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The Role of β-TrCP1 and β-TrCP2 in Circadian Rhythm Generation by Mediating Degradation of Clock Protein PER2

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The mammalian circadian clock proteins undergo a daily cycle of accumulation followed by phosphorylation and degradation. The mechanism by which clock proteins undergo degradation has not been fully understood. Circadian clock protein PERIOD2 (PER2) is shown to be the potential target of F-box protein β-TrCP1, a component of ubiquitin E3 ligase. Here, we show that β-TrCP2 as well as β-TrCP1 target PER2 protein *in vitro*. We also identified β-TrCP binding site (m2) of PER2 being recognized by both β-TrCP1 and β-TrCP2. Luciferase–PER2 fusion system revealed that m2 site was responsible for the stability of PER2. The role of β-TrCP1 and β-TrCP2 in circadian rhythm generation was analysed by real-time reporter assay revealing that siRNA-mediated suppressions of β-TrCP1 and/or β-TrCP2 attenuate circadian oscillations in NIH3T3 cell. β-TrCP1-deficient mice, however, showed normal period length, light-induced phase-shift response in behaviour and normal expression of PER2, suggesting that β-TrCP1 is dispensable for the central clock in the suprachiasmatic nucleus. Our study indicates that β-TrCP1 and β-TrCP2 were involved in the cell autonomous circadian rhythm generation in culture cells, although the role of β-TrCP2 in the central clock in the suprachiasmatic nucleus remains to be elucidated.

Key words: β-TrCP, circadian rhythm, F-box protein, knockout mouse, ubiquitin E3 ligase.

Circadian clock is biological system that regulates physiology and behaviour of most organisms. In mammals, circadian oscillators in suprachiasmatic nuclei (SCNs) of anterior hypothalamus functions as master clock. The master clock integrates the light information and synchronizes the circadian clock of the peripheral tissue cells (*1*). The clock gene is first isolated in *Drosophila*, then many homologous clock genes are identified between *Drosophila* and mammals, including *period (Per)*, *Cryptochrome (Cry)*, *Clock*, *casein kinase 1(CK1)*, *GSK3β* and *E4BP4* (*2–5*). The circadian oscillators are contained within single cell throughout the body and even in immortalized cell lines (*1*, *6*). The oscillators consist of positive and negative transcriptional feedback loops. The basic helix–loop–helix PAS transcription factors, CLOCK and BMAL1 [and/or NPAS2 (*7*)], activate transcription of *Period1* (*Per1*), *Per2*, *Per3*, *Cryptochrome1* (*Cry1*), *Cry2* and *Reb-erba* by binding to E-box enhancer. The PER and CRY proteins act as negative regulators by directly interacting with CLOCK and BMAL1. The REV-ERBα and RORα comprise additional feedback loop by repressing the transcription of *Bmal1*.

In addition to transcriptional feedback regulations, essential role of post-translational regulation has been reported (*8*, *9*). In cyanobacteria, *in vitro* constitution of circadian rhythm does not require transcription or translation but based on phosphorylation cycle (*10*). In *Neurospora*, F-box protein *FWD1*, a component of ubiquitin E3 ligase, is critical to sustain the physiological circadian rhythm by mediating degradation of clock protein FREQUENCY (*11*). The *Drosophila* orthologue of *FWD1*, *Slimb*, is also essential in circadian locomotor activities through the degradation of PER and TIM (*12*, *13*). In Arabidopsis, F-box proteins, ZEITLUPE and FKF1, affect the circadian rhythm indicating that the ubiquitin–proteasome system is essential to generate circadian rhythm (*14*, *15*). Post-translational modifications are also reported in mammals to regulate accumulation, protein interaction and subcellular location of the clock gene products (*8*). Recent reports show that mutation of mouse F-box protein *Fbxl3* leads to prolonged period in locomotion by mediating ubiquitin-dependent degradation of CRY proteins (*16–18*). The role of proteolysis in circadian rhythm generation still remains to be elucidated in mammals.

The mammalian orthologue of *Slimb* is *β-TrCP1* (*FWD1a*/*Fbxw1*/*E3RS*) and *β-TrCP2* (*FWD1b*/*Fbxw11*/*HOS*). The two gene products associate with PER1 (*19*) and β-TrCP1 associate with PER2 (*20*) to degrade the PER proteins through proteasome system, although the

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role of β-*TrCP* genes in circadian rhythm is unclear. Genetic dissection has revealed that *Per2* is one of the indispensable components in clock oscillation (*21*). Cyclic PER2 protein expression is essential for circadian feedback loops in culture cells (*22*). PER2 is destabilized by phosphorylation of casein kinase 1ε (CK1ε) and CK1δ (*23*, *24*). Exogenous PER2 protein expression in mouse fibroblast oscillates without cycling expression of the mRNA that encodes it (*25*), and this oscillation is independent of CRY proteins (*26*). Yet, the mechanism that regulates the cyclic PER2 expression is unclear. And β-TrCP1 and/or β-TrCP2 is possibly responsible for the regulation.

We studied β-TrCP1 and β-TrCP2 in circadian rhythm generation, focusing on degradation of PER2. The suppression of β-*TrCP1* and β-*TrCP2* using small interfering RNA (siRNA) affected circadian clock in NIH3T3. On the other hand, analysis of β-*TrCP1* mutant mouse showed normal period length and light-induced phase-shift response behaviour, and the expression of PER2 in SCN was indistinguishable from control mice.

