DNA repair.

In this thesis, I focused on the regulations for the stability of two clock proteins, PER2 and DBP.

In the first section of this thesis, I investigated the effect of phosphorylation at Ser478 of PER2 on the speed of the circadian clock. This phosphorylation is catalyzed by CK1 and leads to proteasomal degradation of PER2, which is expected to accelerate the clock. However, recent studies have questioned whether the degradation of the core clock component is a critical step in clock regulation. To examine whether this phosphorylation determines the circadian period *in vivo*, I analyzed PER2-Ser478Ala knock-in mice. By monitoring the wheel-running activity, I found that the mutant mice had longer circadian period in behavioral rhythm. PER2-Ser478Ala protein accumulated in both the nucleus and cytoplasm of the mouse liver cells, while *Per2* mRNA levels were almost unaffected. Nuclear PER1, CRY1 and CRY2 proteins also increased, probably due to stabilization of PER2-containing complex. These data suggest that the nuclear levels of these repressors determine the speed of circadian clockwork. Furthermore, temperature compensation of the cellular circadian period was perturbed in mouse embryonic fibroblasts generated from PER2-Ser478Ala::LUC mice. These data show the importance of the PER2 stability control *in vivo* in the circadian clockwork.

以下、5 年以内に雑誌等で刊行予定のため、非公開。

Table of contents

1. General Introduction

Circadian clock is a time-keeping system intrinsically oscillating with a period of ~24 h and entrains a wide variety of physiological functions to daily environmental changes [1]. At the molecular level, an auto-regulatory transcriptional network of intertwined feedback loops composed of clock genes and proteins has been proposed for the core principle underlying the circadian clock (Figure 1). In mammals, two clock proteins, circadian locomotor output cycles kaput (CLOCK) and brain muscle arnt-like 1 (BMAL1), form a heterodimer that binds to the *cis*-regulatory element E-box and promotes transcription of numerous genes including *Period* (*Per1, Per2, Per3*) and *Cryptochrome* (*Cry1, Cry2*). Thereafter, PER and CRY proteins assemble with casein kinase 1 (CK1δ/ε) in the cytosol and the macromolecular complex enters into the nucleus to inhibit the E-box-regulated transcription [2]. Mutant mice lacking one of *Per* or *Cry* genes have stable circadian rhythms whereas *Per1*/*Per2* double knockout mice and *Cry1*/*Cry2* double knockout mice shows arrhythmicity when exposed to constant darkness (DD) [3–5]. On the other hand, deletion of *Per3* gene has little effect on circadian oscillation [5], probably because PER3 lacks the CK1-binding domain [6].

Forward and reverse genetics revealed that circadian dysfunction can be caused by disruption of posttranslational modifications on these key molecular components of the clock, most notably phosphorylation and ubiquitination [7, 8]. The major consequence of ubiquitination is proteolysis by the ubiquitin-proteasome system.

In eukaryotes, the ubiquitin-proteasome system is a conserved proteolytic pathway by which a large proportion of endogenous proteins is degraded [9]. A target protein is labeled for degradation by attachment of a ubiquitin chain(s). 26S proteasome recognizes the ubiquitin chain and degrades the target protein. E1 activating enzymes, E2 conjugating enzymes and E3 ligases are involved in the ubiquitination of substrates (Figure 2). An E1 covalently binds to a ubiquitin moiety in a process that requires ATP hydrolysis, and then the ubiquitin is

transferred to an E2. An E3 functions as a scaffold protein which mediates the interaction between the E2 and substrates, which provides the substrate-specificity to this degradative system. In contrast to the apparently important function of E3, E2 enzymes were thought to serve merely as ubiquitin carriers before, however, accumulating evidences have proposed that E2s also determine the fate of ubiquitinated substrates. The consequence of ubiquitination depends on topology and length of ubiquitin chains [10]. Seven lysine residues and amino terminus in ubiquitin can serve as acceptors for an additional ubiquitin moiety, which generates various types of ubiquitin chains. Among the ubiquitin acceptor sites, K48-linked chains account for about half of all degradative ubiquitin chains. Therefore, the pairing of E2 and E3 determines the product of ubiquitination and the efficiency of proteasomal degradation. Additionally, protein phosphorylation is closely related with the ubiquitination-mediated degradation. A specific array containing one or more phosphorylated residues can form a “phosphodegron” which is bound by an E3 ligase specifically. For example, phosphorylation of the clock protein CRY1 by adenosine monophosphate-activated protein kinase (AMPK) enhances the interaction with an E3 ligase, F-box and leucine-rich repeat protein 3 (FBXL3), leading to proteasomal degradation of CRY1 [11, 12]. *Fbxl3* knockout mice showed a longer circadian period due to the stabilization of CRY proteins. Interestingly, an E3 ligase FBXL21, closely related with FBXL3, appears to have an opposite function to FBXL3 [13, 14]. Deletion of *Fbxl21* attenuates the long-period phenotype of *Fbxl3* knockout mice and FBXL21 stabilizes CRY proteins on the contrary to FBXL3.

Here, I have studied on posttranslational modifications on two clock proteins, PER2 and DBP. For PER2, I investigated the *in vivo* role of a phosphorylation site which destabilizes PER2. And for DBP, I identified a set of enzymes involved in the degradation of DBP.

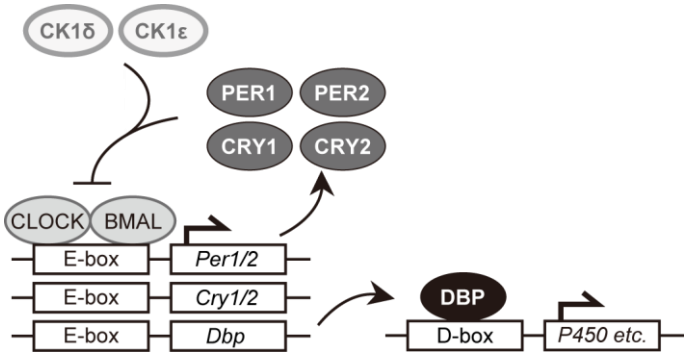

Figure 1. The transcriptional/translational feedback loop in the circadian clockwork
CLOCK-BMAL1 activator complex drives the transcription of numerous genes including *Per1/2*, *Cry1/2*, *Dbp*. PER and CRY proteins assemble with CK1δ/ε in cytoplasm and then enter nucleus to inhibit the transcriptional activity of CLOCK-BMAL1. DBP has a minor role in the oscillation of the clock but regulates daily expression of various genes (*e.g.* cytochrome P450 family genes).

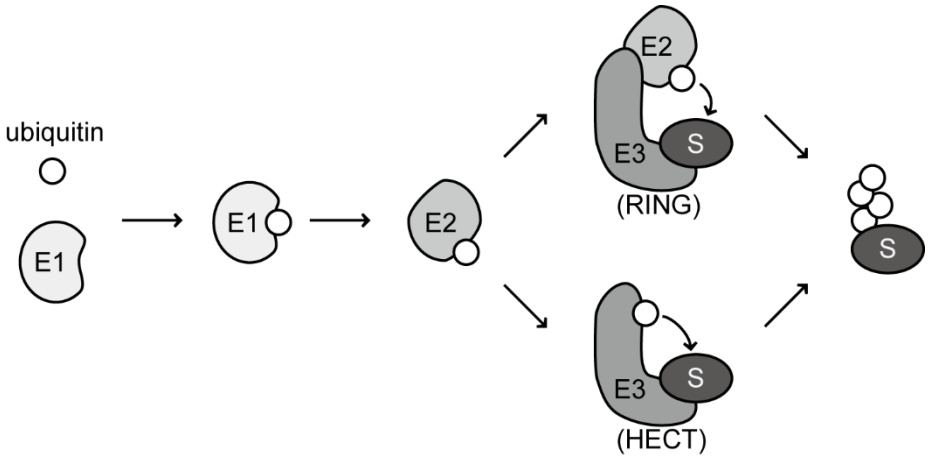

Figure 2. Ubiquitination machinery
E1, E2 and E3 are required for ubiquitination of a substrate (S). A free ubiquitin is covalently conjugated to an E1 activating enzyme in a process which requires ATP hydrolysis. The ubiquitin moiety is then transferred to an E2 conjugating enzyme. Most of E3 ligases are classified into two major groups. A RING (really interesting new gene)-type E3 ligase mediates ubiquitin transfer from the E2 to the substrate, while a HECT (homologues of E6AP carboxy terminus)-type E3 ligase forms a thioester-intermediate with ubiquitin and then attaches the ubiquitin on the substrate.

