Rhythmic control of mRNA stability modulates circadian amplitude of mouse Period3 mRNA

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Abstract
The daily oscillations observed in most living organisms are endogenously generated with a period of 24 h, and the underlying structure of periodic oscillation is an autoregulatory transcription-translation feedback loop. The mechanisms of untranslated region (UTR)-mediated post-transcriptional regulation (e.g., mRNA degradation and internal ribosomal entry site (IRES)-mediated translation) have been suggested to fine-tune the expression of clock genes. Mouse Period3 (mPer3) is one of the paralogs of Period gene and its function is important in peripheral clocks and sleep physiology. mPer3 mRNA displays a circadian oscillation as well as a circadian phase-dependent stability, while the stability regulators still remain unknown. In this study, we identify three proteins – heterogeneous nuclear ribonucleoprotein (hnRNP) K, polypyrimidine tract-binding protein (PTB), and hnRNP D – that bind to mPer3 mRNA 3’-UTR. We show that hnRNP K is a stabilizer that increases the amplitude of circadian mPer3 mRNA oscillation and hnRNP D is a destabilizer that decreases it, while PTB exhibits no effect on mPer3 mRNA expression. Our experiments describe their cytoplasmic roles for the mRNA stability regulation and the circadian amplitude formation. Moreover, our mathematical model suggests a mechanism through which post-transcriptional mRNA stability modulation provides not only the flexibility of oscillation amplitude, but also the robustness of the period and the phase for circadian mPer3 expression.

Keywords: circadian rhythm, post-transcriptional regulation, period, untranslated region, mathematical model.


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A wide range of living organisms from bacteria to humans have the endogenous clock, which governs most of the physiological processes and is necessary for efficient energy metabolism (Reppert and Weaver 2002; Bass and Takahashi 2010; Albrecht 2012). Since the period of the clock is about the length of a day (24 h), it is called a circadian clock. This regular rhythmicity is well known to be an endogenous and self-sustained oscillation. The fundamental molecular structure of biological clocks is a negative feedback loop, and several studies have been performed at transcriptional and post-translational levels to elucidate its function. In mammals, BMAL1 and CLOCK form a heterodimer and induce the synthesis of transcriptional repressor mRNAs that have E-boxes in the promoters of their genes, such as Period (Per), Cryptochrome (Cry), and Rev-erb α (Gekakis et al. 1998; Jin et al. 1999; Kume et al. 1999; Preitner et al. 2002). The repressor proteins PER and CRY form a heterodimer and repress the activity of BMAL1/CLOCK (Sangoram et al. 1998; Griffin et al. 1999), and REV-ERB α represses Bmal1 transcription (Ueda et al. 2002; Guiquimond et al. 2005). Moreover, several post-translational regulators, such as casein kinase 1 δ/ε (Akashi et al. 2002; Lee et al. 2009), protein phosphatase 1 (Lee et al. 2011a; Schmutz et al. 2011), sirtuin 1 (SIRT1) (Asher et al. 2008; Nakahata et al. 2008), F-box protein β-transducin repeat-containing protein (Shirogane et al. 2005; Ohsaki et al. 2008), F-box/LRR-repeat protein 3 (FBXL3) (Busina et al. 2007; Siepka et al. 2007), and FBXL21 (Dardente et al. 2008; Hirano et al. 2013; Yoo et al. 2013) have been identified for their roles in the regulation of clock proteins and alterations of behavior.

Besides transcriptional and post-translational regulation, recent studies in a genome-wide scale reveal that other types of gene regulation may have roles in the rhythmic expression of clock genes (Koike et al. 2012; Menet et al. 2012; Morf et al. 2012). These include mRNA polyadenylation (Kojima et al. 2012), RNA-methylation-dependent RNA processing (Sanchez et al. 2010), and mRNA untranslated region (UTR)-mediated post-transcriptional regulation, such as mRNA degradation via the 3’-UTR, internal ribosomal entry site (IRES)-mediated translation via the 5’-UTR, and 3’-UTR-mediated translational regulation (Kojima et al. 2007). Several destabilizer proteins for clock gene mRNAs have been identified. These proteins are thought to accelerate the degradation of clock mRNAs and decrease their amplitude of circadian oscillation (Kim et al. 2005; Woo et al. 2008, 2010). Other proteins, identified as IRES trans-acting factors (ITAFs), enhance translation in a cap-independent manner. They trigger IRES-mediated translation of clock genes, as well as one tumor-suppressor gene, p53 (Kim et al. 2007, 2010, 2013; Lee et al. 2011b). In addition to RNA-binding proteins, microRNAs have also been suggested as post-transcriptional regulators that control circadian rhythms (Cheng et al. 2007; Chen et al. 2013; Lee et al. 2013; Du et al. 2014).

In mammals, Per gene is widely expressed throughout every organ. All of the three paralogs, Per1, Per2, and Per3, display typical circadian mRNA oscillations and Per1 and Per2 are essential in the negative feedback loop via formation of heterodimer with Cry1 and Cry2. However, they differentially function in various tissues. Per1 and Per2 are indispensable in the suprachiasmatic nucleus, a master clock located in the brain (Bae et al. 2001), while Per3 is important for the period and the phase of peripheral clocks located in pituitary, lung, adrenals, and so forth (Pendegast et al. 2012). The expression of Per1 is entrained by external photic stimuli through the retinohypothalamic tract (RHT)-cyclic adenosine monophosphate (cAMP) pathway and by the BMAL1/CLOCK-mediated modulation through the auto-regulatory transcriptional feedback loop (Wilsbacher et al. 2002). In contrast, the expression of Per3 is influenced only by the latter (Albrecht et al. 2001; Travnickova-Bendova et al. 2002). Furthermore, it is known that the polymorphism in Per3 gene is related to the diurnal preference (Parsons et al. 2014), sleep homeostasis (Hasan et al. 2014), and delayed sleep phase syndrome (DSPS) (Ebisawa et al. 2001).

Despite the importance in gene expression and sleep physiology, little is known about how Per3 mRNA is regulated. Additionally, it remains unclear how circadian oscillation of Per3 mRNA is affected through the regulation of its stability compared to Per1 and Per2 (Woo et al. 2008). Recent studies demonstrated that 3’-UTR-mediated mRNA degradation is essential for mouse Per3 (mPer3) mRNA oscillation (Kwak et al. 2006). Specifically, heterogeneous nuclear ribonucleoprotein (hnRNP) Q was identified as a modulator for 5’- and 3’-UTR-mediated phase-dependent translation, which is coupled to mPer3 mRNA decay (Kim et al. 2011). Nonetheless, there is no evidence of trans-acting factors that bind directly to the cis-acting element in the 3’-UTR of mPer3 mRNA and lead to mRNA decay.