MATERIALS AND METHODS

DNA Constructs and Primers—The cDNA constructs of rat PER2 and mouse CK1ε were a gift K. Miyazaki. The full-length mouse β-TrCP1 or β-TrCP2 was tagged with FLAG. For dominant negative form of β-TrCP, amino acid positions 144–199 or 140–195 were eliminated to make β-TrCP1ΔF and β-TrCP2ΔF (*27*), which lacks F-box domain, and therefore cannot recruit Skp1. Mutagenesis of PER2 were constructed by PCR. The amino acid sequences were exchanged from 92TSGCSS97 to 92TIGCSI97 (m1) or 477SSGYGS482 to 477SNG YGN482 (m2).

Immunoprecipitation—For immunoprecipitation assay, COS1 cells were transfected with cDNA constructs (myc-PER2, FLAG- β-TrCPs, V5-CK1ε). Twenty hours after the transfection, proteasome inhibitor MG132 (30 mM) was added 3 h before harvesting. The cells were lysed in 10 mM Tris–HCl, 1 mM EDTA, 150 mM NaCl, 1% NP40, protease inhibitor Complete (Roche). Total protein was determined by DC Protein Assay (Bio-Rad). The cell lysate that contained 1 mg of protein was mixed with 1 μg of anti-myc (Roche) or anti-FLAG (Sigma) antibody combined with 25 μl of A/G Agarose (Santa Cruz) and was allowed to form a complex for overnight at 4°C with rotation. The complex was rinsed three times with lysis buffer and then boiled in SDS sampling buffer. Western blotting was performed with anti-FLAG or anti-myc antibody to detect the protein input and protein precipitation.

Analysis of PER2 Degradation—For PER2 degradation analysis in COS1 cells, myc-PER2 and CK1ε were co-transfected with FLAG-tagged β-TrCP1, β-TrCP2, β-TrCP1ΔF or β-TrCP2ΔF. Empty FLAG vector was used as a control. Twenty hour after the transfection, cycloheximide was added (20 nM) and incubated for 0, 3, 6 or 9 h before harvesting. After a brief rinse in PBS, cells were dissolved in sampling buffer. Samples with the same amount of cell were separated in 5.5% SDS polyacrylamide gels, and the amount of PER2 was detected by western blotting analysis with anti-myc antibody.

For PER2 degradation analysis in NIH3T3 cells, full length of rat Per2 was fused to N-terminal region of luciferase driven under SV40 promoter (Luc::rPER2). Luc::rPER2 reporters, CK1ε and phRL-TK were co-transfected and cultured more than 48 h. To stop the protein synthesis, cycloheximide (20 nM) was added and incubated for 0, 2, 4 or 6 h before harvesting. After a brief rinse in PBS, cells were lysed and dual luciferase assay was performed (Promega). Luminescents untreated with cycloheximide (0 h) were set to 100 and the percent stability after the inhibition of protein synthesis was described. Experiments were repeated for more than three times.

Western Blotting Analysis—The antibody was diluted as follows: anti-FLAG (Sigma) 1 : 5000, anti-myc (Roche) 1 : 3000, anti-GFP (Clontech) 1 : 1000.

Real-time Monitoring System of Circadian Clock—Real-time luciferase was essentially described previously (*5*). Reporter construct of mouse *Per2* promoter in which luciferase was conjugated with destabilization motif, PEST, (*mPer2*-Luc-PEST) was provided by Dr Y. Nakajima. Reporter construct and siRNA were co-transfected by PolyFect (Qiagen) to NIH3T3 cells. The concentration of siRNA was set to 20 nM, and Fluorescent Oligo (Invitrogen) was used as a control. Twenty-four hour after the transfection, NIH3T3 was stimulated with 100 nM synthetic glucocorticoid [dexamethasone(Dex)] for 2 h to reinitiate the circadian rhythm. Real-time monitoring of fluorescent emission was performed by Kronos (ATTO) that count the fluorescence for 1 min in every 10 min. To ensure the health condition, the cells were re-stimulated by Dex 6 days after the first stimulation, and we observed the reinstated emission peak in siRNA-treated and control cells (data not shown).

For *smoothed* data, date from 2 h average was plotted. To measure the amplitude of the rhythm, *detrended* data was acquired by subtraction of smoothed data from 24 h average. Amplitudes between peak and trough were compared while amplitude of control siRNA was set to 100% in every circadian peak. Experiments were repeated six times.

Evaluation of FWD1a and FWD1b si-RNA—Validated β-TrCP1 siRNA (Btrc-MS202454) was purchased from Invitrogen Inc. β-TrCP2 siRNA was designed by Dr M. Miyagishi (Tokyo University). 5′-GUAAUGGAACGUCAU CUGUGAUUGU-3′ and 5′-ACAAUCACAGAUGACGUU CCAUUAC-3′, the double-stranded Stealth™ siRNAs were synthesized (Invitrogen). Fluorescent Oligo (Invitrogen) was used as a negative control. Around 10 or 20 nM of siRNA were transfected in mouse cells by X-treamGENE siRNA transfection reagent (Roche). Forty-eight hours after the transfection, specific inhibition of endogenous mRNA was checked by RT–PCR with primer pairs of GAPDH, β-TrCP1 and β-TrCP2. Primers for β-TrCP1 were 5′-GCGAACCCCCTAGGAAGATA-3′ and 5′-GCTGGCAGTGCAGTTATGAA, and for β-TrCP2 were 5′-CATGCCCAGTGTCAGATGTC-3′ and 5′-CTGGC ATCCAGGTAGGAGAG-3′. The primer pair amplified full length and alternative spliced form that skipped exon3 of

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β-TrCP2 (Supplementary Fig. S1A). Both the forms of β-TrCPs were continuously expressed in all circadian times (data not shown). COS1 cells in which FLAG–β-TrCP1 or FLAG–β-TrCP2 were overexpressed were transfected with si-RNAs. Specific inhibition of β-TrCP1 and β-TrCP2 protein synthesis was checked by anti-FLAG antibody (Supplementary Fig. S1B).