2. Analysis of PER2-S478A knock-in mice

2.1 Introduction

CK1 determines the clock speed via the PER2 stability control

Casein kinase 1 (CK1) is an evolutionally conserved core clock component [8]. The first mammalian clock mutant was reported in Syrian hamster in 1988 [15], and more than a decade later, a mutation of CK1ε (*tau*) was identified in these mutant animals [16]. This *tau* mutation is semidominant and *tau* homozygous animals exhibit markedly shortened locomotor rhythms with a period of 20 h [15, 16]. A few years before the identification of *tau* mutation, a series of *doubletime* (*dbt*, *ck1* homolog in Drosophila) mutants were reported to have shorter, longer or disrupted clocks in Drosophila [17, 18]. More recently, T44A and H46R mutation in human CK1δ causes Familial Advanced Sleep Phase (FASP) syndrome and shortens the circadian period [19, 20]. In all organisms, the primary target of phosphorylation is PER [16–20], therefore how CK1 regulates PER function has been investigated enthusiastically. In 2001, human PER2-S662G mutation (the FASP site mutation) was identified in patients suffering from FASP syndrome [21]. This mutation results in hypophosphorylation of PER2 by CK1ε [21], leading to destabilization of PER2 protein [22, 23]. hPER2-S662G transgenic (Tg) mice also exhibit the FASP phenotype and shorter circadian period of locomotor activity [24]. The downstream residues of PER2 Ser659 (Ser662 in human) coincide with CK1 consensus motif pSxxS, where pS is phosphorylated serine, x is any amino acid and the second serine residue is phosphorylated by CK1 [25], while the position -3 of the FASP site is lysine residue. Thus, another kinase (frequently referred as “the priming kinase”) had been presumed to mediate the phosphorylation of the FASP site. Surprisingly, recent studies revealed that CK1δ/ε are the priming kinases [26, 27]. To sum up, CK1δ/ε phosphorylate the FASP site and downstream serine residues, leading to the PER2 stabilization and circadian period lengthening.

On the other hand, there were conflicting data which showed that CK1δ/ε promote degradation of PER proteins [28, 29]. In addition, pharmacologic inhibition of CK1 consistently lengthened the circadian period in animals [30–32] and in plants [33], suggesting CK1 catalyzes another phosphorylation besides the FASP site. In 2005, it was reported that CK1δ/ε promote beta-transducin repeat-containing homologue protein (β-TrCP)-dependent degradation of PER2 through phosphorylation of PER2 Ser478 (the β-TrCP site) [30] and that PER1 is also degraded by β-TrCP [34].

Phosphoswitch mechanism by CK1 regulates the PER2 stability

Although the bidirectional actions of CK1δ/ε against the PER2 stability are the key reactions in the clockwork, the mechanism in which the FASP phosphorylation stabilizes PER2 protein had not been addressed. In 2015, David Virshup and colleagues demonstrated that phosphorylations of the FASP site and the adjacent serine residues prevent the phosphorylation of the β-TrCP site, namely the “phosphoswitch” regulation [35]. They further proposed that this phosphoswitch supports the temperature compensation of the circadian period, the mechanism that maintains a stable circadian period despite changes in ambient temperature [27, 35–37]. This idea originally stemmed from the observation that the kinase activity of CK1δ/ε on synthetic peptides is temperature-insensitive [31, 38]. At lower temperature, the kinase activity of CK1δ/ε on the β-TrCP site is relatively higher than that on the FASP site, which results in destabilization of PER2 protein and acceleration of the clockwork. To date, however, there is no evidence that the β-TrCP site is phosphorylated *in vivo* to control the circadian period. To test this, I analyzed two knock-in mouse lines harboring Ser478Ala mutation of PER2 protein. I found that the PER2-Ser478Ala (PER2-S478A) mutation lengthened the circadian period of behavioral rhythms and that the PER2-S478A mutant protein accumulated in the mouse liver probably due to stabilization of PER2 protein. These data provide the first *in vivo* evidence showing the importance of the β-TrCP site in setting the speed of the circadian clock.

2.2 Materials and Methods

Mice

Mice used in this study (C57BL/6) were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals at The University of Tokyo. The animals were maintained in a light-tight chamber at a constant temperature (23°C ± 1°C) and humidity (55% ± 10%). Mice were housed in cages with access to food (CLEA Japan) and tap water *ad libitum*. PER2-S478A mice and PER2-S478A::LUC mice were generated by Dale Cowley (TransViragen/UNC Chapel Hill Animal Models Core, USA).

For genotyping, genome DNA prepared from the tail were analyzed by the polymerase chain reaction with the primers Per2-ScF2 (5'- CGGGT CTCTC TGTGC ACTCT TG-3'), Per2-ScFwt (5'- CACAG CGGCT CCAGT GG-3'), Per2-ScRmut (5'- AGGCT CCCAT AGCCA GCTG-3') and Per2-ScR3 (5'- GCTTC TCAGG GAGAG GAACA G-3') for both PER2-S478A and PER2-S478A::LUC.

Behavioral experiments

Six- to ten-week-old male mice were individually housed in cages equipped with running wheels and wheel revolutions were recorded. After entrained to the 12-h /12-h LD cycle for more than two weeks, mice were released into constant darkness (DD) under for 24 days or longer. The number of wheel revolutions in 5-min bins were analyzed with ClockLab software (Actimetrics) to assess their spontaneous locomotor activities. The data obtained from days 11–24 after the start of DD were used for determination of the circadian period of the locomotor activity rhythms by using a chi-square periodogram procedure.

Cell culture and real-time monitoring of rhythmic gene expression

MEFs generated from PER2::LUC mice and PER2-S478A::LUC mice and HEK293T cells (American Type Culture Collection) were cultured at 37°C under 5% CO_2, 95% air in Dulbecco's modified Eagle's medium (SIGMA, D5796-500ML) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and

10% fetal bovine serum. To monitor the bioluminescence from luciferase, cells were entrained by pulse treatment of 0.1 μM (final concentration) dexamethasone for 30 min, and then cultured in recording media [phenol-red free Dulbecco's modified Eagle's medium (SIGMA, D2902) supplemented with 10% fetal bovine serum, 3.5 g/l glucose, 25 U/ml penicillin, 25 μg/ml streptomycin, 0.1 mM luciferin, and 10 mM HEPES-NaOH; pH 7.0]. The bioluminescence signals were continuously recorded for 5-10 days at 37ºC in air with Dish Type Luminescencer, LumiCycle (Actimetrics).

RT-qPCR

Tissues from 7–8-week-old male mice were collected every 4 h from 38 h after the beginning of DD (projected circadian time (CT)). ~50 mg livers were lysed with 1 ml TRIzol reagent (Invitrogen, 15596018), and total RNA was prepared with the RNeasy Mini Kit (Qiagen, 74104) according to the manufacturer's protocol. From purified RNA, cDNA was generated by Go Script Reverse Transcriptase (Promega, A5003) with both anchored (dT) 15 primer and random oligo primers. The expression levels of genes were examined by using GoTaq Master Mix (Promega, A6010) and StepOnePlus Real-Time PCR Systems (Applied Biosystems). The primers used in this study were shown in Table 1.

Preparation of nuclear and cytosolic fractions of mice liver

The nuclear proteins and cytosolic proteins were isolated as previously described [39]. The mouse tissue (1 g wet weight) was washed with ice-cold Hank's Balanced Salt Solution (SIGMA, H8264-500ML) and homogenized on ice with 11 ml of ice-cold buffer A [10 mM HEPES-NaOH (pH 7.8), 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 4 μg/ml aprotinin, 4 μg/ml leupeptin, 50 mM NaF, and 1 mM Na_3VO_4]. The homogenate was centrifuged (5 min each, 700 xg), and the supernatant was used as "cytosolic fraction". the resultant precipitate was rinsed by buffer A and centrifuged (5 min, 700 xg). The precipitate was resuspended in 2 ml of ice-cold buffer C [20 mM HEPES-NaOH (pH 7.8), 400 mM NaCl, 1 mM EDTA, 5

mM $MgCl_2$, 2% (v/v) glycerol, 1 mM DTT, 1 mM PMSF, 4 μg/ml aprotinin, 4 μg/ml leupeptin, 50 mM NaF, and 1 mM Na_3VO_4]. After being gently mixed at 4°C for 1 h, the suspension was centrifuged (30 min each, 21,600 xg) twice, and the final supernatant was used as “nuclear fraction”. Samples were mixed with SDS-PAGE sample buffer [final concentration; 10 mM Tris-HCl, 2 mM EDTA, 2% SDS, 6% glycerol, 50 mM DTT, 0.02% CBB R-250; pH 6.8] and incubated at 95°C for 5 min.

Immunoprecipitation

Immunoprecipitation was performed as previously described [39] with some modifications. Liver nuclear extract was mixed with 2 volumes of buffer D1 [20 mM HEPES-NaOH (pH 7.8), 5.5 mM NaCl, 1 mM EDTA, 6.5% (v/v) glycerol, 1.5% (v/v) Triton X-100, 1 mM DTT, 1 mM PMSF, 4 μg/ml aprotinin, 4 μg/ml leupeptin, 50 mM NaF, and 1 mM Na_3VO_4] and incubated at 4°C for 30 min with anti-CLOCK antibody [39] (CLNT1, Medical & Biochemical Laboratories, D334-3). Protein G-Sepharose 4 Fast Flow (Amersham Biosciences) was then added to this mixture, and the resultant was mixed by gentle rotation for 1 h at 4°C. The beads were collected by centrifugation for 5 min at 2,400 x g as the immunoprecipitates.