The theoretical analysis using mathematical models has been widely used to analyze the complex phenomena in biological systems. Mathematical models provide theoretical insight and systemic understanding with less need for further experiments. Various mathematical models have been designed to simulate a range of biological systems from a single cell to a tissue. The latter models consist of highly interconnected neurons and are used to describe circadian oscillations in bacteria, flies, and mammals (Leloup et al. 1999; Forger and Peskin 2003; Leloup and Goldbeter 2004; Bernard et al. 2007). Although they could explain the robustness of 24-h-period against various external perturbations, few of them except a recent study, describing nonsense-mediated decay (NMD) by auto-regulated RNA-binding protein in the circadian rhythm of plants (Schmal et al. 2013), include post-transcriptional effects despite significant experimental evidence that post-transcriptional controls contribute to clock mRNA oscillations. Furthermore, most of the mathematical models describing circadian rhythms lack direct correspondence to biological experiments.
Here, we identify trans-acting factors that contribute to 3′-UTR-mediated stability regulation of mPer3 mRNA. We show that the binding of a stabilizer or a destabilizer to mPer3 3′-UTR influences its stability and oscillation profile. Moreover, with the help of a mathematical model based on our experimental results, we demonstrate that the modulation of mRNA stability by 3′-UTR-binding proteins is critical for the amplitude of circadian mPer3 mRNA oscillation.

Methods

Plasmids
The pcNAT reporter containing full-length mPer3 3′-UTR and pSK vectors containing serially deleted mPer3 3′-UTR for in vitro transcription were used as described previously (Kwak et al. 2006). Each of UTR-containing constructs was inserted into the EcoRI/Xhol restriction enzyme site and linearized by digestion with Xhol. For purification of Glutathione S-transferase (GST)-tagged recombinant proteins, the coding regions of mouse hnRNP K and polypyrimidine tract-binding protein (PTB) isoform 1 and 2 were amplified and digested with SalI/NorI, and cloned into SalI/NorI sites of pGEX-4T-3 vector (GE Healthcare Life Sciences, Uppsala, Sweden).

Cell culture and dexamethasone treatment
NIH-3T3, Neuro2A, and HEK293A cells were maintained in Dulbecco’s modified Eagle’s medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (HyClone) and 1% penicillin–streptomycin (Welgene, Daegu, Republic of Korea) in a humidified atmosphere containing 5% CO2 at 37°C. The circadian phase of NIH-3T3 cells was synchronized by 100 nM 7.6), 75 mM KCl, 2 mM MgCl2, 0.1 mM EDTA, 4% glycerol, 20 U recombinant RNasin ribonuclease inhibitor (Promega), and 4 µg of tRNA for 20 min at 30°C. After incubation, the mixtures were UV irradiated on ice for 15 min with a CL-1000 UV-crosslinker (UVP, Upland, CA, USA), and unbound RNAs were digested with 5 µL RNase cocktail [2.5 µL of RNase A (100 mg/mL; Roche Diagnostics, Indianapolis, IN, USA) and 2.5 µL of RNase T1 (100 U/mL; Roche Diagnostics)] for 50 min at 37°C. UV cross-linked proteins were analyzed by autoradiography after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For immunoprecipitation, RNase-digested lysates were incubated with IgG or specific antibodies for 16 h at 4°C. After incubation, protein G agarose beads (Roche Diagnostics) were added and further incubated for 3 h at 4°C. Washed beads were analyzed by SDS-PAGE and autoradiography. For streptavidin-biotin RNA affinity purification, biotin-UTP-labeled RNA was transcribed from the Xhol-linearized pSK-mPer3 3′-UTR plasmid using T7 RNA polymerase. About 1 µg of pre-cleared cytoplasmic extracts from NIH-3T3 cells were incubated with biotinylated-mPer3 3′-UTR RNAs in a dialysis buffer containing 10 mM HEPES (pH 7.4), 90 mM KOAc, 1.5 mM MgOAc, 2.5 mM DTT, and tRNA for 30 min at 23°C. The binding mixtures were subjected to streptavidin resin adsorption for 16 h at 4°C. Resin-bound proteins were analyzed by SDS-PAGE.

DNA-immunoprecipitation
Immunoprecipitation was performed with a buffer containing 125 mM KCl, 20 mM HEPES (pH 7.4), 0.5 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.05% NP-40, and Rnasin. About 1 µg of pre-immune serum or specific antibodies were incubated with RNase-free lysates for 2 h at 4°C. After incubation, 50 µL of protein G agarose beads was added and further incubated 16 h at 4°C. Washed beads were analyzed by SDS-PAGE followed by immunoblot or subjected to RNA isolation using TRI reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer’s instructions.

Transient transfection and RNA interference
For siRNA transfection into NIH-3T3 cells, a microporator (DigitalBio, Seoul, Republic of Korea) was used according to the manufacturer’s instructions with a condition of 1235 V, 20 ms, and 2 pulses. For pcNAT plasmid transfection, Neuro2A and HEK293A cells were seeded in 6-well plates at a density of 1 × 106 cells per well and incubated for 24 h before transfection. Transfection was performed using Metafectene (Biontex, München, Germany) according to the manufacturer’s instructions. About 24 h after incubation, cells were harvested for further experiments. siRNAs for endogenous hnRNP K and hnRNP D knockdown were purchased from Dharmacon, Lafayette, CO, USA (siGENOME SMARTpool HNRPK M-048992091 and HNRPD M-042940-00). siRNA for endogenous PTB knockdown was synthesized as follow: siPTB 5′-ACA CCUGUGCCUAGCAAUATT-3′ (Bioneer, Daejeon, Republic of Korea).