Knockout Mice of β-TrCP1—β-TrCP1 knockout mice has been reported previously (*28*).

The knockout mouse was crossed with C57/BL6. The heterozygote offspring were intercrossed to get the siblings of wild-type, β-TrCP1 heterozygote and β-TrCP1 homozygote mice for the analysis. For the behaviour analysis, the male mice of 2- to 6-months old were caged individually with food and water available *ad libitum*. Drinking behaviour was recorded for period and phase-shift analysis, and then was analysed by Chronobiology Kit Analysis (Standard Software Systems). After being accustomed in constand light conditions (LD) (ZT0: 8:00 a.m., light on; ZT10: 20:00 p.m., light off), mouse was transferred to constant dark conditions (DD) as the light was kept off after ZT0 (CT0). The first of DD condition, onset time was measured as onset phase angle to see the reactions to the darkness. The following 2 weeks after DD condition without the first and second days were used for period analysis.

To see the phase-shift response to light, Achoff type-II analysis was performed. Mice entrained to LD conditions were stimulated by light for 4 h: ZT8–12, ZT12–16 or ZT20–24. Then they were let for free-runs in DD conditions. The 4 h of light stimulation is effective for the phase-shift analysis. The animals were treated as approved by Institution of Animal Care and Use Committee of AIST for the ethical use of experimental animals.

Immunohistochemistry—Mice were housed individually while monitoring drinking locomotion. Mice were kept in DD for more than a week before sampling. CT was estimated by its locomotion. The method of immunohistochemistry has been described previously (*29*). The mouse brain at CT4, CT16 or ZT16 was freshly dissected and frozen on dry ice. Frontal SCN slice was made by cryostat of 12 μm thick section, attached to silanized slides. Specimen was fixed in 10% formalin; first anti-PER2 antibody (ADI) was diluted 1 : 800; enhanced by ABC kit (Vector); developed in DAB and H_2O_2.

RESULTS

Phosphorylated PER2 is the Degradation Target of β-TrCP1 and β-TrCP2—To examine the effect of β-TrCP1 and β-TrCP2 on PER2 protein stability or degradation, PER2, CK1ε and β-TrCP were coexpressed in COS1 cells and then the expression of PER2 was examined by western blotting analysis after the interruption of protein synthesis with administration of cycloheximide. The expression level of phosphorylated PER2 was decreased by coexpression of β-TrCP1 or β-TrCP2. The result indicated that both β-TrCP1 and β-TrCP2 targeted to phosphorylated form of PER2. The idea was further examined by the dominant negative form of β-TrCPs; β-TrCP1ΔF and β-TrCP2ΔF cannot interact with ubiquitin E3 component Skp1. As expected, the expression of β-TrCP1ΔF or β-TrCP2ΔF led to accumulation of phosphorylated PER2 compared to unphosphorylated form of PER2 (Fig. 1).

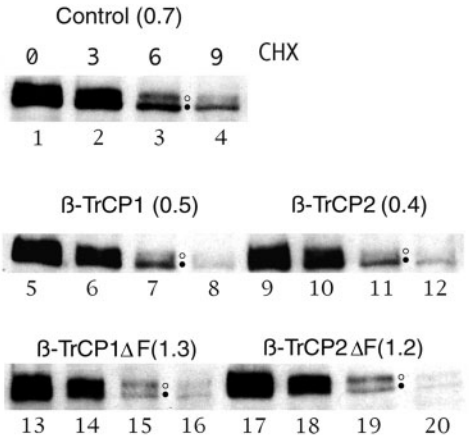

Fig. 1. **Phosphorylated PER2 is the target of β-TrCP1 and β-TrCP2**. Myc-PER2, CK1ε and FLAG–β-TrCPs were co-transfected in COS1. Empty FLAG vector was used as a control. Cycloheximide (CHX) was added as indicating hours before harvesting. PER2 was detected by anti-myc antibody by western blotting analysis. Numbers in parentheses indicate the ratios of phosphorylated form of PER2 (open circle) to unphosphorylated form of PER2 (closed circle). Compared to the control, phosphorylated PER2 is less abundant when β-TrCP1 or β-TrCP2 are over expressed. Phosphorylated PER2 excess the unphosphorylated PER2 when dominant negative β-TrCP1ΔF or β-TrCP2ΔF are expressed.

Identification of the β-TrCP Binding Site in PER2—There were several candidate sites in PER2 protein being recognized by β-TrCP proteins (Fig. 2A), although the typical β-TrCP1 consensus binding motif (DpSGXXpS; pS represents candidate serine-phosphorylation site) did not exist. We especially focused on two sites. One site was 92-TpSGCSpS-97, because this sequence was conserved between PER1 and PER2, and β-TrCP1 and β-TrCP2 were reported to associate with PER1 through this site (*19*). The other site was 477-SpSGYGpS-482, since 477S and 497G is reported to be essential for PER2 to associate with β-TrCP1 (*20*). We mutated the candidate serine-phosphorylation sites (m1: S93, S97; m2: S478, S482). Immunoprecipitation assay revealed direct association of PER2 with β-TrCP1 and β-TrCP2 (Fig. 2B). The bindings of β-TrCP1 and β-TrCP2 to PER2 were abrogated by m2 mutation (Fig. 2C, lanes 3 and 7). Thus, the β-TrCP1 binding site of PER2 is also functional for the β-TrCP2 binding. On the other hand, the mutation of m1, which was conserved between β-TrCPs binding site of PER1 had little effect for the binding (Fig. 2C, lanes 2 and 6). PER2 protein without CK1ε overexpression could associate with β-TrCP1 and β-TrCP2 too (Fig. 2B, lanes 2 and 7). We assumed that these bindings were due to phosphorylation of PER2 by endogenous kinases within NIH3T3.