Antibodies and immunoblot analysis

Nuclear and cytosolic proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore). The membranes were incubated at 37°C for 1 h in a blocking solution {1%[w/v] skim milk in TBS [50 mM Tris-HCl, 140 mM NaCl, 1 mM $MgCl_2$ (pH 7.4)]} and then probed with primary antibodies in the blocking solution overnight at 4°C. After washed three times with the blocking solution, the membranes are incubated for 1 h at 37°C with a secondary antibody conjugated with horse radish peroxidase. The signals were visualized by an enhanced chemiluminescence detection system (PerkinElmer Life Science). The blot membranes were subjected to densitometric scanning and the band intensities were quantified using ImageQuant TL (GE Healthcare). The primary antibodies used in this study are as follows; anti-Raf-1 (C-12, Santa Cruz Biotechnology, sc-133), anti-ATF2 (C-

19, Santa Cruz Biotechnology, sc-187), anti-CLOCK [39] (CLSP3, Medical & Biological Laboratories, D333-3), B1BH2 anti-ARNTL [39] (B1BH2, Medical & Biological Laboratories, D335-3), anti-PER1 (Medical & Biological Laboratories, PM091), anti-PER2 (Medical & Biological Laboratories, PM083), anti-CRY1 (Medical & Biological Laboratories, PM081), anti-CRY2 (Medical & Biological Laboratories, PM082), anti-DBP (Medical & Biological Laboratories, PM079) and anti-NR1D1 (Medical & Biological Laboratories, PM092). These primary antibodies are detected by following secondary antibodies conjugated with horseradish peroxidase: anti-mouse IgG, anti-rabbit IgG, anti-guinea pig (Kirkegaard & Perry Laboratories, 5220-0341, 5220-0337, 5220-0366, respectively).

Degradation assay

CRY1::LUC or CRY2::LUC [14] was co-expressed with myc-PER2 or myc-PER2-S478A [40] in HEK293T cells. After 48 h from transfection, 100 μg/ml cycloheximide (Nakalai tesque) was added and cells were incubated in LumiCEC (Churitsu) for monitoring bioluminescence or harvested for immunoblotting.

To calculate half-lives of CRY1::LUC and CRY2::LUC, each bioluminescence data was fitted to two-component exponential curve;

$$f(x) = a_1(1/2)^{(x/\tau_1)} + a_2(1/2)^{(x/\tau_2)} + b,$$

where x represent time, a_i, b and τ_i are constant value such as initial quantities, baseline and half-lives, respectively. These values were computed by linear least square fittings.

The instantaneous half-life of PER2 were calculated by a collaborator Jae Kyoung Kim. The bioluminescence data were divided into segments of 1h with a sliding window, which was moved at increments of 0.1 h as done in Zhou et al [35]. Then, each segment of PER2 decay curve is fitted to the exponential curve.

2.3 Results

PER2 mutation at Ser478 lengthens the period of behavioral rhythms

Two key phosphorylation sites, the β-TrCP site and the FASP site, are important for the regulation of the PER2 stability (Figure 4A). The phosphoswitch model also predicts that a mutation of PER2 at the β-TrCP site should stabilize PER2 protein and lengthen the circadian period *in vivo*. To test this, I analyzed a mouse strain which carries PER2 Ser478Ala (PER2-S478A) mutation introduced by the collaborators (Figure 4A). PER2-S478A homozygotes appeared to be morphologically normal and fertile and were born at a normal Mendelian ratio. The mutant mice showed robust wheel-running activity rhythms under a 12 h light: 12 h dark (LD) cycle, and the rhythms were in phase with the LD cycle (Figure 5B) in contrast to the FASP mutant mice [24]. However, when exposed to constant darkness (DD), the mutant mice exhibited ~1 h longer period of the locomotor activity rhythms (24.58 ± 0.08 h) than wild-type (23.67 ± 0.06 h) (Figure 5C). This is the first *in vivo* demonstration that PER2 Ser478 is involved in the determination of the circadian period.

Excessive accumulation of PER2-S478A mutant in the mouse liver

When overexpressed in fibroblasts, PER2-S478A mutant protein was more stable than wild-type PER2 protein [35]. In this study, I examined the endogenous *Per2* mRNA levels and PER2 protein levels in the PER2-S478A mice liver. Livers were collected at 4-h intervals during the second day in DD (Figure 6A). Temporal expression of *Per2* mRNA was time-of-day dependent in both wild-type and PER2-S478A mice and there was no significant difference between the genotypes (Figure 6B). In contrast, the PER2 protein levels were ~2-fold higher at CT22 in PER2-S478A mice in both the nucleus and the cytoplasm when compared with wild-type (Figure 6C, D). Thus, the S478A mutation stabilizes PER2 protein *in vivo* similar to what was seen in *in vitro* overexpression assays [35]. It is worth noting that the abundance of PER2-S478A protein was still ambient at CT34 and CT14 in contrast to upregulation of the peak levels of PER2-S478A protein, suggesting that phosphorylation of

Ser478 is required for time-of-day specific degradation, and that other degradation mechanism(s) may work at other times.

PER2-S478A mutation causes stabilization of CRY proteins

PER2 has been proposed to act as a scaffold protein for formation of a stable multi-protein complex containing PER1, CRY1/2 and CK1δ/ε and this assembly in the cytoplasm is thought to be rate-limiting for nuclear translocation of these circadian repressors [41–43]. To clarify the influence of accumulation of PER2-S478A protein on these clock proteins, I examined the nuclear and cytoplasmic abundance of the circadian repressors in the livers of wild-type and mutant mice by immunoblotting. At CT22, when PER2 protein levels were maximum (Figure 6C, D), the cytosolic protein abundance of PER1 and CRY2 was also increased (Figure 7A). The nuclear levels of PER1, CRY1 and CRY2 proteins were also increased in the PER2-S478A mice liver with a several-hours delay (Figure 7B). Given that *Per1*, *Cry1* and *Cry2* mRNA levels were not upregulated (Figure 7C), these proteins may be stabilized posttranslationally in PER2-S478A mice. Thus, stabilized PER2 increases the abundance of the entire circadian repressive complex.

Previous studies have proposed that CRY proteins are stabilized by PER2 due to overlapping of the interface between PER2 and CRY proteins and between CRYs and FBXL3, an E3 ubiquitin ligase that promotes proteolysis of CRYs [44–46]. I hypothesized that CRY proteins were stabilized in response to elevation of PER2 protein abundance in the PER2-S478A mutant liver, resulting in accumulation of CRY proteins (Figure 7A, B). To assess this possibility, I measured the half-lives of CRY1::LUC and CRY2::LUC fusion proteins in HEK293T cells by monitoring the decrease of bioluminescence after treatment of cycloheximide (CHX) to stop new protein synthesis. PER2 and PER2-S478A similarly slowed the degradation of both CRY1::LUC and CRY2::LUC in a dose-dependent manner (Figure 8A). To calculate the half-lives, I tried to fit the decay curves to a single-term exponential model but failed because of the poor fittings (Figure 8B (i)). Therefore, I applied two-term exponential model and observed the data were well explained by this model (Figure 8B (ii)). This result indicates

that CRY proteins can exist at least in two states with different stability. The half-life of unstable form (τ_1) seemed to be independent of the dose of PER2 expression plasmid, while that of stable form (τ_2) was significantly increased in a PER2 dependent manner (Figure 8A, C). This result suggests that some CRY proteins with high turnover rate are sequestered from PER2. The balance between two states of CRY2 was unaffected by PER2 expression, and that of CRY1 was slightly affected (Figure 8D). The expression levels of PER2 were comparable between PER2 and PER2-S478A (Figure 8E), probably because the endogenous CK1δ/ε cannot destabilize overexpressed PER2 proteins as previously described [35]. These data indicate that PER2 and PER2-S478A equally stabilize the stable form of CRY proteins and that increased accumulation of PER2 stabilized CRY proteins in the PER2-S478A mouse liver.

Notably, stabilization and promoted nuclear entry of CRY1 and CRY2 in PER2-S478A mice result in the maintained nuclear protein abundance of CRY1 and CRY2 even at CT34 (Figure 7B) when a large proportion of PER2-S478A protein was degraded (Figure 6C). This suggests that CRY proteins may be degraded more slowly than PER proteins and can repress CLOCK-BMAL1 transcriptional activity even after PER proteins disappear from the nucleus, consistent with a previous study [47]. In fact, the interaction between CRY1 and CLOCK was enhanced in PER2-S478A mice when compared with wild-type at CT26-34 (Figure 9). Furthermore, prolonged repression of mRNA expressions were observed for typical E-box-regulated genes, such as albumin D-site binding protein (*Dbp*), *Nr1d1* (also known as *Rev-erbA*) and *Nr1d2* (also known as *Rev-erbB*) in the PER2-S478A mutant liver (Figure 10A). The expression profiles of RRE-regulated genes *Bmal1, Clock,* and *Cry1* were also delayed (Figure 10A, 7C). These profiles are consistent with the longer period phenotype of the PER2-S478A mice. Concomitantly, nuclear abundance of DBP and NR1D1 proteins was phase-delayed in the PER2-S478A mouse liver (Figure 10B). Overall, my data indicate that the mutation of the β-TrCP site leads to nuclear accumulation of full circadian repressive complex and prolonged downregulation of E-box-regulated genes.