Subcellular fractionation and immunoblot analysis
Cytoplasmic lysates were prepared from NIH-3T3 cells using hypotonic-lysis buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl2, 1 mM DTT, 0.2 mM PMSF, and 2.5% NP-40 at 4°C. Nuclei were obtained by centrifugation, and washed twice in the hypotonic-lysis buffer to avoid contamination of cytoplasmic proteins. Nuclear lysates were prepared using extraction buffer containing 20 mM HEPES (pH 7.9), 450 mM NaCl, 1.5 mM MgCl2, 1 mM DTT, 0.2 mM PMSF, and 0.2 mM EDTA (pH 8.0)
at 4°C. Both lysates were followed by repeated freeze-thaws for complete disruption. Immunoblot analysis was performed using polyclonal anti-PTB, polyclonal anti-Per3, anti-hnRNP D (Millipore Corporation, Bedford, MA, USA), anti-14-3-3ζ, anti-Lamin-B (Santa Cruz Biotechnology, Dallas, TX, USA), anti-hnRNP K (Abcam, Cambridge, UK) and monoclonal anti-FLAG (Sigma, St Louis, MO, USA), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Millipore Corporation) primary antibodies and horseradish peroxidase-conjugated rat-, rabbit-specific (Bethyl Laboratories, Montgomery, TX, USA), and mouse-specific (Thermo Scientific, Rockford, IL, USA) secondary antibodies. Proteins were analyzed using SUPLEX enhanced chemiluminescence (ECL) solution kit (NeuroneX, Daegu, Republic of Korea) and LAS-4000 chemiluminescence detection system (Fujifilm, Tokyo, Japan), according to the manufacturer’s instructions.

Northern blot analysis
Total RNA was isolated by using TRI reagent according to the manufacturer’s instructions. About 10 mg of total RNA was size-separated by 1% agarose gel electrophoresis containing 0.66 M formaldehyde, transferred to nylon membranes (Pall Corporation, Port Washington, NY, USA), and hybridized with a randomly primed probe labeled with [32P]dCTP (PerkinElmer). For detection of reporter mRNA, rat serotonin N-acetyltransferase (NAT)-coding region was used as probe. Radioactivity was analyzed by autoradiography.

Quantitative real-time reverse-transcription (RT) PCR
Total RNA was isolated by using TRI reagent and reverse transcribed using oligo-dT and ImProm-II reverse-transcription system (Promega) according to the manufacturer’s instructions. The amount of mRNA was analyzed by quantitative real-time PCR (Applied Biosystems, Foster City, CA, USA) with the FastStart Universal SYBR Green Master Mix (Roche Diagnostics). A comparative Ct method was used for quantification.

Pulse-chase with 5-bromouridine
Pulse-chase with 5-bromouridine (5-BrU) was performed as described previously (Paulsen et al. 2014) with minor modifications. Briefly, cells were incubated in conditioned medium including 2 mM of 5-BrU (Sigma-Aldrich, St Louis, MO, USA) for 30 min at 37°C. After incubation, rinse plate with phosphate-buffered saline (PBS) twice, then the medium was changed by conditioned medium including 20 mM uridine (Sigma). Cells were harvested and subjected to RNA isolation using TRI reagent. Heat-denatured total RNA was incubated in gentle rotation for 1 h at 23°C with 2 μg of anti-BrU antibody (BD Pharmingen, BD Biosciences, San Jose, CA, USA) in diethylpyrocarbonate (DEPC)-treated PBS containing 0.1% bovine serum albumin (BSA) and 40 U RNasin. 5-BrU-labeled RNA captured by anti-BrU antibody was immunoprecipitated by Protein G agarose, followed by phenol-chloroform extraction and EtOH down. Pellet was resuspended in DEPC-treated water and analyzed by quantitative real-time reverse-transcription PCR.

Mathematical modeling
To model a circadian clock with a fixed period against mRNA stability modulation, we considered two negative feedback loops based on the previous mathematical model with several modifications (Gonze and Goldbeter 2006). The model assumed that each of loops for transcriptional activator (stabilizing loop) and repressor (core loop) consists of three variables – mRNA (M), cytoplasmic protein (Pc), and nuclear protein (Pn) – and nuclear protein represses the synthesis of mRNA. Clock proteins (PCc, PCn, PCBC, and PCnBC) shuttle between nuclear and cytoplasm, and clock gene mRNAs (MPC and MPnBC) and cytoplasmic proteins (PCp and PCnBC) are degradable. In addition, the nuclear protein in the stabilizing loop (PCnBC) induces the transcription of mRNA in the core loop (MPc). The equations in the model are as follows:

\[
\frac{d[MBC]}{dt} = v_{abc} \frac{K_{ABC} M_{BC}^{nBC} + [P_{BC}]^{nBC}}{K_{ABC} + [P_{BC}]} - v_{ubc} \frac{M_{BC}}{K_{ubc} + M_{BC}}
\]

(1)

\[
\frac{d[PC_{BC}]}{dt} = k_{ubc}[M_{BC}] - v_{ubc} \frac{[PC_{BC}]}{K_{ABC} + [P_{BC}]} - k_{ABC}[P_{BC}]
\]

(2)

\[
\frac{d[P_{BC}]}{dt} = k_{ubc}[P_{BC}] - k_{ABC}[P_{BC}] + k_{out}[P_{BC}]
\]

(3)

\[
\frac{d[M_p]}{dt} = v_{ap} \frac{K_{AP} [P_{BC}]^{nPC} + [P_{BC}]}{K_{AP} + [P_{BC}]} - v_{ap} \frac{M_p}{K_{ap} + M_p}
\]

(4)

\[
\frac{d[P_{nBC}]}{dt} = k_{out}[P_{BC}] - k_{out}[P_{nBC}]
\]

(5)

The squared brackets indicate the concentration of associated components, and the variables in the equations represent the following species: MBC, transcriptional activator mRNA; PCBC, cytoplasmic transcriptional activator protein; PCnBC, nuclear transcriptional activator protein; MPc, transcriptional repressor mRNA; PCp, cytoplasmic transcriptional repressor protein; PnP, nuclear transcriptional repressor protein. The parameter values for the model are from ones used in previous studies (Gonze and Goldbeter 2006) with additional parameters reflecting the nature of two interlocked feedback loops as follows: vabc = 2.15, KABC = 1, nBC = 4, vubc = 0.505, KanBC = 0.5, kABC = 0.5, vABC = 1.4, KBC = 0.13, KanBC = 0.5, KinBC = 0.6, vab = 0.1, Kp = 10, vap = 0.2, KAP = 0.2, kout = 1, vout = 0.15, kinBC = 0.5, koutBC = 0.6. To test the effect of stable or unstable mRNA in the model, vap was halved or doubled. Simulation was performed using MATLAB (The Mathworks, Natick, MA, USA), and the ordinary differential equations were numerically analyzed using the 4th-order Runge-Kutta method.