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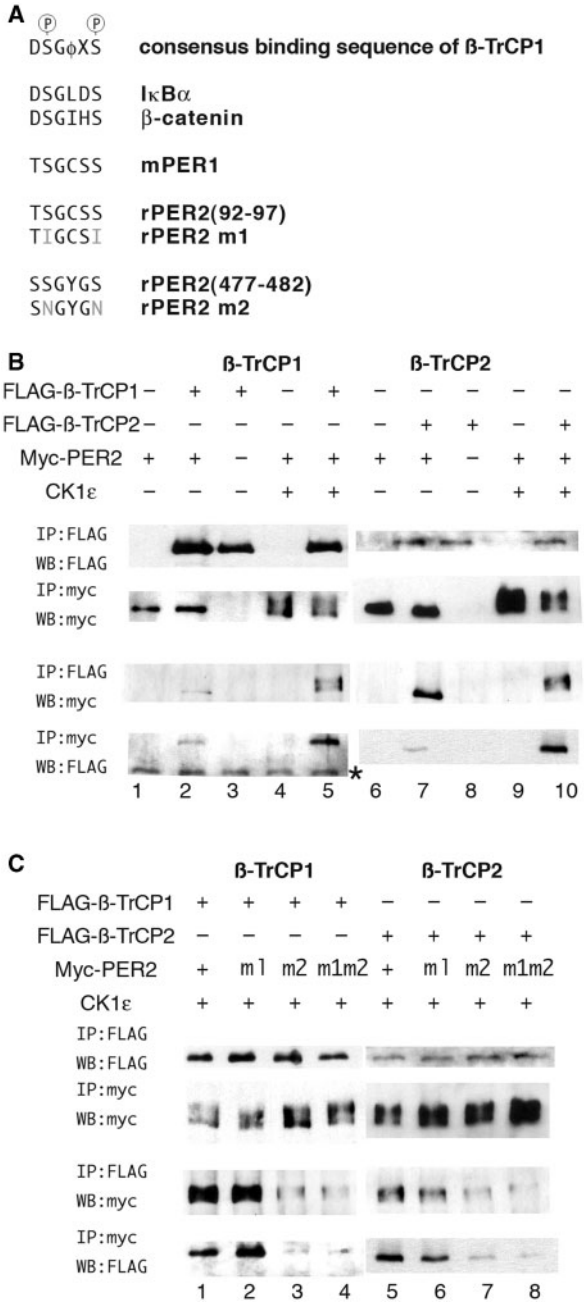

Fig. 2. **β-TrCP1 and β-TrCP2 associate with PER2.** Interaction of PER2 with β-TrCP1 or β-TrCP2 was analysed by immunoprecipitation. (A) Candidates of rat PER2 (rPER2) phospho-degron site, m1 and m2, being recognized by FWD1 proteins. Sequence motifs of other genes recognized by β-TrCP1 are also shown. (B) β-TrCP1 and β-TrCP2 can interact with PER2. PER2 is phosphorylated by CK1ε and shifted to upper position making a broader band (compare lanes 1, 2 and 4, 5). β-TrCP1 and β-TrCP2 can associate with phosphorylated form of PER2 and vice versa (lanes 5 and 10). Association of PER2 with β-TrCP1 and β-TrCP2 without CK1ε is also seen (lanes 2 and 7). Non-specific binding of anti-FLAG antibody is marked by asterisk. (C) Binding of β-TrCP1 and β-TrCP2 to PER2 depend on the target sequence. Mutation m2 abolish most of the binding to both β-TrCP1 and β-TrCP2, and vice versa. On the other m1 mutation has less effect compared to the intact PER2.

We tried to examine the stability of PER2 protein in clock gene oscillating cells by luciferase–PER2 fusion system (*25*, *30*). Luciferase–PER2 fusion reporter (Luc::rPER2, Fig. 3A) was transfected to NIH3T3 cells, and then bioluminescence was monitored to measure the amount of the protein after interruption of protein synthesis by cycloheximide treatment. The luminescent blunted quickly when a protein degradation motif (degron), PEST, was fused to the luciferase (Luc::PEST), indicating that luciferase fusion system could be utilized for the search of degron motif. The luminescent was decreased rapidly when full length of rat PER2 was fused, implying that destruction motif in PER2 could destabilize Luc::rPER2. The mutation of the putative β-TrCP target site of PER2, m2, increased the stability of Luc::rPER2 protein, although the m1 mutation had little effect, if any, on the stability (Fig. 3B). The stability of Luc::rPER2 m1m2 was as stable as luciferase control. These results indicated that m2 site that was essential for binding of β-TrCPs (Fig. 2C) was critical for the destabilization of PER2 (Fig. 3B).