S478A mutation of PER2 compromises temperature compensation of the circadian period

The phosphoswitch model arose from the observation of the three-phase decay of PER2::LUC [35]: an initial rapid decay (the first phase), a plateau-like slow decay (the second phase), and finally, a rapid terminal decay (the third phase). This was visualized by continual monitoring of bioluminescence signals from PER2::LUC in MEFs. To investigate how the S478A mutation affects the three-phase degradation *in vivo*, the S478A mutation was introduced into the PER2::LUC allele (PER2-S478A::LUC) which encodes PER2 protein fused with firefly luciferase [48]. I analyzed the circadian rhythm of the wheel running activity, and found that the period of behavioral rhythm of PER2-S478A::LUC mice was significantly longer than PER2::LUC control mice (Figure 11A, B), similar to that seen in the PER2-S478A mice (Figure 5). Mouse embryonic fibroblasts (MEFs) were established from both PER2-S478A::LUC and PER2::LUC control mice by the collaborators. They observed that the three-phase degradation was perturbed in PER2-S478A::LUC mutant MEFs, consistent with the role of Ser478 phosphorylation for the phosphoswitch (Figure 12A, B).

Zhou and colleagues proposed this phosphoswitch has a role in temperature compensation of the circadian period [35], which is a key feature of the circadian clock [37]. While the rate of most enzymatic reactions doubles or triples when temperature is increased by 10°C (*i.e.*, temperature coefficient, Q_{10} = 2-3), the circadian period is relatively unaffected by fluctuations of temperature, and in fact is often over-compensated, with a $Q_{10} < 1.0$. Their idea is that CK1δ/ε phosphorylate preferentially at Ser478 at lower temperature to accelerate the degradation of PER2, which counteract decrease of other reactions in the clockwork. To test this, I examined if disruption of the phosphoswitch by S478A mutation affects the temperature compensation. While there was a significant clone-to-clone variability, the period length of the PER2-S478A::LUC MEFs was consistently longer than that of control MEFs at 30°C (Figure 13A, B). Further, Q_{10} values of the circadian period tended to be higher

in the PER2-S478A::LUC MEFs than the PER2::LUC MEFs, suggesting that temperature compensation was compromised by the S478A mutation (Figure 14B). These data strengthen the importance of the β-TrCP site in the phosphoswitch-regulated temperature compensation.

Collectively, these data demonstrate that the β-TrCP site of PER2 may contribute to the temperature compensation of the circadian period *in vivo*.

2.4 Discussion

The PER2 phosphoswitch does function *in vivo*

CK1δ/ε set the speed of the circadian clock by modulating PER protein stability. While mutation at one CK1-targeting site (the FASP site) causes a shortened circadian rhythm [21, 24], it has not been addressed whether mutation at the other target site (the β-TrCP site) alters the circadian period. In this thesis, I showed that the period of behavioral rhythm was lengthened by introducing Ser-to-Ala mutation into the β-TrCP site. Mice with the PER2-S478A mutation showed significantly longer circadian period in behavioral rhythm than control mice (Figure 5). This period lengthening was similar to those observed in mice treated with a CK1 inhibitor [32] or in transgenic mice carrying human PER2-S662D, a phospho-mimic mutant of the FASP site [24]. My data propose that phosphorylation of the β-TrCP site virtually acts as the competing process against the phosphorylation of the FASP site and that this phosphoswitch regulation controls the circadian period. Although the *Per2* mRNA levels were not significantly changed in PER2-S478A::LUC mice, the peak amounts of PER2 protein were increased both in cytosol and nucleus (Figure 6), consistent with disruption of the Ser478 phosphodegron.

Other kinases may also contribute to the PER2 stability control

CK1δ and CK1ε may not be the only kinases which can regulate PER2 stability. For example, a chemical screen unveiled that CK1α regulates the PER2 stability in mammalian clockwork [49]. This effect of CK1α may not be so strong because CK1α binds to PER proteins with a much lower affinity than CK1δ/ε do [49]. In *Drosophila*, CK1α itself does not destabilize PER2, but enhances PER degradation elicited by DBT (the CK1δ/ε homolog in *Drosophila*) [50], implicating that mammalian CK1α is also a modulatory kinase for the PER2 stability control. CK2 also cooperates with CK1δ/ε to promote PER2 proteolysis, while CK2 alone phosphorylates PER2 at Ser53, which also destabilizes PER2 protein [51]. PKCα [52] and GRK2 [53] regulate the nuclear entry of PER2 and might also affect the stability of PER2. In this thesis, I

showed that PER2-S478A mutation upregulated PER2 protein levels posttranslationally probably due to attenuation of CK1-mediated degradation. Furthermore, the circadian period was lengthened by this mutation both in cells and in mice. In fungi, CK1 also regulates the protein stability of FREQUENCY (FRQ) which plays an analogous function to mammalian PER. FRQ is progressively phosphorylated and destabilized by CK1 [54, 55]. Loss of function of CK1 lengthened circadian period as well [56]. However, recent studies demonstrated that the binding affinity between CK1 and FRQ, instead of FRQ stability, well explained the circadian period of various *frq* mutants and proposed that CK1-mediated phosphorylation of other clock proteins is crucial [57, 58]. CK1δ/ε in mammals may play a dual role for regulating the speed of circadian clock; one is for the PER2 stability and the other is for the phosphorylation of CLOCK [42].

PER2-S478A mutation alters the oscillation of the circadian clock

In this study, these data are consistent with the model illustrated in Figure 13, in which PER2 Ser478 phosphorylation governs decay of the repressive complex which determines re-activation timing of the E-box-dependent transcription. The peak amount of PER2 protein is regulated by two counteracting phosphorylation. The PER2-S478A mutation disrupts the balance between the phosphorylation levels of two sites and results in excessive accumulation of PER2 (Figure 6), upregulating the nuclear abundance of PER1 and CRY proteins (Figure 7). PER1 and PER2 are then degraded by an undefined pathway which does not require PER2 Ser478 phosphodegron. After PER proteins degraded, CRY1 and CRY2 still remain in nucleus and repress the transcriptional activity of CLOCK-BMAL1 activator complex without PERs [47, 59, 60], also contributing to the lengthened period phenotype.

The phosphoswitch has a role in temperature compensation of the period

Although temperature compensation of the circadian period was first described more than 60 years ago [37], the molecular mechanism underlying this characteristic is poorly understood. Mathematical models predicted that

opposing reactions contribute to the temperature-compensated clockwork [61], although the processes which virtually act in this mechanism have not been addressed. The collaborators previously proposed the PER2 phosphoswitch may act as the temperature-compensating process in mammalian clockwork [35]. Here, I showed that temperature compensation of the period tended to be attenuated by the S478A mutation (Figure 13). The period difference between two genotypes was larger at lower temperature, which is consistent with the idea that the β-TrCP site is more susceptible to phosphorylation at lower temperature than the FASP site [35]. My data provides the *in vivo* evidence that the phosphoswitch contributes to the temperature compensation. On the other hand, the circadian period was still compensated in temperature changes (Figure 13), suggesting Ser478 phosphodegron is not the only regulation for temperature compensation. Indeed, the collaborator demonstrated the three-phase degradation was compromised but still observed in PER2-S478A::LUC MEFs (Figure 12), indicating another phosphodegron within PER2 is also involved in the phosphoswitch regulation. A potential degron motif TpSGCSpS is conserved in PER1 (aa121-126) and PER2 (aa92-97), while the β-TrCP site (477-SpSGYGpS-482 in PER2, the primary degron) is not present in PER1 [34]. In PER2, the primary degron has a major role for β-TrCP-mediated proteolysis and the second degron has a minor role because its contribution to PER2 degradation was reported when the primary degron was mutated [62]. Therefore, the second degron may contribute to the alternative degradation pathway in PER2-S478A mice.

Implication of other degradation pathway

Despite the disruption of the β-TrCP site, I observed rapid degradation of PER2-S478A mutant protein in mouse liver during the dark-light transition (Figure 6). The second phosphodegron may somewhat contribute to this degradation, other regulations have been implicated in this process. Oncoprotein MDM2 was recently reported to promote proteasomal degradation of PER2 protein independent of its phosphorylation status [63]. Additionally, PER2 exists in a complex with PER1, and it is possible that PER2 degradation

may coincide with β-TrCP-dependent degradation of PER1.