Statistical analysis
Two-way ANOVA was performed to analyze the knockdown effect of different siRNAs throughout circadian times, followed by Bonferroni post hoc test using GraphPad Prism 6.03 for Windows (GraphPad Software Inc., La Jolla, CA, USA). The p values below 0.05 were considered significant. The results are expressed as mean ± SEM. CircWave (courtesy of Dr Roelof A. Hut) was used to analyze the rhythmicity of circadian mPer3 mRNA expression (Statistics calculated by CircWave is summarized in Table S3).
Results

Circadian phase-dependent mPer3 mRNA degradation
To examine the phase-dependent mPer3 mRNA stability regulation by 3'-UTR-binding proteins, we disturbed the transcription of mPer3 mRNA at different circadian phases. After Dex. treatment, mPer3 mRNA exhibited a periodic oscillation (Fig. 1a); we observe a peak at 24 h after synchronization and a trough at 36 h after synchronization. When treated with actinomycin D (Act.D) at 28 h after synchronization (declining phase) or at 40 h after synchronization (rising phase), mPer3 mRNA displayed different decay kinetics. The decay rate of mPer3 mRNA normalized to mouse Ribosomal protein L32 (mRpl32) at the declining phase was much faster than the decay rate at rising phase (Fig. 1b). In contrast, the decay rate of mouse TATA-box-binding protein (mTbp) mRNA was constant in both cases (Fig. 1c). Since the 3'-UTR is believed to be important for mRNA stability and mPer3 mRNA has a known cis-acting element in 3'-UTR which is essential for its stability (Kwak et al. 2006), we assume that certain proteins bind to the cis-acting element in mPer3 3'-UTR and regulate the mPer3 mRNA stability in a circadian phase-dependent manner.

Identification of mPer3 3'-UTR-binding proteins
To identify specific proteins that bind to mPer3 3'-UTR, a set of deletion constructs covering the cis-acting element identified in the previous study (Kwak et al. 2006) was generated (Fig. 2a). Cytoplasmic lysates were prepared from NIH-3T3 cells and subjected to in vitro binding assays. Protein bands of 57 and 43 kDa (p57 and p43) were almost disappeared when incubated with a construct that lacks the cis-acting element (Fig. 2b, lane 5); even though p57 was dramatically decreased when 1056 nucleotides were removed from 3' of the full-length construct (Fig. 2b, lanes 1 and 2). This result indicates that the region of nucleotides 382–561 is indeed a

Fig. 1 mPer3 mRNA is degraded in a circadian phase-dependent manner. (a) Part of the circadian expression of endogenous mPer3 mRNA. NIH-3T3 cells were synchronized and harvested at indicated times. Total RNA was isolated and analyzed by quantitative real-time reverse-transcription (RT) PCR. The amount of mPer3 mRNA was normalized to the level of mTbp mRNA, and the initial amount of mPer3 mRNA was arbitrarily set to 1. Error bars represent the SEM of three independent experiments. (b and c) Decay kinetics of endogenous mPer3 (b) and mTbp (c) mRNA. NIH-3T3 cells were synchronized by dexamethasone (Dex.) treatment. About 28 or 40 h after synchronization, actinomycin D (Act.D) was treated to the cells for the decay kinetics of mPer3 mRNA in the declining (filled circles and solid line) or the rising (open squares and dashed line) phase, respectively. Vehicle (DMSO)-treated control for each of mRNAs are shown in right panels. The amount of mPer3 (b) and mTbp (c) mRNA was analyzed by quantitative real-time RT PCR and normalized to the level of mRpl32 mRNA. The initial amount of mPer3 (b) and mTbp (c) mRNA was arbitrarily set to 1. Error bars represent the SEM of three independent experiments (*p < 0.05; n.s. not significant).
Fig. 2 Heterogeneous nuclear ribonucleoprotein (hnRNP) K, polypyrimidine tract-binding protein (PTB), and hnRNP D bind to mPer3 3'-UTR. (a) Schematic representation of pcNAT reporters containing either full-length mPer3 3'-UTR or its fragments. Stop codon of serotonin N-acetyltransferase (NAT)-coding region and nucleotide numbers of each constructs are shown. Cis-acting elements are indicated as gray squares. (b) UV cross-linking analysis between radiolabeled mPer3 3'-UTR serial deletion constructs and cytoplasmic lysates prepared from NIH-3T3 cells. 32P-labeled RNA-protein complexes were UV cross-linked, digested by RNase and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The arrowheads on the right indicate the 57 (p57) and 43 (p43) kDa proteins bound to the cis-acting element. Molecular mass markers are shown on the left in kilodaltons. (c) UV cross-linking analysis between radiolabeled mPer3 3'-UTR serial deletion constructs and glutathione S-transferase (GST)-tagged recombinant proteins. The arrowhead on the right represents GST-tagged mouse hnRNP K. Molecular mass markers are shown on the left in kilodaltons. (d) Streptavidin-biotin RNA affinity purification analysis between biotin-labeled mPer33'-UTR full-length construct and cytoplasmic lysates prepared from NIH-3T3 cells. Fivefold and 10-fold-unlabeled RNAs were co-incubated for competition of the interaction (lanes 4 and 5). Lamin-B and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as a nuclear and a cytoplasm marker, respectively.
putative-binding site of mPer3 mRNA stability modulators. Using MALDI-TOF mass spectrometry with streptavidin-biotin RNA purified samples, p57 corresponded to two proteins, identified as hnRNP K and PTB, and p43 was identified as hnRNP D. The binding was confirmed by UV cross-linking and immunoprecipitation with specific antibodies (Figure S1a, lanes 7 to 10). Using autoradiography, we found that RNase-digested 32P-labeled mPer3 3′-UTR full-length fragments co-immunoprecipitated with hnRNP K and PTB with an apparent molecular weight of 57 kDa. Likewise, 32P-labeled fragments co-immunoprecipitated with hnRNP D at a molecular weight of 43 kDa. GST-tagged recombinant proteins were used to confirm cis-acting element-specific binding. In addition to hnRNP K, all isoforms of mouse PTB (PTB1; PTBP1 isoform 1 and PTB2; PTBP1 isoform 2, not a neuronal PTB – nPTB or PTBP2) and hnRNP D (hnD37, hnD40, hnD42, and hnD45) were purified by GST pull-down. The samples were then subjected to UV cross-linking with the set of radiolabeled mPer3 3′-UTR deletion constructs. The autoradiographs showed that hnRNP K, all isoforms of PTB, and hnRNP D bind to various regions on mPer3 3′-UTR. Moreover, all they bind directly to the cis-acting element in mPer3 3′-UTR, while purified GST protein did not bind to mPer3 3′-UTR (Fig. 2c, Figure S1b). Binding between mPer3 3′-UTR and hnRNPs was also verified by streptavidin-biotin RNA affinity purification analysis. Cytoplasmic hnRNP K, PTB, and hnRNP D were bound to biotin-labeled full-length mPer3 3′-UTR, and their specific binding to mPer3 3′-UTR was confirmed by competition analysis with unlabeled 3′-UTR, that gradually decreased as the amount of competitor was increased (Fig. 2d). These results suggest that hnRNP K, PTB, and hnRNP D directly bind to the cis-acting element in mPer3 3′-UTR.