β-TrCP1 and β-TrCP2 are Essential for the Robust Oscillation of the Circadian Clock—To examine the role of β-TrCP1 and β-TrCP2 in circadian rhythm generation, rhythmic expression of *mPer2* were monitored by *mPer2*-Luc reporter in real time. The reporter construct and siRNA (Supplementary Fig. S1) were simultaneously transfected to NIH3T3, then the cells were stimulated with synthetic glucocorticoid, Dex, to synchronize the rhythm. The level of *mPer2*–Luc activity during the oscillation was reduced by suppression of β-TrCP1, β-TrCP2 and both genes (Fig. 4A). The activity was reduced by administration of β-TrCP1, β-TrCP2 or both siRNAs (Fig. 4A graph). A total of 36 circadian amplitude of *mPer2* from six cases were analysed. The amplitude was reduced compared to the control by suppression of β-TrCP1, β-TrCP2 or both genes (Fig. 4B). The reduction of the amplitude was statistically significant in β-TrCP1, β-TrCP2 or both siRNAs. The reduction was $74.7 \pm 14.7\%$ in β-TrCP1 siRNA, $55.0 \pm 24\%$ in β-TrCP2 siRNA and $37.7 \pm 9.3\%$ in both β-TrCPs. The dampened circadian oscillation could be reinstated by re-stimulation of Dex (data not shown), indicating that the results were not due to the impairment of cell viability. The period lengths of *mPer2*–Luc were 22.98 ± 0.53 h (mean ± SEM, $n = 19$) in controls, 23.39 ± 0.56 h in β-TrCP1 siRNA, 23.05 ± 0.64 h in β-TrCP2 siRNA and 23.27 ± 0.73 h in both β-TrCPs (Fig. 4C). The differences of the period lengths were not significant between control and β-TrCP siRNAs by analysis of variance.

We studied the intracellular localization of β-TrCP2 in NIH3T3 cells by using GFP–β-TrCP2 conjugate. The main expression of GFP–β-TrCP2 was in cytosol, while in some cells, the expressions were in nucleus (Supplementary Fig. S4).

The Circadian Locomotor Behaviour is not Affected in β-TrCP1-Deficient Mice—β-TrCP1 mRNAs were ubiquitously expressed in adult mice tissues, including the SCNs (data not shown). The β-TrCP1 homozygote mice were reported to be fertile (*28*). To determine the role of β-TrCP1, we monitored circadian drinking behaviour. β-TrCP1 homozygote and β-TrCP1 heterozygote mice

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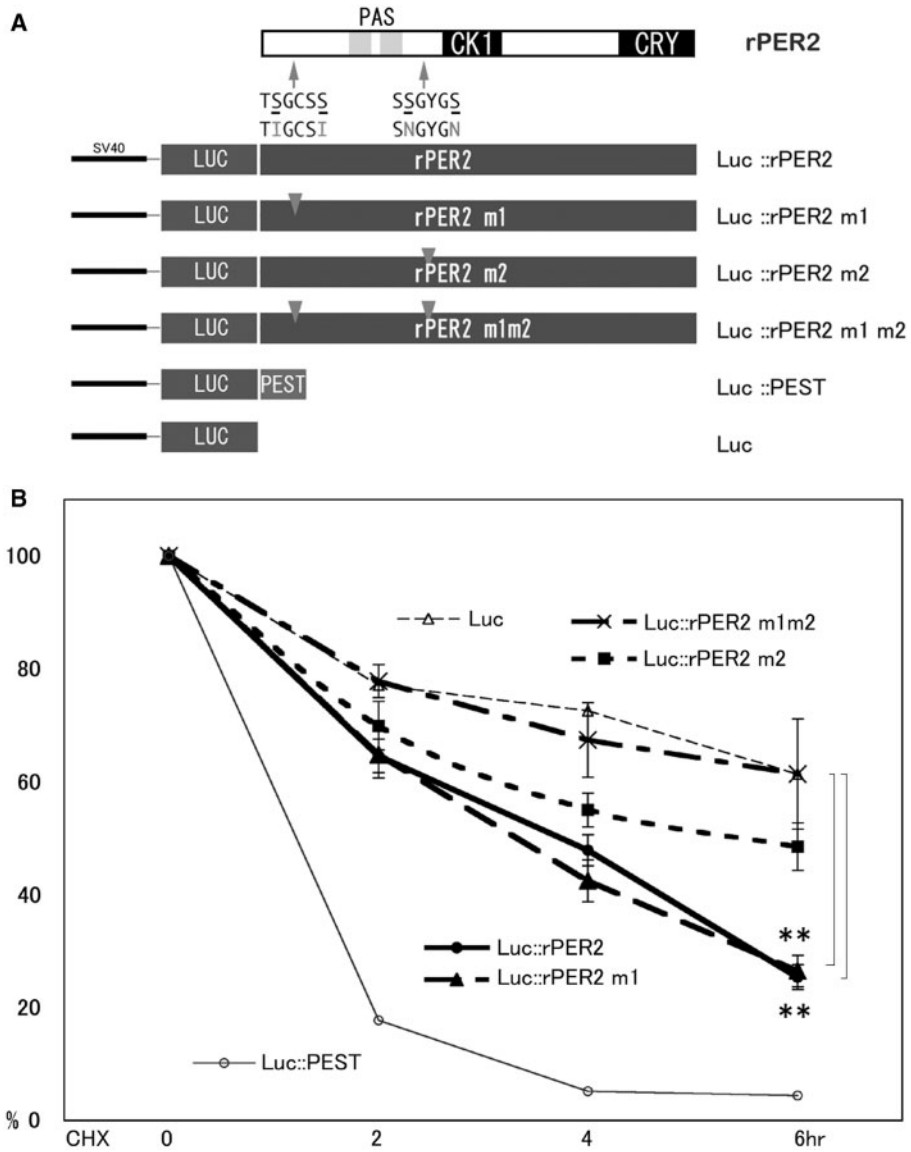