Phosphorylation by CK1 is not so simple reaction

To understand the PER2 stability control, the regulation for the activity of CK1δ/ε must be considered. CK1δ/ε have at least three different types of substrates: priming (FASP), primed (downstream residues of the FASP site), and priming-independent (degron) sites. Phosphorylation on all these sites seem to be temperature-compensated in *in vitro* kinase assay while Q_{10} values are slightly different [31, 38]. The mechanism underlying temperature compensated phosphorylation on a “primed” peptide is relatively well understood. In brief, a primed phosphopeptide has more affinity to CK1 at higher temperature, which decreases the turnover rate and may also cause dephosphorylation of the product [38]. In this reaction, an anion binding site unique to the CK1 family is important for the interaction between a phosphorylated residue and CK1. Mutation of CK1 or treatment of a small molecule abrogating this interaction compromises temperature-insensitive phosphorylation and perturb temperature compensation of the clock [38]. On the other hand, temperature compensation of phosphorylation on an unphosphorylated peptide is due to enhanced enzyme-substrate interaction at lower temperature, although structural bases for this mechanism is not defined [38]. Furthermore, it was recently reported that a two-state conformational switch in the activation loop of CK1 determines the preference of phosphorylation sites. The “loop down” conformation is more stable in wild-type CK1 and this conformation facilitates phosphorylation of the FASP region. On the other hand, the “loop up” conformation facilitates phosphorylation of the β-TrCP site [40], while it is unclear what toggles this switch *in vivo*. One possible regulation is alternative splicing of *Csnk1d* mRNA which encodes CK1δ. *Csnk1d* gene encodes two splicing isoforms of CK1δ, CK1δ1 and CK1δ2 [26, 27]. The C-terminal tails of CK1δ1 and CK1δ2 are slightly different, which affects the phosphorylation activity towards the FASP site. The molar ratio of two CK1δ isoforms is tissue-specific but not oscillating during the day [26]. The splicing of CK1δ might be affected by some external stimuli such as

temperature. Future studies on detailed mechanisms underlying the PER2 stability control will help to understand the temperature compensation of the circadian clock.

2.5 Figures and Table

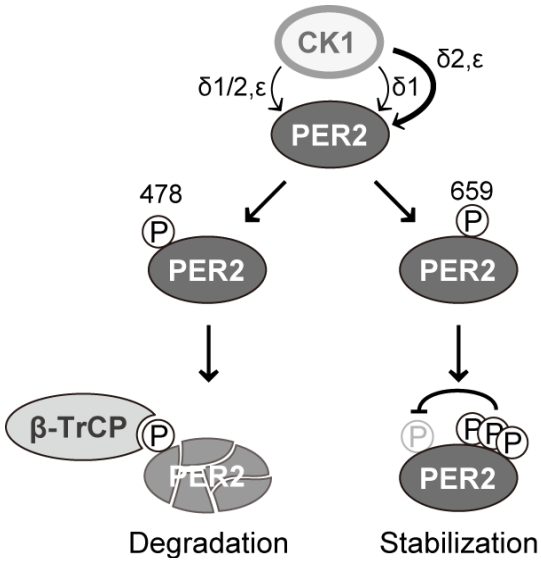

Figure 3. The stability of PER2 protein is regulated by the phosphoswitch
PER2 is phosphorylated by CK1δ/ε. Phosphorylation of Ser478 (the β-TrCP site) leads to β-TrCP-dependent proteolysis, whereas phosphorylation of Ser659 (the FASP site) triggers progressive phosphorylation by CK1δ/ε in the motif pSxxSxxS. This multi-phosphorylation inhibits phosphorylation of the β-TrCP site. CK1ε and a recently identified CK1δ2 splice isoform more efficiently phosphorylate Ser659 than CK1δ1 (see Discussion).

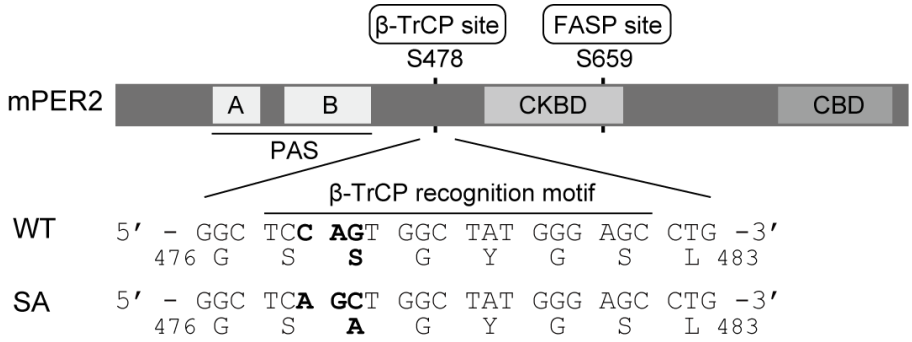

Figure 4. Generation of PER2-S478A mice
Schematic showing of the functional domains of PER2 (upper). The genome sequences around the β-TrCP recognition motif in wild-type (WT) and PER2-S478A (SA) knock-in mouse with encoded amino acid sequences are shown (lower). PAS, Per-Arnt-Sim domain; CKBD, Casein kinase-binding domain; CBD, CRY-binding domain

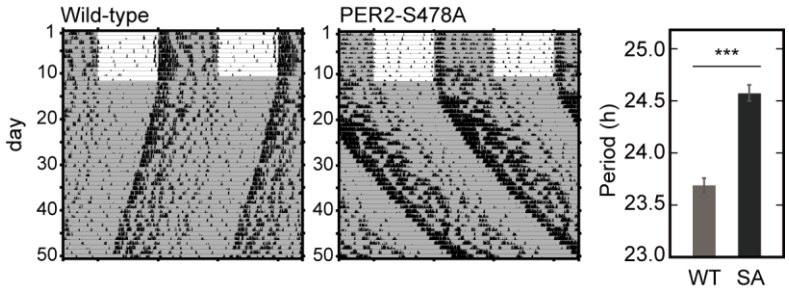

Figure 5. Wheel running activities of PER2-S478A homozygous mice
(left) Representative recordings of the rotation of running wheel are shown. PER2-S478A homozygous mice and littermate wild-type mice were entrained to LD for two weeks or longer and then exposed to DD.
(right) The circadian period of the activity rhythms under the DD was determined from locomotor activity in days 11–24 after transferred to DD by a chi-square periodogram procedure. Mean values ± SEM obtained from ten wild-type (WT) mice and twelve PER2-S478A (SA) mice are given. *** p = 4.2 x E-8, two-sided Student's t-test.

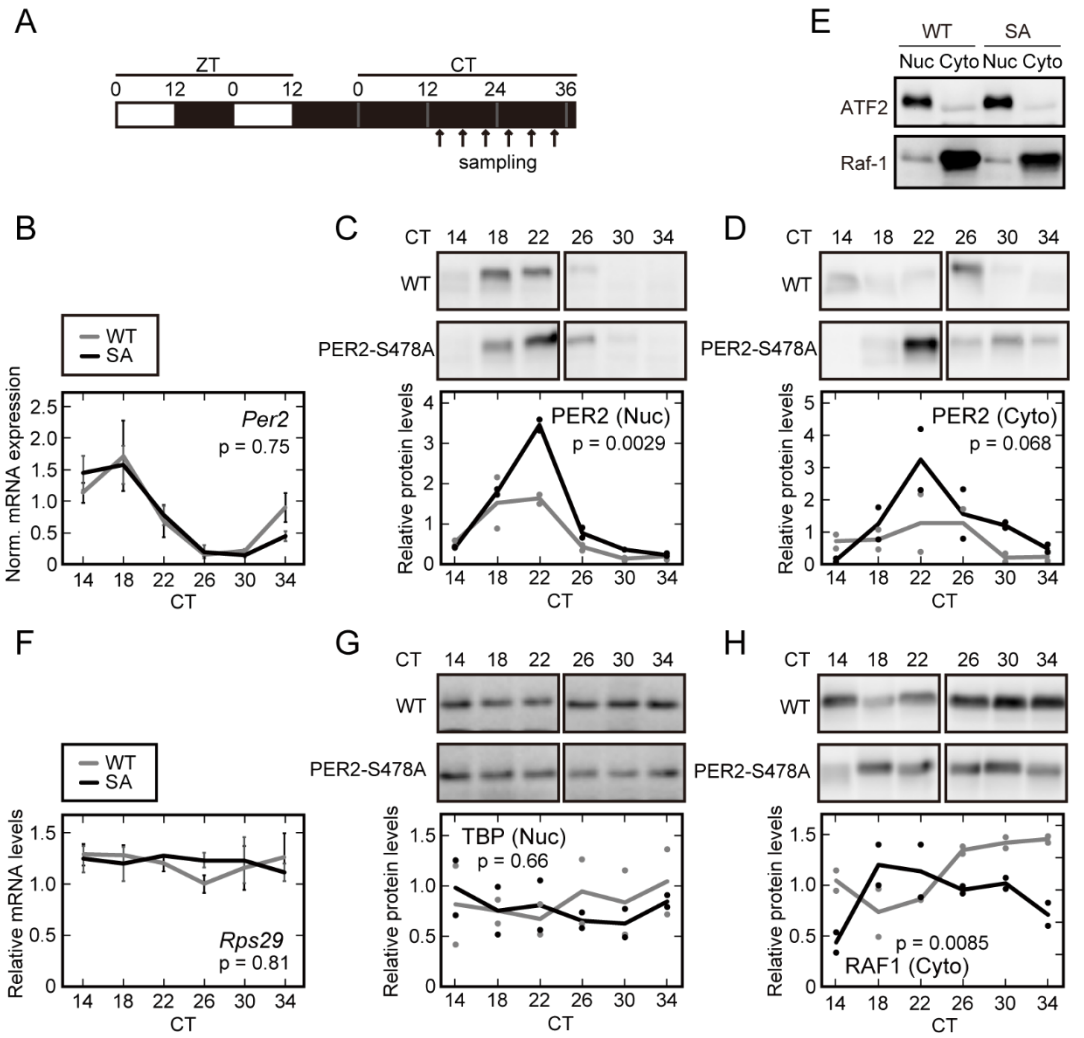

Figure 6. PER2 expression levels are post-transcriptionally upregulated in PER2-S478A mouse liver

(A) Scheme of the experiment examining the daily expression profile of proteins. Also see Materials and Methods for details. ZT, zeitgeber time, CT, circadian time.