Role of mPer3 3′-UTR-binding proteins in mRNA stability modulation

In the previous report, nucleotides 382–561 in mPer3 3′-UTR was identified as the cis-acting element for post-transcriptional mRNA decay (Kwak et al. 2006), and we identified hnRNP K, PTB, and hnRNP D as the cis-acting element-binding proteins. To examine their role in mPer3 mRNA stability regulation, Neuro2A and HEK293A cells were transiently transfected with siRNAs for knockdown (Fig. 3a) or with expression vectors for over-expression (Fig. 3b) of each identified trans-acting factors, respectively. Furthermore, using NAT reporter plasmids without or with full-length mPer3 3′-UTR (NAT or NAT 3′-UTR), we investigated the stability of reporter mRNA-containing mPer3 3′-UTR. Northern blot analysis revealed that the knockdown of mPer3 3′-UTR-binding proteins differentially influenced the stability of mPer3 3′-UTR-containing reporter mRNA. Compared to non-targeting siRNA (siCon) treatment (Fig. 3a, lanes 1 and 2 in lower panel), the knockdown of hnRNP K (sihnK) accelerated reporter mRNA degradation (Fig. 3a, lanes 3 and 4 in lower panel). On the other hand, the knockdown of hnRNP D (sihnD) attenuated reporter mRNA degradation (Fig. 3a, lanes 7 and 8 in lower panel), while the knockdown of PTB (siPTB) exhibited little effect on reporter mRNA stability (Fig. 3a, lanes 5 and 6 in lower panel).

The knockdown effect of mPer3 3′-UTR-binding proteins on the stability of mPer3 mRNA was validated by 5-BrU pulse-chase analysis. Neuro2A cells were transiently transfected with siRNAs for knockdown of each of trans-acting factors (Figure S2a). Using the reporter plasmids pulse labeled with 5-BrU, we obtained the consistent result with northern blot analysis that 6 h after chase, the knockdown of hnRNP K dramatically reduced the amount of reporter mRNA (Figure S2b, lanes 5 and 6) compared to the treatment of non-targeting control siRNA (Figure S2b, lanes 3 and 4), while the knockdown of hnRNP D did not make any decrease in the amount of reporter mRNA (Figure S2b, lanes 9 and 10).

The over-expression of mPer3 3′-UTR-binding proteins also displayed the differential reporter mRNA stability regulation. The amount of NAT reporter mRNA lacking mPer3 3′-UTR remained as a constant level after 6-h-actinomycin D treatment as reported previously (Kwak et al. 2006) (Fig. 3b, lanes 1 and 2 in lower panel). Compared to mock transfection (Fig. 3b, lanes 3 and 4 in lower panel), the over-expression of hnRNP K (FLAG-hnK) decelerated the degradation of reporter mRNA-containing mPer3 3′-UTR (Fig. 3b, lanes 5 and 6 in lower panel). On the other hand, the over-expression of hnRNP D (FLAG-hnD) accelerated reporter mRNA degradation (Fig. 3b, lanes 9 and 10 in lower panel), while the over-expression of PTB (FLAG-PTB) showed no effect on reporter mRNA stability (Fig. 3b, lanes 7 and 8 in lower panel). From these knockdown and over-expression analyses, we suggest that hnRNP K stabilizes and hnRNP D destabilizes mPer3 3′-UTR-containing reporter mRNA, while PTB has no effect on the stability.