Fig. 3. **Destabilization site of PER2 is sequence specific**. (A) Reporter constructs used for the degradation analysis in NIH3T3 cell. Luciferase under SV40 promoter was used as a control. Luc::rPER2 is a conjugate of luciferase and full length of rat PER2 under SV40 promoter. Luc::PEST is for evaluation of this system. Mutations in PER2, m1 and m2, are the same as in Fig. 2. (B) Luminescents without the inhibition of protein synthesis (0 h) were set to 100%. The Student's *t*-test reveals that the stability of Luc::rPER2 and Luc::rPER2 m1 were statistically different from Luc::PER2 m1m2. $P < 0.01$. The difference between Luc:rPER2 m2 and Luc::rPER2 m1m2 was not significant. Error bars represent SEM.

were normally entrained to an environmental LD cycle as wild-type siblings. Onset phase angle on the first day of DD was calculated to see the dark response. The onsets were all normal among siblings (Fig. 5A). Circadian period of wild-type, β-TrCP1 heterozygote and β-TrCP1 homozygote knockout mice were identical among siblings, 23.79 ± 0.10 h (mean ± SEM, $n = 7$); 23.76 ± 0.03 h, $n = 10$; 23.80 ± 0.03 h, $n = 8$ (Fig. 5C), with robust circadian behaviour (Fig. 5B).

To determine light-induced phase shift, Aschoff type II was performed; mice maintained to LD were stimulated by light for 4 h before release into DD. Four hours of light administration at ZT12–16 (ExT12–16) caused a phase delay (Supplementary Fig. S2A). Exposure to light at ZT20–24 (ExT20–24) caused a phase advance (Supplementary Fig. S2B). Light at ZT8–12 (ExT8–12) on dead zone time had no effect (Supplementary Fig. S2C). The phase delays and phase advances in the activity rhythm were normal in β-TrCP1-deficient mice (Supplementary Fig. S2D and E).

Immunohistochemistry of PER2 proteins in SCN revealed the expression of PER2 at ZT16, CT16 or CT4 in SCN was oscillating normally in β-TrCP1 heterozygote and in the homozygote mice (Supplementary Fig. S3). The normal expression of PER2 in SCN of β-TrCP1-deficient mice might be reflected by normal circadian

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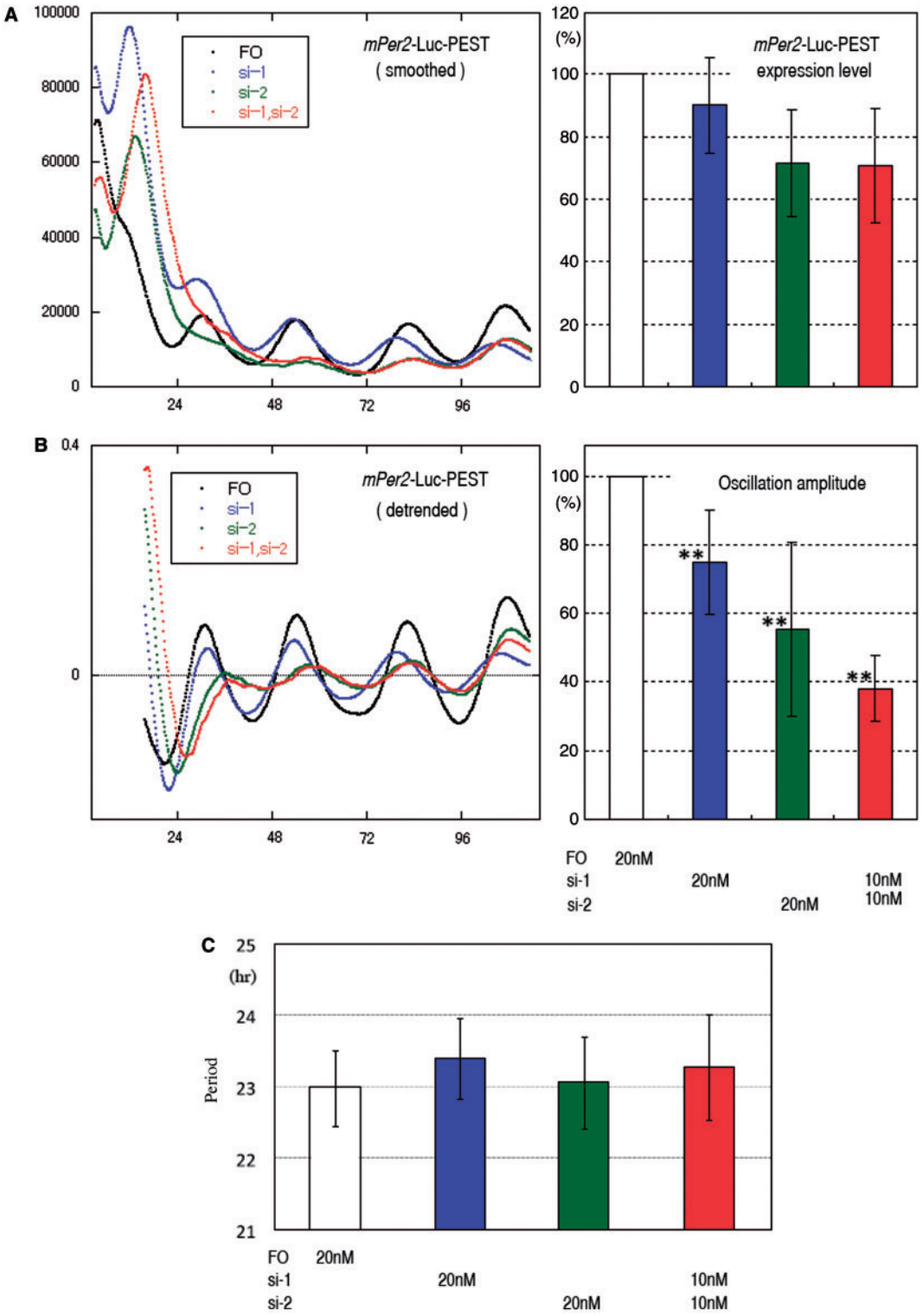