(B) Daily expression profile of Per2 mRNA normalized by Rps29. Livers were harvested at 4-h intervals and lysates were analyzed by real-time RT-qPCR. Mean values ± SEM obtained from three animals of each genotype are shown. Statistical significance was determined for genotype by two-way ANOVA.

(C, D) Daily expression profile of PER2 proteins in liver. Liver nuclear extracts (C, Nuc) and cytoplasmic lysates (D, Cyto) were prepared at 4-h intervals and subjected to SDS-PAGE and immunoblotting. Data are represented as dots for individual experiments and as lines for means. Statistical significance was determined for genotype by two-way ANOVA.

(E) Fractionation controls. Liver nuclear and cytoplasmic fractions at CT26 were subjected to immunoblotting. ATF2 and Raf-1 are detected as nucleic and cytoplasmic markers, respectively.

(F, G, H) Loading control for mRNA (F, Rps29), nuclear fraction (G, TBP) and cytoplasmic fraction (H, RAF1). Statistical significance was determined for genotype by two-way ANOVA.

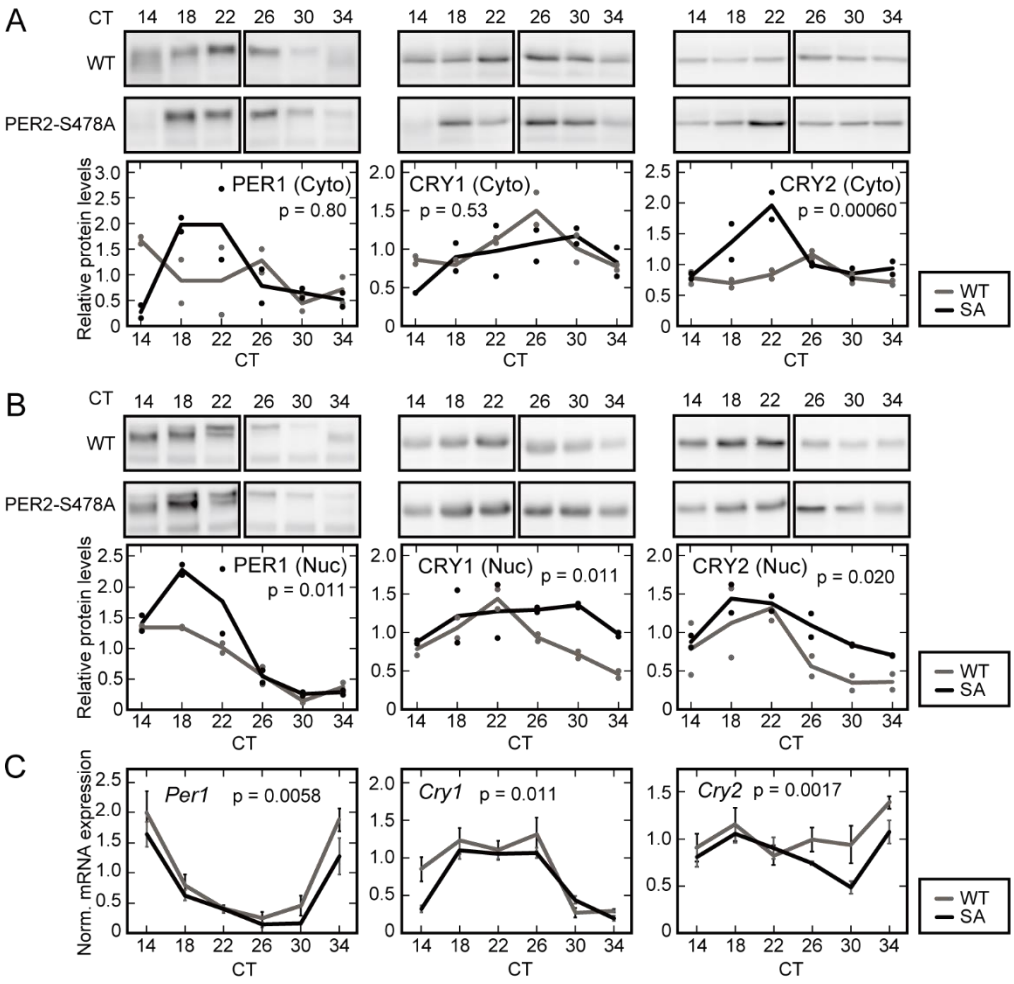

Figure 7. The components of the circadian repressor complex are accumulated in PER2-S478A mice liver

(A, B) Daily expression profiles of PER1, CRY1 and CRY2. Liver cytosolic lysates (A, Cyto) and nuclear extracts (B, Nuc) were prepared at 4-h intervals and subjected to SDS-PAGE and immunoblotting with indicated antibodies. Data are represented as dots for individual experiments and as lines for means. Statistical significance was determined for genotype by two-way ANOVA.

(C) Daily expression profiles of *Per1*, *Cry1* and *Cry2* mRNA. Livers were harvested at 4-h intervals and subjected to real-time RT-qPCR. Mean values ± SEM obtained from three animals of each genotype are given. Statistical significance was determined for genotype by two-way ANOVA.

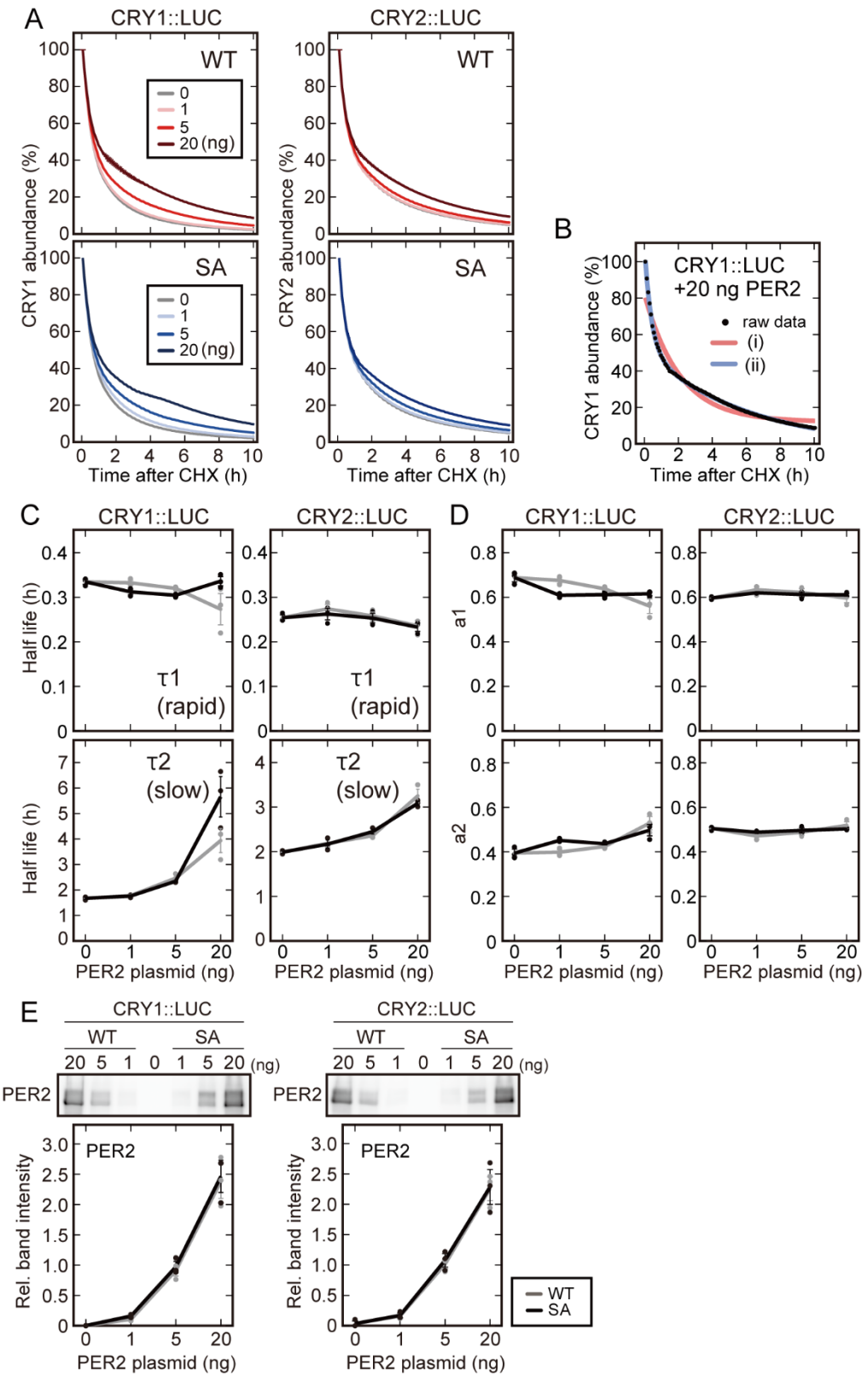
A
CRY1::LUC
CRY2::LUC
WT
SA
0
1
5
20 (ng)
CRY1 abundance (%)
CRY2 abundance (%)
Time after CHX (h)
B
CRY1::LUC
+20 ng PER2
raw data
(i)
(ii)
C
D
Half life (h)
τ1
(rapid)
τ2
(slow)
a1
a2
PER2 plasmid (ng)
E
PER2
Rel. band intensity
WT
SA