Interaction between binding proteins and mPer3 mRNA 3′-UTR

Most of the hnRNPs shuttle between cytoplasm and nucleus, and post-transcriptional mRNA degradation is believed to occur in cytoplasm (Dreyfuss et al. 2002; Bevilacqua et al. 2003). For this reason, we hypothesized that the cytoplasmic abundance of hnRNP K, PTB, and hnRNP D is important in each protein’s ability to modulate temporal stability regulation of target mRNAs. Immunoblot analysis with specific antibodies showed the rhythmic expression of cytoplasmic mPer3 3′-UTR-binding proteins (Figure S3a). During a circadian period synchronized by Dex. treatment, the amount of cytoplasmic hnRNP K was began to increase after 24 h and began to decrease after 36 h after synchronization. The phase of cytoplasmic PTB was similar to that of hnRNP K albeit less dramatic (Figure S3a), but the phase of cytoplasmic hnRNP K and PTB were reciprocal compared to the
mPer3 mRNA oscillation (Figs S3a and 1a). On the other hand, the phase of cytoplasmic hnRNP D, a trough at 28 h and a peak at 44 h after synchronization, was slightly delayed compared to the phase of hnRNP K and PTB. This result led us to hypothesize that hnRNP K and PTB are acting in the declining phase (from the peak to the trough) of mPer3 mRNA, and hnRNP D is acting in the rising phase (from the trough to the peak) of mPer3 mRNA. To elucidate the rhythmic interaction between mPer3 mRNA and 3'UTR-binding proteins, an in vitro binding assay with synchronized cytoplasmic lysates harvested at different points along the circadian phases was performed. Radiolabeled mPer3 mRNA, and hnRNP D is acting in the rising phase (from the trough to the peak) of mPer3 mRNA. To elucidate the rhythmic interaction between mPer3 mRNA and 3'UTR-binding proteins, an in vitro binding assay with synchronized cytoplasmic lysates harvested at different points along the circadian phases was performed. Radiolabeled mPer3 mRNA and hnRNP D were transiently transfected with siRNAs (siCon for non-targeting, sihnK for hnRNP K, siPTB for polypyrimidine tract-binding protein (PTB), and sihnD for hnRNP D). About 24 h after incubation, the cells were secondarily transfected with a reporter plasmid that mPer3 3'UTR is inserted after serotonin N-acetyltransferase (NAT)-coding region (NAT 3'-UTR). Total RNA was isolated at indicated times after actinomycin D (Act.D) treatment, and analyzed using radiolabeled DNA probes against the NAT-coding sequence. The knockdown of corresponding proteins was confirmed with antibodies against hnRNP K, PTB, and hnRNP D. First-order decay constants and mRNA half-lives were calculated and shown (lower right).
3′-UTR full-length and cis-acting element was co-incubated with the cytoplasmic lysates of 28 h (a descent point of mPer3 mRNA oscillation) and 40 h (an ascent point) after synchronization followed by UV cross-linking. The binding pattern between mPer3 mRNA and 3′-UTR-binding proteins was not significantly different for both constructs (Figure S3b). The same result was observed when the cytoplasmic lysates were co-incubated with biotin-labeled mPer3 3′-UTR full-length or cis-acting element followed by streptavidin-biotin RNA affinity purification (Figure S3c). Since in vitro transcribed constructs were used, we performed an RNA-immunoprecipitation analysis, which has the advantage of observing the interaction between endogenous mRNA and protein. Cytoplasmic lysates prepared from Dex.-treated NIH-3T3 cells, and endogenous hnRNP K, PTB, and hnRNP D were immunoprecipitated with specific antibodies. Immunoprecipitated samples were subjected to RNA isolation and quantitative real-time reverse-transcription (RT) PCR for mPer3. Interestingly, the amount of hnRNP K-bound mPer3 mRNA was slightly increased in the lysates prepared 24 h after synchronization that mPer3 mRNA was maximally abundant. The amount of hnRNP D-bound mPer3 mRNA was increased in the lysates collected 36 h after synchronization, the trough in mPer3 mRNA expression (Figure S3d).

**Role of RNA-binding proteins in circadian mPer3 mRNA expression**

To verify the effect of stability regulation on the circadian mRNA oscillation, we analyzed endogenous mPer3 mRNA oscillation according to the individual knockdown of hnRNP K, PTB, and hnRNP D. NIH-3T3 cells were transiently transfected with siRNAs for non-targeting (siCon), hnRNP K (sihnK), polypyrimidine tract-binding protein (PTB) (siPTB), or hnRNP D (sihnD) at 24 h before synchronization, then incubated for 48 additional hours (Fig. 4a). Compared to the transfection with non-targeting siRNA (siCon), the knockdown of hnRNP K significantly lowered the peak amplitude of mPer3 mRNA oscillation (p = 0.0225), while the period and the phase of circadian oscillation remained unchanged.

**Fig. 4 Knockdown of heterogeneous nuclear ribonucleoprotein (hnRNP) K and hnRNP D affects the amplitude of circadian mPer3 mRNA oscillation. (a)** Immunoblot analysis for knockdown of trans-acting factors. NIH-3T3 cells were transiently transfected with siRNAs for non-targeting (siCon), hnRNP K (sihnK), polypyrimidine tract-binding protein (PTB) (siPTB), and hnRNP D (sihnD) at 24 h before synchronization. Cells were synchronized by dexamethasone (Dex.) treatment and harvested at indicated times. Proteins were analyzed by antibodies against hnRNP K, PTB, and hnRNP D. Glycereraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control.

(b) Temporal expression of mPer3 mRNA affected by the knockdown of hnRNP K. NIH-3T3 cells were transiently transfected with siRNAs for non-targeting (siCon; filled diamonds and solid line) or hnRNP K (sihnK; open squares and dashed line) 24 h before synchronization. Cells were synchronized by Dex. treatment and harvested at indicated times. Total RNA was isolated and analyzed by quantitative real-time reverse-transcription (RT) PCR. The amount of mPer3 mRNA was normalized to the level of mTbp, and the initial amount of mPer3 mRNA with siCon was arbitrarily set to 1. Error bars represent the SEM of four independent experiments (*p < 0.05; **p < 0.01; ***p < 0.001). (c) Temporal expression of mPer3 mRNA affected by knockdown of PTB. NIH-3T3 cells were transiently transfected with siRNAs for non-targeting (siCon; filled diamonds and solid line) or PTB (siPTB; open triangles and dashed line) 24 h before synchronization. Experimental procedures were same as described in panel (b). Error bars represent the SEM of five independent experiments. (d) Temporal expression of mPer3 mRNA affected by knockdown of hnRNP D. NIH-3T3 cells were transiently transfected with siRNAs for non-targeting (siCon; filled diamonds and solid line) or hnRNP D (sihnD; open circles and dashed line) 24 h before synchronization. Experimental procedures were same as described in panel (b). Error bars represent the SEM of ten independent experiments (*p < 0.05).
(Fig. 4b and Table S3). A dose-dependent sihnK treatment showed a dramatic decrease in mPer3 mRNA oscillation (Figure S4a). This result indicates that hnRNP K is required not only for mPer3 mRNA stabilization but also for amplitude formation. In contrast, the knockdown of PTB did not make any change in the period, phase, and amplitude of circadian mPer3 mRNA oscillation (Fig. 4c and Table S3, p = 0.9877). Likewise, a dose-dependent siPTB treatment displayed no significant alteration in circadian mPer3 mRNA oscillation despite a little fluctuation (Figure S4b). The effect of hnRNP D knockdown was restricted to the first period (Fig. 4d and Table S3, p = 0.0033). The amplitude of mPer3 mRNA oscillation was significantly increased only at the first peak. A dose-dependent sihnD treatment also showed that the amplitude of mPer3 mRNA oscillation was gradually increased only at the first peak, consistent with the initial experiment (Figure S4c). This result indicates that hnRNP D participates in the destabilization of mPer3 mRNA, as well as the reduction in its circadian amplitude. The knockdown of hnRNP K and hnRNP D showed a partly opposite effect on circadian mPer3 mRNA oscillation that the knockdown of hnRNP K decreased the amplitude of mPer3 mRNA oscillation and the knockdown of hnRNP D increased it at the first peak. We examined whether the double knockdown recovered the amplitude of mPer3 mRNA oscillation. NIH-3T3 cells were transiently transfected with siRNAs for hnRNP K, hnRNP D, and mix of two at 24 h before synchronization, and then incubated cells for 48 additional hours (Figure S5a). The oscillation of mPer3 mRNA treated with siRNAs for non-targeting (siCon), hnRNP K (sihnK), and hnRNP D (sihnD) was consistent with the initial experiments (Fig. 4). Interestingly, the mPer3 mRNA treated with siRNAs for both hnRNP K and hnRNP D (sihnKD) oscillated between mPer3 mRNA of shhnK and that of shhnD except a single time point (Figure S5b). mPer3 mRNA of shhnKD failed to recover to that of siCon, because the knockdown effect of hnRNP K on the amplitude of mPer3 mRNA oscillation was greater than that of hnRNP D (Fig. 4b and d).