Fig. 4. **Suppression of β-TrCP1 and β-TrCP2 affect the circadian rhythm**. mRNA rhythm of *mPer2* and *mBmal1* was measured in NIH3T3 using *mPer2*-Luc-PEST reporter. Circadian rhythm was synchronized by stimulation of Dex for 2 h, and then the medium containing luciferin was replaced. (A) One example of smoothed data of *mPer2*-Luc expression. Experiences were repeated by six times, and the expression levels of Per2 in the third circadian peaks were compared (right graph). (B) Plot of detrended data (see MATERIAL AND METHODS section). Total 36 of circadian amplitudes, peak–trough substractions, from the six cases was calculated. Control amplitude was set to 100% (right graph). The amplification reduced by β-TrCPs siRNA treatments. **: $P < 0.01$. (C) Circadial period lengths of *mPer2*-Luc-PEST reporter. The differences are not significant by analysis of variance. Concentrations of siRNA are shown at the bottom of graph. Error bars in graphs represent SEM. FO: Fluorescent Oligo, control si-RNA; si-1: β-TrCP1 siRNA; si-2: β-TrCP2 siRNA.

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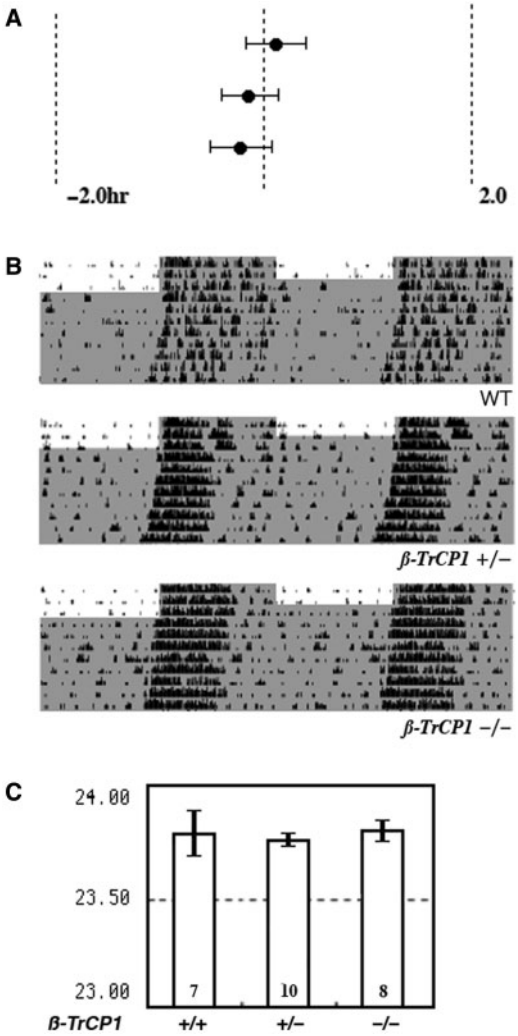

Fig. 5. **Locomotion period of knockout mice.** Locomotion 2 weeks after the transfer to DD condition were used to period Chin square analysis. (A) Onset on the first day in DD condition. Light responses are indistinguishable among mice. (B) Examples of drinking behaviour in double plot. Siblings of wild-type, β-TrCP1 heterozygote and the homozygote knockout mice are shown. (C) Period length does not differ between the control siblings and the knockout siblings. Numbers in the bar indicate the experimental number of mice. Error bars represent SEM.

behaviours. Thus, β-TrCP1 is dispensable for circadian rhythm generation *in vivo*. The results may imply more important role of β-TrCP2 in mice.

DISCUSSION

In the present study, we showed that both β-TrCP1 and β-TrCP2 are involved in the molecular clock by the direct association with PER2 proteins. We demonstrated that the transient knockdown of β-TrCP1 and β-TrCP2 continuously decreased expression levels of *mPer2* in culture cells. The reduced *Per2* transcription is provably caused by accumulated PER2 protein that associates the heterodimer, CLOCK and BMAL1, to suppress the transcription of *Per2*. It should be noted that the suppression of β-TrCPs severely dampened the clock gene transcriptional oscillations, suggesting that β-TrCP-mediated protein degradation is essential for the transcription and translation-based feedback loop of the mammalian circadian clock as well as that of the *Drosophila* (*12*, *13*). The questions still remain if PER2 is solely responsible for the attenuation of the rhythm. β-TrCP1 proteins also degrade clock protein, PER1, at least (*19*). There may be more target clock proteins other than PER1 and PER2 that associate with β-TrCP1 and/or β-TrCP2 essential for rhythm generations. Down-regulation of β-TrCP1 and expression of dominant negative β-TrCP1 is reported to lengthen circadian rhythm in culture cells (*31*). In the present study, the knockdown effects of β-TrCPs on period length were not significantly different. The discrepancy probably comes from the different effects on the circadian amplitude of these studies, because the peak–trough amplitude of *mPer2*–Luc activity was severely dampened in the present study but not significant in the previous study (*31*). The results together with previous study (*31*) indicate that both β-TrCP1 and β-TrCP2 are involved in circadian rhythm generation in culture cells.

