Two *Ck1δ* transcripts regulated by m6A methylation code for two antagonistic kinases in the control of the circadian clock

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The N'-methylation of internal adenosines (m6A) in mRNA has been quantified and localized throughout the transcriptome. However, the physiological significance of m6A in most highly methylated mRNAs is unknown. It was demonstrated previously that the circadian clock, based on transcription-translation negative feedback loops, is sensitive to the general inhibition of m6A. Here, we show that the Casein Kinase 1 Delta mRNA (*Ck1δ*), coding for a critical kinase in the control of circadian rhythms, cellular growth, and survival, is negatively regulated by m6A. Inhibition of *Ck1δ* mRNA methylation leads to increased translation of two alternatively spliced *Ck1δ* isoforms, *Ck1δ*1 and *Ck1δ*2, uncharacterized until now. The expression ratio between these isoforms is tissue-specific, *Ck1δ*1 and *Ck1δ*2 have different kinase activities, and they cooperate in the phosphorylation of the circadian clock protein PER2. While *Ck1δ*1 accelerates the circadian clock by promoting the decay of PER2 proteins, *Ck1δ*2 slows it down by stabilizing PER2 via increased phosphorylation at a key residue on PER2 protein. These observations challenge the previously established model of PER2 phosphorylation and, given the multiple functions and targets of *Ck1δ*, the existence of two isoforms calls for a re-evaluation of past research when *Ck1δ*1 and *Ck1δ*2 were simply *Ck1δ*.

Since 2012, the N'-methylation of internal adenosines (m6A) has regained tremendous interest due to the use of affinity immunoprecipitation of methylated mRNA coupled with next-generation RNA-Seq (1, 2), confirming earlier reports (3, 4). m6A accounts for 50% of all methylated nucleotides in polyadenylated mRNA (5) and for 80% of all base methylations (3). It is the most prevalent internal mRNA modification in higher eukaryotes and is widely conserved from yeast to mammals (6, 7). m6A has been involved in the regulation of mRNA splicing, processing, and translation (8, 9) and is required for survival and development in multiple species (10–12).

The circadian clock is composed of clock genes interlocked in transcription-translation negative feedback loops (TTFL) regulating their own expression (13). This molecular oscillator, robust in normal conditions yet sensitive to perturbations affecting gene expression and RNA processing, allowed us to demonstrate the importance of m6A for its function (14), but which clock genes are directly regulated by m6A remains unknown.

While the core TTFL has been the focus of early circadian research, protein phosphorylation was quickly shown to be essential for molecular timekeeping (15, 16). Phosphorylation of clock proteins is mediated mainly by the Casein Kinases 1 Delta and Epsilon. The first human inherited circadian syndrome to be described, the Familial Advanced Sleep Phase Syndrome (FASPS), originates from a mutated *Ck1δ* (17) or a mutated *Ck1δ*-target serine in PER2 protein (18), causing early sleep onset and offset. The first circadian mutation to be discovered in a mammalian model is a mutation in *Ck1ε* (16, 19).

Here, we show that the 3'-UTR of *Ck1δ* is heavily m6A-methylated and negatively controls the translation of two *Ck1δ* isoforms, uncharacterized until now. These two *Ck1δ* isoforms are expressed in a tissue-specific manner and antagonistically regulate the circadian clock.

**Results**

The 3'-UTR of *Ck1δ* is Methylated. To identify clock-related transcripts regulated by m6A, we quantified m6A levels in mouse liver mRNA at two time points, CT4 and CT16 (CT is circadian time, and CT0 and CT12 correspond to the beginning of the rest and the active phase, respectively). Significant m6A peaks were found at CT4 (8,414) and at CT16 (8,821). Most peaks (72%) were detected at both time points (Dataset S1). Several transcripts involved in circadian regulation showed significant peaks at CT4 and CT16 (SI Appendix, Fig. S1A). Since Clock (20), Ddbp (21), *Ck1δ* (17), and Flod2 (22, 23) are potent regulators of the circadian clock, we decided to study their expression in mouse liver 4 h after the lights were turned on (CT4).

**Significance**

The most abundant modification in mRNA is the N'-methylation of internal adenosines (m6A), but m6A's physiological function is unknown for most mRNAs. Here we show that Casein Kinase 1 Delta mRNA (*Ck1δ*), coding for a critical kinase in the control of circadian rhythms, is regulated by m6A. When m6A is inhibited, the expression of two *Ck1δ* isoforms, uncharacterized until now, increases due to enhanced translation. This increase in *Ck1δ*1 leads to a slower clock because of increased phosphorylation of the clock protein PER2 at a key residue, leading to the stabilization of PER2 protein.


The authors declare no conflict of interest.