Altogether, our results demonstrate that hnRNP K and hnRNP D control not only the stability of mPer3 mRNA but also the amplitude of its circadian oscillation in an opposite manner, while PTB has no significant function.

**Theoretical analysis between mPer3 mRNA stability and its circadian oscillation**

To confirm theoretically the effect of post-transcriptional mRNA stability regulation on its circadian oscillation, we developed a mathematical model consisting of two interlocked negative feedback loops as described in Methods. Each of the loops were called a stabilizing loop (for transcriptional activators: Bmal1 and Clock) and a core loop (for transcriptional repressors: Per3) as described previously (Emery and Reppert 2004). They contained parameters for three variables that mRNA, cytoplasmic protein, and nuclear protein for each loop (Fig. 5a). The simulation result from the model with a suitable choice of parameters showed the circadian oscillations with a period of about 24 h for all components in each of loops (Fig. 5b, solid line). In addition, the model showed the circadian expression of cytoplasmic and nuclear mPER3 proteins and their phase relationship, as observed experimentally (data not shown). To investigate the alteration in circadian mPer3 oscillation as the function of its stability, we increased or decreased the maximum rate of mPer3 mRNA degradation (v_{mP}). As expected, the amplitude of mPer3 mRNA oscillation was increased when stable (v_{mP} was decreased, Fig. 5b; dotted line), which corresponds to the knockdown of hnRNP D (Figs 3a and 4d). Likewise, the amplitude of mPer3 mRNA oscillation was decreased when unstable (v_{mP} was increased, Fig. 5b; dashed line), which corresponds to the knockdown of hnRNP K (Figs 3a and 4b). Interestingly, the period of mPer3 mRNA oscillation was constant even if the stability of mPer3 mRNA was perturbed (Fig. 5d), while the amplitude showed a reciprocal relationship with the degradation rate of mRNA (Fig. 5c and Figure S6). This result cannot be obtained from a single negative feedback loop model, which shows a dependency between mRNA stability and the period of oscillation (Figure S7). Based on the results from the model, we can theoretically confirm the effect of post-transcriptional mRNA degradation on its oscillation profile, consistent with the results of experiments. Furthermore, we find that an additional negative feedback loop is required for the robustness of circadian period against the post-transcriptional modulation of mRNA stability.

**Discussion**

The circadian expression of clock genes originates from the interlocking negative feedback loops. A number of studies have focused on the transcriptional and the post-translational regulation of clock genes to describe their rhythmic expression. In other words, the rhythmic synthesis of clock gene mRNA through the rhythmic turnover of repressor proteins or the rhythmic dimerization of transcriptional activator proteins is considered as the core process in circadian rhythms. However, recent studies suggest that post-transcriptional regulation including mRNA decay and IRES-mediated translation also contributes to the clock gene expression. We suggest here that the circadian phase-dependent clock gene mRNA degradation modulated by RNA-binding proteins is important for the circadian expression of clock genes.

hnRNPs, one family of RNA-binding proteins, are well known for their function on RNAs during the process of mature mRNAs from pre-RNAs. In general, hnRNPs immediately bind to newly synthesized RNAs in the nucleus, and they participate in the preventing of RNA degradation, splicing of immature RNAs, and transporting of RNAs to cytoplasm (Dreyfuss et al. 2002). Moreover, several hnRNPs have been identified to have a post-transcriptional role in the stability
control of clock gene mRNAs. hnRNP R, hnRNP Q, and hnRNP L accelerate Nat mRNA degradation (Kim et al. 2005); PTB and hnRNP D destabilize Per2 and Cry1, respectively (Woo et al. 2008, 2010). In this study, we identified three proteins that bind to the cis-acting element located in the 3′-UTR of mPer3 mRNA. Among them, hnRNP

Fig. 5 Mathematical model for circadian mPer3 expression shows the relation between mRNA stability and circadian oscillation. (a) Scheme of the model that consists of two negative feedback loops. The model is based on the effect of nuclear clock proteins (PNbc and PNp) as a transcriptional repressor of themselves (Mbc and Mbc) and the effect of nuclear clock protein in the stabilizing loop (PNbc) as a transcriptional activator of clock mRNA in the core loop (Mbc). Clock mRNAs (Mbc and Mbc) and cytoplasmic clock proteins (PCp and PCbc) are spontaneously degraded with their own decay rate. (b) mRNA decay kinetics and corresponding oscillation from the model. (Left) The initial amount of Mbc was set to 1, and Mbc with various maximum degradation rates were decreasing for 6 h. The dotted line indicates stable Mbc (small vMbc) and the dashed line indicates unstable Mbc (large vMbc). (Right) The amount of Mbc with corresponding maximum degradation rates described in panel (a) was oscillating for 72 h. (c) Bifurcation diagram as the function of the maximum mRNA degradation rate vMbc. The curve shows the maximum and minimum amount of mRNA in a steady state. A stable limit cycle is appeared in the wide range of the vMbc, and the basal level of mRNA is decreased as the value of vMbc is increased. The x-axis indicates the degradation rate of mRNA (vMbc), and the y-axis indicates the amplitude of mRNA oscillation in a logarithmic scale. (d) Bifurcation diagram of period as the function of the maximum mRNA degradation rate vMbc. The curve shows the period of mRNA oscillation with the corresponding parameter value in a steady state. The x-axis indicates the degradation rate of mRNA (vMbc), and the y-axis indicates the period of mRNA oscillation.
K and hnRNP D are characterized as a stabilizer and a destabilizer for mPer3 mRNA, respectively (Fig. 6). However, the underlying mechanism how hnRNPs regulate the stability of target mRNAs remains unclear. A possible destabilizing mechanism by hnRNP D was suggested in the recent study about the RNA-dependent interaction of guanosine triphosphate-binding protein1 (GTPBP1) (Woo et al. 2011). Since GTPBP1 directly binds to the components of exosome complex, which is 3’ to 5’ exoribonuclease, the binding of hnRNP D to mPer3 mRNA 3’-UTR may result in the exosome-mediated mRNA degradation. In contrast, the stabilizing mechanism by hnRNP K is not fully understood despite some experimental observations that hnRNP K stabilizes ribosomal RNA in human pancreatic ductal epithelial cells (Wen et al. 2012) and extracellular signal-regulated kinases (ERK)-mediated cytoplasmic accumulation of hnRNPK increases the stability of thymidine phosphorylase mRNA (Chen et al. 2009). We suggest three possible stabilizing mechanisms of hnRNP K. First, hnRNP K shares a binding site with hnRNP D, therefore the binding of hnRNP K on target mRNA disturbs the binding of hnRNP D. Alternatively, the binding of hnRNP K on target mRNA may interfere with the recruitment of GTPBP1-associated exosome components by hnRNP D. The third possibility is that hnRNP K has an exosome-independent stabilizing mechanism.