β-TrCP1 locates in nucleus by binding to pseudo-substrate hnRNP-U, while β-TrCP2 locates only in cytosol in HeLa cells (*32*). By utilizing GFP–β-TrCP2 fusion protein, we found that about 60% of β-TrCP2 restricted in cytosol regardless in circadian time in NIH3T3 cell (Supplementary Fig. S4). Since PER2 is expressed both in nucleus and in cytosol, the importance of β-TrCP2 in circadian rhythm generation may due to different intracellular expression patterns. PER and CRY proteins are known to form a complex that is transferred to the nucleus (*33*, *34*), and the complex is resistant to ubiquitin-dependent degradation (*35*). This is also supported by the finding that FASPS mutation that disrupts CRY1 binding leads to destabiization of PER2 in nucleus (*36*). Thus, the main target of PER2 degradation is in cytosol where β-TrCP2 but not β-TrCP1 is abundant.

In addition to the difference of intracellular localization, there may be some fundamental difference between β-TrCP1 and β-TrCP2. For example, β-catenin and hnRNP-U exclusively associate with β-TrCP1, and PRLR is exclusively degraded by β-TrCP2 (*32*, *37*, *38*), whereas both β-TrCP1 and β-TrCP2 share many similar target proteins [such as IkBα (*27*, *39*), NF-κB1 (*40*), CDC25A (*41*), PER1 (*19*) and PER2 (*20*), and this study]. Our *in vitro* study showed that both β-TrCP1 and β-TrCP2 associated with PER2, but the role of the two genes may not be equal in circadian rhythm generations because of the different intracellular location or different target.

Phosphorylation is a key step for the degradation of PER proteins (*23*, *24*, *42*, *43*). Mutation of PER2 S662 that is phosphorylated by CK1 (*36*, *44*) causes familial advanced syndrome (FASPA) in human (*45*). Mutation of CK1ε and CK1δ also lead to shortened period and phase advance in hamster and human (*46*, *47*). We have shown that phosphorylation is essential for association of PER2 and β-TrCP proteins through m2 site that is different

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from FASPS region. M2 site is not the target of CK1δ *in vitro* (*48*). It is unclear whether CK1ε directly phosphorylates m2 site or other kinase is involved. In our study, the degradation speed of PER2 in dominant negative β-TrCP expressing COS1 cell seemed faster than that in the control cells. One possible underlying mechanism of this phenomenon is that dominant negative form of β-TrCPs inhibit protein phosphatase 1 (PP1). The m2 site might be targeted by PP1 that stabilize PER2 protein (*49*). β-TrCPs binding site of PER1 is reported (*19*) Since this site was conserved between PER1 and PER2, we studied if this PER2 site (m1) is also important for the stabilization. In our study, mutation of PER2 m1 site did not abolish binding of β-TrCP1 or β-TrCP2 (Fig. 2C) nor changed the stability of PER2 (Fig. 3). On the other hand, mutation of the three amino acids (T1: T92A, S93A, S97A) is reported to stabilize PER2 protein and overexpression of T1 using Flp-In system leading to disruption of circadian rhythm (*31*). The difference may be due to the different method. They found that T1 protein is stably expressed in NIH3T3 cell line, while we studied the decay of luciferase using Luc::PER2 fusion constructs. PER2 destabilization site is reported, which is the target of β-TrCP1 (*20*). In our immunoprecipitation assay, this m2 site was essential for the binding of β-TrCP2 as well as β-TrCP1.

F-box protein Fbxl3 is reported to be essential for robustness of circadian rhythm by mediating degradation of CRY proteins (*16–18*). Fbxl3 might be indirectly involved in regulating the stability of PER2. Because PER2 associates with CRY proteins (*34*), the complex becomes resistant to ubiquitin-dependent degradation (*35*). Thus, CRY proteins possibly regulate cyclic degradation of PER2 proteins, although recent reports indicate that binding of PER2 and CRY proteins is not essential for cyclic expression of PER2 protein (*26*), and cyclic expression of CRY proteins are unnecessary for circadian clock function (*50*).

Our results represent that β-TrCP1 and β-TrCP2 are involved in circadian rhythm generation in culture cells. Important role of β-TrCP1, whereas β-TrCP1 is dispensable for sustaining circadian locomotor behaviour. The discrepancy of β-TrCP1 effect between culture-based rhythm generation and circadian behaviour *in vivo* might be due to the difference of peripheral clocks and master clock located in the SCN. Although circadian clock system is cell-sustained molecular mechanism that is persisting in many different tissues and culture cells, there are fundamental differences of the circadian clock between SCN and culture cells (*9*, *51*). For example, *Clock* gene is indispensable for the oscillation of peripheral clocks but not for that in the SCN, as CLOCK and NPAS2 have redundant functions in the SCN but not in other tissues (*52*). In β-TrCP1 homozygote deficient mice, other proteolysis mechanisms, such as β-TrCP2 and Fbxl3 may substitute the role for sustaining the circadian rhythm generations. Conditional knockout of β-TrCP2 and/or double knockout of β-TrCP1 and Fbxl3 might reveal the important role β-TrCP1 and β-TrCP2 for circadian rhythm generations *in vivo* (*29*).

SUPPLEMENTARY DATA

Supplementary data are available at *JB* online.

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CONFLICT OF INTEREST

None declared.

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