Figure 8. PER2 stabilizes CRY1::LUC and CRY2::LUC
(A) Bioluminescence from CRY1::LUC (left) or CRY2::LUC (right) are shown. PER2 (upper) or PER2-S478A (lower) was overexpressed with CRY1::LUC or CRY2::LUC in HEK293T cells. CHX was added 48 h after transfection. Mean values ± SEM obtained from three replicates are given.
(B) Representative recording of bioluminescence is shown (dot). The decay of bioluminescence was fitted to single-term exponential model (i) or to two-term exponential model (ii).
(C) Half-lives of rapid decays (τ_1, upper) and that of slow decays (τ_2, lower) are calculated by two-term exponential model. Mean values ± SEM obtained from three replicates are given.
(D) Coefficients of rapid and slow decays are shown as a1 and a2, respectively. Mean values ± SEM obtained from three replicates are given.
(E) The expression of PER2 and PER2-S478 were detected by western blotting with anti-PER2 antibody. Mean values ± SEM obtained from three biological replicates are given.

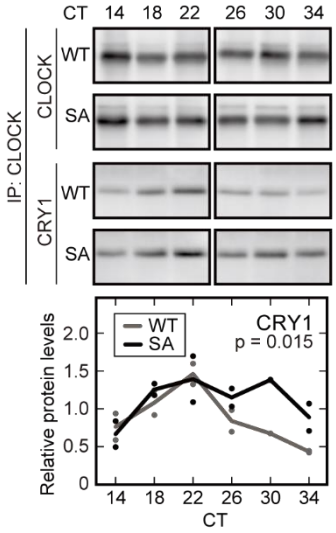

Figure 9. Circadian recruitment of CRY1 on CLOCK
Schematic showing of the functional domains of PER2 (upper). The genome sequences around the β-TrCP recognition motif in wild-type (WT) and PER2-S478A (SA) knock-in mouse with encoded amino acid sequences are shown (lower). PAS, Per-Arnt-Sim domain; CKBD, Casein kinase-binding domain; CBD, CRY-binding domain

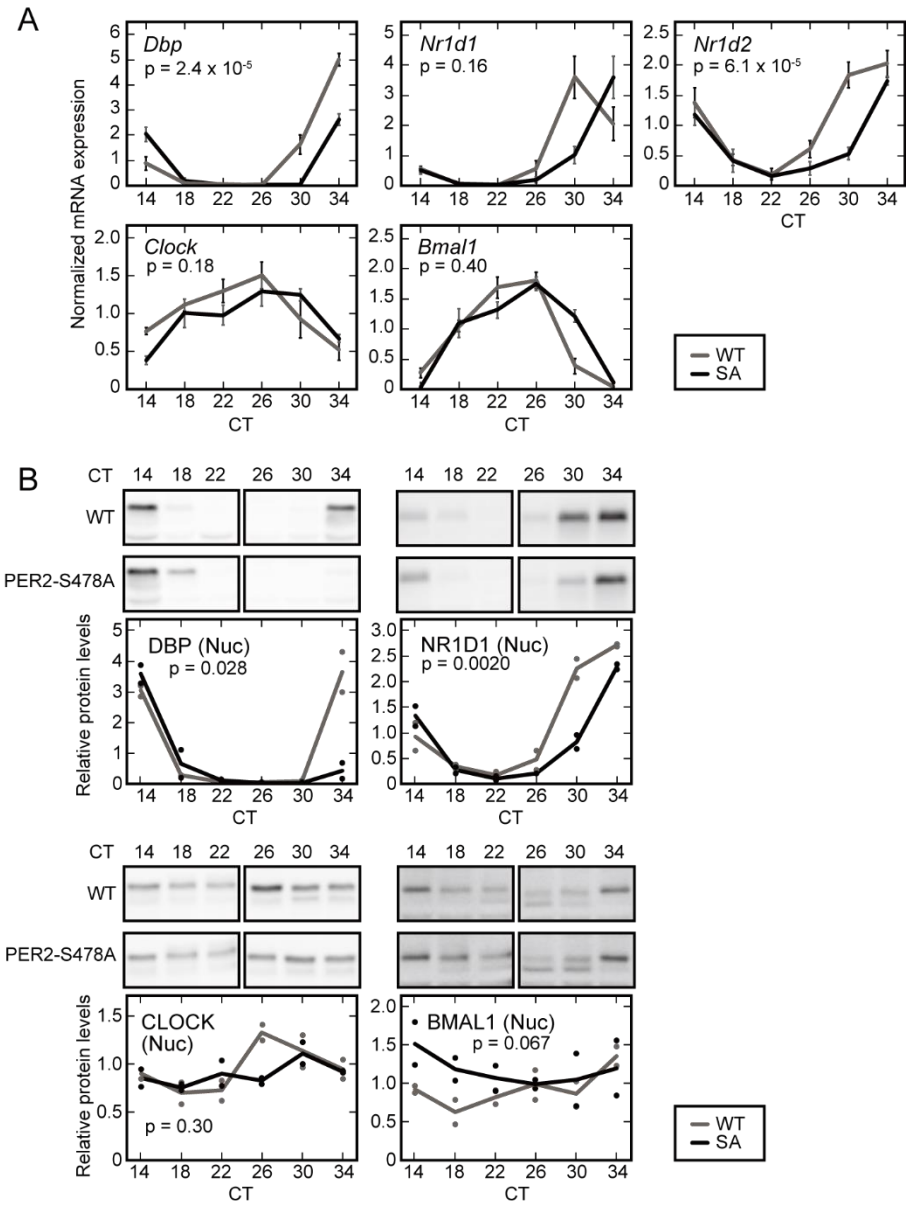

Figure 10. The PER2-S478A mutation alters the expression profiles of clock genes in liver

(A) Daily expression profiles of clock genes. Livers were harvested at 4-h intervals and subjected to real-time RT-qPCR. Mean values ± SEM obtained from three animals of each genotype are given. Statistical significance was determined for genotype by two-way ANOVA. (B) Daily expression profiles of clock proteins in liver nucleus. Liver nuclear extracts prepared every 4 h were subjected to SDS-PAGE and immunoblotting with indicated antibodies. Data are represented as dots for individual experiments and as lines for means. Statistical significance was determined for genotype by two-way ANOVA.

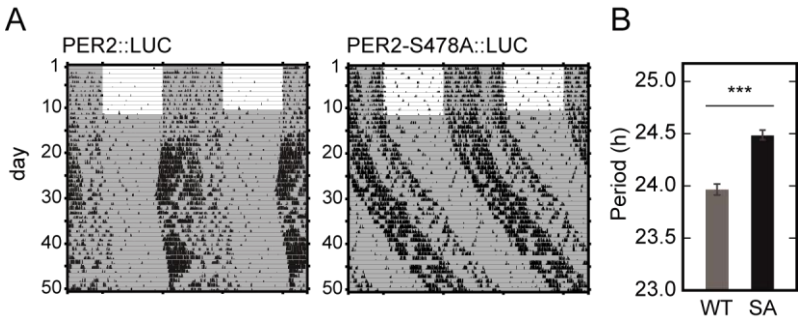

Figure 11. Wheel running activities of PER2-S478A::LUC mice
(A) Representative recordings of the rotation of running wheel are shown. PER2::LUC and PER2-S478A::LUC homozygous mice were entrained to LD for two weeks or longer and then transferred to DD.
(B) The circadian period of the activity rhythms under DD was determined from locomotor activity in days 11–24 after transferred to DD by a chi-square periodogram procedure. Mean values ± SEM obtained from ten PER2::LUC mice (WT) and ten PER2-S478A::LUC (SA) mice are given. *** p = 5.6 x E-7, two-sided Student's t-test.