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m6A Regulates the Expression of Two CK1δ Isoforms. To investigate the role of m6A in the control of CK1δ expression, we treated mouse embryonic fibroblasts (MEFs) with Deazanoplacin A (DZnep), an inhibitor of methylation known to inhibit m6A and elongate the circadian period length in these cells (14, 25), and observed an increase in CK1δ proteins (Fig. 1A). This was opposite to our expectations, since available evidence published so far in mammals has associated higher CK1δ expression with a faster clock (26). While replicating these results, we noticed that, under certain conditions during migration of proteins in the acrylamide gel, two bands, not one, were seen on the membrane, both bands increasing under DZnep treatment (Fig. 1A). By comparing these two bands with the bands obtained from cells transfectected with CK1δ expression vectors, we later identified the two bands as two isoforms of CK1δ (Fig. 3), CK1δ1 (415 aa) and CK1δ2 (409 aa), reported in databases but of functions unknown, sharing 99.75% identity with human CK1δs, and conserved in virtually all vertebrates (SI Appendix, Fig. S1 C and D). The only difference between these isoforms is a small 63-bp exon in mouse, retained in CK1δ2 immediately upstream of the 3′-UTR and containing a STOP codon (SI Appendix, Fig. S1B), thus coding for the smaller kinase.

To confirm that the results above were dependent upon mRNA m6A inhibition, we silenced in MEFs the catalytic subunit of the mRNA m6A methyltransferase, Mettl3 (27, 28), and measured CK1δ protein levels by immunoblotting. Again, two specific bands were detected. Silencing of Mettl3 increased the intensity of the two bands, demonstrating m6A negatively regulates CK1δ expression (Fig. 1B). At the mRNA level, Mettl3 silencing mildly increased CK1δ and had little effect on CK1δ2 (Fig. 1C) and significantly delayed the initial steps in CK1δ1 and CK1δ2 degradation (SI Appendix, Fig. S24). Surprisingly, the half-life of CK1δ2 was significantly longer than that of CK1δ1, which we confirmed in a longer time course (SI Appendix, Fig. S2B).

Since the increase in CK1δ1 under m6A inhibition cannot be readily explained by changes in steady-state transcript levels or mRNA stability, we measured the impact of Mettl3 silencing on translation of CK1δ transcripts by polysome fractionation (Fig. 1D). Consistent with a recent report showing that m6A attenuates
translation efficiency (29). Mettl3 silencing significantly increased the amount of Ck1δ transcripts only in fractions containing the heavier polyosomes, while Ck1ε was not significantly affected.

The 3'-UTR of Ck1δ transcripts is over 2,000 nucleotides long, but its function in the regulation of Ck1ε expression is unknown. We designed reporter mRNAas in which the luciferase coding sequence was flanked by the 5'- and 3'-UTRs of Ck1δ. To closely mimic Ck1δ and Ck1ε, the end of the coding sequence of each Ck1δ was also included downstream of the luciferase STOP codon. We also designed Ck1δ luc transcripts lacking most of the 3'-UTR. These reporter mRNAs were transcribed in vitro and then added to reticulocyte lysates to assess the efficiency of their translation by luminometry (SI Appendix, Fig. S2C). The deletion of the 3'-UTR caused a significant increase in Ck1δ luc (approximately sixfold) and Ck1δ luc (approximately threefold) luminescence, demonstrating the role of the 3'-UTR as a negative regulator of Ck1ε translation.

Ablation of Ck1δ m6A Methylation by CRISPR-Cas9 in Vivo. Next, to investigate the physiological role of the methylated locus in the 3'-UTR of Ck1δ, we generated a mutant mouse strain with a 43-bp deletion in the genomic region corresponding to the methylated peak (Fig. 2A). This deletion does not affect the coding sequence but removes the N6-methylated adenosine at GGM6ACA, located previously by screening of m6A sites in brain and liver transcriptomes (30). The consequences of this mutation on Ck1δ m6A levels, mRNA, and protein expression were investigated in the liver, since it is known to have high mRNA m6A levels (2). As expected, Ck1δ transcripts had lower m6A levels in -43/-43 animals compared with wild type (Fig. 2B), but the level of Ck1δ mRNAs was not significantly affected (Fig. 2B). In contrast, Ck1δ protein levels increased significantly: homozygous -43 mutant mice had higher Ck1δ levels in the liver and the brain (Fig. 2C), despite an inverted Ck1δ/Ck1δ ratio (Fig. 3), and had a longer period (23.97 ± 0.02 h) of locomotor activity behavior rhythms compared with wild-type mice (23.64 ± 0.08, Fig. 2D). Although noncoding, this mutation, weakening m6A-dependent posttranscriptional processes limiting Ck1δ expression, was sufficient to cause an observable increase in Ck1δ proteins, accompanied by a change in circadian locomotor activity behavior. How an increase in the expression of endogenous Ck1δ is able to elongate period length is investigated in the next part of this work.

Ck1δ1 and Ck1δ2 Are Two Different Kinases. Data presented above go against expectations based on period elongation observed in liver explants from Ck1δ knock-out mice (31) and on Ck1δ overexpression causing a shorter period (26). The overexpression studies, however, did not specify which isoform was used. The existence of two isoforms has been suggested before in a circa-adian context (32), but has been ignored until now. Surprisingly, since Ck1δ is considered canonical and has been the de facto focus of past works, Ck1δ2 is the major isoform in the liver, where both transcripts show relatively constant levels across 24 h (Fig. 3A). In the suprachiasmatic nucleus of the hypothalamus (SCN), this relationship is reversed (Fig. 3B; see also Fig. 2C), and the molar ratio of Ck1δ2/Ck1δ1 is tissue-specific (Fig. 3C).