Stability modulation by hnRNP K and hnRNP D affects the oscillation profile of mPer3 mRNA. In this study, we identified that hnRNP K knockdown decreases the amplitude of mPer3 mRNA oscillation through two full circadian periods (Fig. 4b). Alternatively, hnRNP D knockdown increases the amplitude of mPer3 mRNA oscillation at the first peak (24 h after Dex. treatment), but the amplitude of mPer3 mRNA nears normal levels during the second peak (48 h after Dex. treatment) as shown in Fig. 4d. This observation seems to be the result of the differential effect of 3’-UTR-binding proteins on the paralogs of clock gene mRNAs. Previously, a study suggested that the negative regulators of circadian clock, such as Per, Cry, and Rev-erb, have their own paralogs that exhibit structural or functional homology (Per1, Per2, and Per3; Cry1 and Cry2; Rev-erb a and Rev-erb b). Moreover, it is believed that the disruption of clock gene expression is compensated and recovered by its paralogs; hence the robustness of circadian oscillations can be achieved (Baggs et al. 2009). In our study, the knockdown of hnRNP K lowers the overall amplitude of mPer3 mRNA oscillation for 48 h. It is likely that the effect of hnRNP K knock-down widely influences the overall clock gene paralogs; therefore the circadian amplitude of mPer3 mRNA oscillation becomes entirely dampened. In contrast, the knockdown of hnRNP D decreases the amplitude of the first peak but slightly increases that of the second peak of mPer3 mRNA oscillation. It is likely that the effect of knock-down at the first peak became diminished and recovered during the second circadian period. In this case, the perturbation of

Fig. 6 Proposed model for the rhythmic modulation of mPer3 mRNA stability and its circadian oscillation by heterogeneous nuclear ribonucleoprotein (hnRNP) K and hnRNP D. Proposed model for rhythmic degradation of mPer3 mRNA modulated by hnRNP K, polypyrimidine tract-binding protein (PTB), and hnRNP D. The transcription of mPer3 mRNA is induced by the stabilizing loop of transcription activators (e.g., Bmal1 and Clock). mPER3 protein shuttles between cytoplasm and nucleus, and the only nuclear mPER3 protein represses its transcription. hnRNP K, PTB, and hnRNP D are mPer3 3’-UTR-binding proteins and they regulate the stability of mPer3 mRNA. Their binding to mPer3 mRNA is constant; however, they recruit the RNA decay machinery (e.g., exosome) rhythmically through an unknown mechanism. (hnK, hnRNP K; hnD, hnRNP D; AAA, poly(A) tail).
mPer3 mRNA stability seems to be compensated by mPer1 or mPer2. From these results, we conclude that the effect of post-transcriptional mRNA stability regulation is buffered by the feedback loops consisting of clock genes and their paralogs.

Several studies have reported the role of mRNA 3′-UTR-binding proteins and their cytoplasmic accumulation that influences mRNA stability and its oscillation profile in post-transcriptional way. For instance, the amount of cytoplasmic PTB strongly affects the amplitude and marginally affects the phase of mPer2 mRNA (Woo et al. 2008). Furthermore, the amount of cytoplasmic hnRNP D controls the amplitude and phase of mCry1 mRNA to some extent (Woo et al. 2010). In this study, hnRNP K and hnRNP D influenced the stability and oscillation amplitude of mPer3 mRNA in an opposite manner, while no alteration of period and phase was observed. Our results from the mathematical model suggest the underlying mechanism that an additional negative feedback loop is required for the robustness of a circadian period. The authors have no conflict of interest to declare.

**Acknowledgments and conflict of interest disclosure**

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All experiments were conducted in compliance with the ARRIVE guidelines. The authors have no conflict of interest to declare.

**Supporting information**

Additional supporting information may be found in the online version of this article at the publisher’s web-site:

**Figure S1.** hnRNP K, PTB, and hnRNP D bind to nucleotide 382-561 in mPer3 mRNA 3′-UTR.

**Figure S2.** hnRNP K and hnRNP D modulate the stability of mPer3 3′-UTR-containing reporter mRNA.

**Figure S3.** mPer3 mRNA interacts with cytoplasmic hnRNPs.

**Figure S4.** Dose-dependent treatment of siRNAs for hnRNP K and hnRNP D affect the amplitude of circadian mPer3 mRNA oscillation.

**Figure S5.** Knockdown of hnRNP K and hnRNP D oppositely affects the amplitude of circadian mPer3 mRNA oscillation.

**Figure S6.** Mathematical model for circadian mPer3 expression shows the relation between mRNA stability and circadian oscillation.

**Figure S7.** Single negative feedback loop model for circadian mPer3 expression.

**Table S1.** Primer sequences for GST-tagged recombinant protein cloning.

**Table S2.** Oligo sequences for quantitative real-time RT PCR.

**Table S3.** Statistical parameters calculated by Circwave.

**References**


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