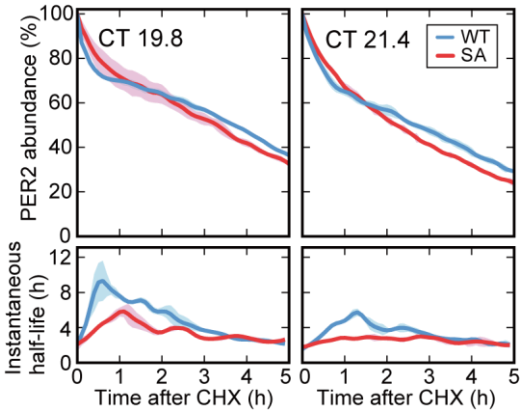

Figure 12. The PER2-S478A mutation perturbs the phosphoswitch (performed by Rajesh Narasimamurthy)
Bioluminescence from PER2::LUC (WT) and PER2-S478A::LUC (SA) MEFs are shown. MEFs were synchronized with dexamethasone and the bioluminescence was continually measured in the Lumicycle. CHX was added at indicated time points. Time after pulse treatment of dexamethasone is referred as Circadian Time (CT). The instantaneous half-life is calculated by fitting the exponential curve to the small segment of the decay curves (see Materials and methods for details). Shadows indicate standard error of the mean values of three to four biological replicates.

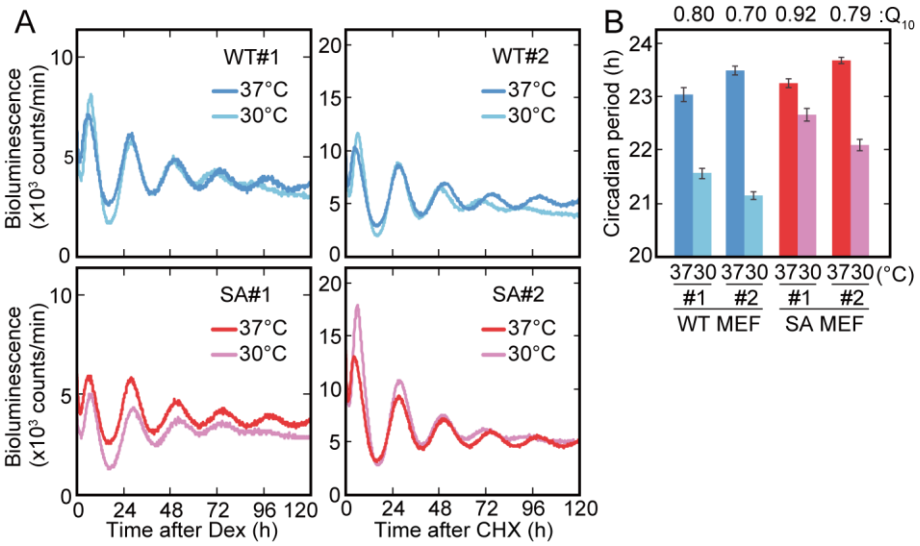

Figure 13. The mutation of PER2 at Ser478 affects the temperature compensation of circadian period

(A) Representative recordings of bioluminescence from PER2::LUC or PER2-S478A::LUC MEFs (two lines each) are shown. MEFs were synchronized with dexamethasone and the bioluminescence was then continually measured in the Lumicycle.

(B) The circadian period of cellular rhythms was calculated. Q_{10} values of each cell line were shown above the bars. Mean values ± SEM obtained from three PER2::LUC (WT) MEFs and three PER2-S478A::LUC (SA) MEFs are given.

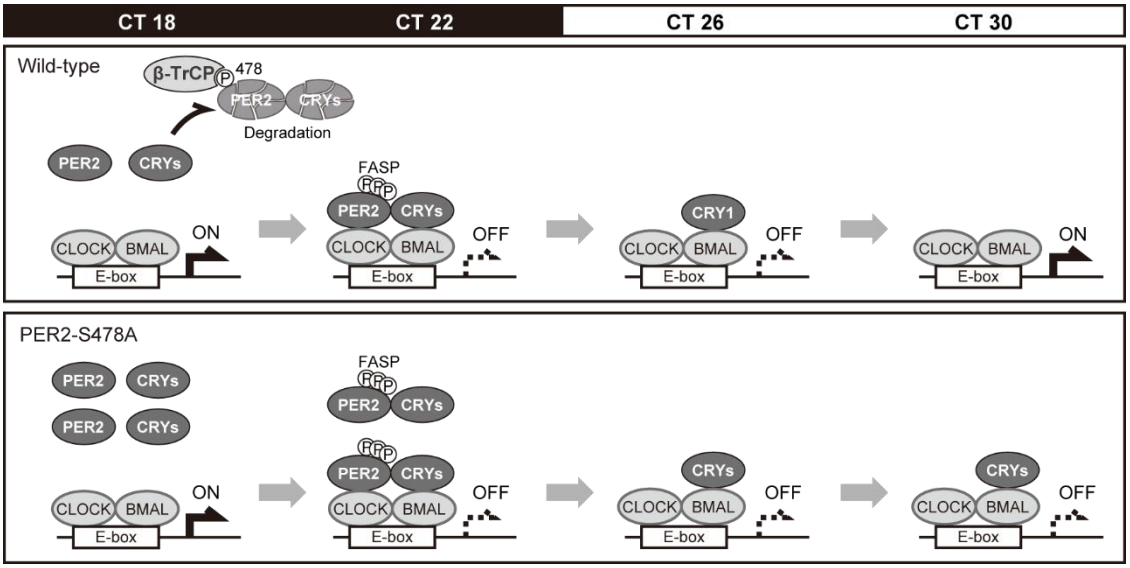

Figure 14. PER2-S478A mutation lengthens the circadian period
Negative components of clock, PER2 and CRY proteins, accumulate during night and are degraded before CT30 in wild-type. In contrast, PER2 and CRY proteins are stabilized and excessively accumulate around CT22 in PER2-S478A mutants. PER2 proteins are then degraded via unknown degradative pathway by CT26, whereas CRY proteins remains in nucleus to repress the activity of the CLOCK-BMAL complexes.

Table 1. Primer sequences for RT-qPCR

Primer	Sequence (5'-3')
mRps29-Fw	TGAAGGCAAGATGGGTCAC
mRps29-Rv	GCACATGTTCAGCCCGTATT
mPer1-Fw	CAGGCTAACCAGGAATATTACCAGC
mPer1-Rv	CACAGCCACAGAGAAGGTGTCCTGG
mPer2-Fw	GGCTTCACCATGCCTGTTGT
mPer2-Rv	GGAGTTATTTCGGAGGCAAGTGT
mCry1-Fw	CCCAGGCTTTTCAAGGAATGGAACA
mCry1-Rv	TCTCATCATGGTCATCAGACAGAGG
mCry2-Fw	GGGACTCTGTCTATTGGCATCTG
mCry2-Rv	GTCACTCTAGCCCGCTTGGT
mClock-Fw	CCTATCCTACCTTGGCCACACA
mClock-Rv	TCCCGTGGAGCAACCTAGAT
mArntl-Fw	GCAGTGCCACTGACTACCAAGA
mArntl-Rv	TCCTGGACATTGCATTGCAT
mDbp-Fw	AATGACCTTTGAACCTGATCCCGCT
mDbp-Rv	GCTCCAGTACTTCTCATCCTTCTGT
mNr1d1-Fw	CGTTCGCATCAATCGCAACC
mNr1d1-Rv	GATGTGGAGTAGGTGAGGTC
mNr1d2-Fw	ACGGATTCCCAGGAACATGG
mNr1d2-Rv	CCTCCAGTGTTGCACAGGTA

3. 5 年以内に雑誌等で刊行予定のため、非公開。

4. Conclusions

In summary, I have described the importance of posttranslational modifications destabilizing the highly oscillating proteins *in vivo*.

First, I demonstrated PER2-S478A mutant mice have a slower clock due to stabilization of the repressive complex composed of PER1, PER2, CRY1 and CRY2. This is the first demonstration for the *in vivo* role of the β-TrCP site in the clockwork. By analyzing rhythms of cellular clock, this regulation is important for the temperature compensation of the circadian period which is one of the most important characteristics of the clock. This feature is presumed to be tightly associated with the core oscillatory mechanism, therefore it is difficult to obtain mutant animals which have robust but not-temperature-compensated clocks. This hypothesis-driven forward genetic study provides a new foundation for future research on the temperature compensation.

以下、5 年以内に雑誌等で刊行予定のため、非公開。

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6. List of Abbreviations

PER	Period
DBP	Albumin D-site binding protein
CLOCK	Circadian locomotor output cycle kaput
BMAL1	Brain muscle arnt-like 1
CRY	Cryptochrome
CK1	Casein kinase 1
FASP	Familial advanced sleep phase
β-TrCP	Beta-transducin repeat-containing homologue protein
LD	Light dark cycle
DD	Constant darkness
MEF	Mouse embryonic fibroblast
LUC	Luciferase
CHX	Cycloheximide
Dex	Dexamethasone

5 年以内に雑誌等で刊行予定のため、非公開。

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