Both Ck1δs can interact with PER2 (Fig. 3D), but no evidence of Ck1δ1/Ck1δ2/PER2 complexes was observed. To test the role of Ck1δs in the circadian clock, immortalized MEFs prepared from PER2−/−LUCIFERASE knock-in reporter mice (33) were stably transfected with Ck1δ1 or Ck1δ2 expression vectors. Overexpression of Ck1δ1 led to a shorter period (Fig. 3E) compared with colonies stably transfected with the empty vector. In contrast, Ck1δ2 led to an ~1-h period elongation. While both isoforms could phosphorylate PER2 (Fig. 3F), the intensity of the phosphorylated bands was higher with Ck1δ2 and Ck1δ1 + Ck1δ2, suggesting higher kinase activity for Ck1δ2 and/or faster PER2 degradation with Ck1δ1. A picture emerges in which Ck1δ isoforms may not simply be two redundant kinases but may phosphorylate PER2 differently and in cooperation.

Work on Ck1δ and −ε have shown that PER2 phosphorylation can either accelerate or decelerate the clock depending on which residue is targeted (15, 34, 35). pS478 in mice leads to faster degradation of PER2 by the ubiquitin ligase β-Trcp (36–38), while pS659, named FASPs because its mutation in humans (S662G) causes FASPS, stabilizes PER2 and elongates period (16, 18, 34, 34, 38–40). S659 and three to four nearby downstream residues are phosphorylated in vivo, but work in vitro using Ck1δ or Ck1ε has shown that Ck1 needs a priming phosphorylation at S659 by an unknown “priming” kinase before phosphorylating downstream residues in the cluster, which is necessary for PER2 stabilization. In vitro experiments concluding that Ck1δ could not phosphorylate S659, however, used a truncated Ck1δ containing only the N-terminal kinase domain and thus gave no insights into the respective functions of Ck1δ isofoms (41).

Ck1δ1 and Ck1δ2 Lead to Different PER2 Phosphorylation Patterns. To gain insights into the respective functions of Ck1δs, we refined a previously published mathematical model of the regulation of PER2 by branched phosphorylation fates (38) to allow the rate of each phosphorylation step to be independently tuned (SI Appendix, Fig. S3A). As explained in SI Appendix, Supplemental Results, our model predicted that Ck1δ2 could be the priming kinase. To test this possibility, we transfected PER2−/−LUC MEFs with the vector mixes used in Fig. 3E, immunoprecipitated MYC-PER2, and
quantified PER2 phosphopeptides after trypsin digestion. Tetra-, tri-, di-, and unphosphorylated FASPS peptides were detected (Fig. 4A, 4I Appendix, Fig. S4, and Dataset S2). The amount of unphosphorylated FASPS peptide was highest without CK1δs, and lowest with CK1δs together. The diphosphorylated peptide, always detected without pS659, had similar levels with or without CK1δs. The level of triphosphorylated peptides showed some variations between replicates but was always highest when both CK1δ and CK1δ2 were present. Remarkably, the tetraphosphorylated FASPS peptide, with confirmed pS659 (SI Appendix, Fig. S4B), showed the most pronounced isoform-dependent differences in abundance among all PER2 phosphopeptides detected (SI Appendix, Fig. S4A and Dataset S2). It was undetected without CK1δ.

**Fig. 3.** CK1δ1 and CK1δ2 are antagonistic. CK1δ1 and CK1δ2 mRNA levels in the liver (A) and the SCN (B) across a circadian day, with opposite CK1δ1/CK1δ2 ratios mainly due to different levels of CK1δ1. Mean ± SEM analyzed by two-way ANOVA, n = 3, significant for CK1δ1 vs. CK1δ2 (P < 0.0001) and for time, only in the liver for CK1δ2 (P < 0.05). (C) Tissue-specific CK1δ1/CK1δ2 ratios measured in six different tissues. Analyzed by one-way ANOVA, P < 0.0001, followed by Bonferroni post hoc, a vs. b vs. c vs. d, indicating at least P < 0.05 significance. Results shown are mean ± SD of n = 2. (D) Immunoprecipitation shows that both CK1δ1-αMYC and CK1δ2-αHA interact with 5xMYC-PER2. Two CK1δ1-tag:CK1δ2-tag:5xMYC-PER2 ratios were used at 1:1 and 1:1.3. (E) Stable overexpression of CK1δ1 and CK1δ2 in PER2::luc MEFs leads to period shortening and lengthening, respectively. (Inset) Period analyzed by one-way ANOVA, P < 0.001, with a vs. b vs. c in Bonferroni post hoc at least P < 0.05; results shown are mean ± SEM of n = 4 monoclonal cell lines for each group. (Top) CK1δ and ACTIN immunoblots for each cell line. (F) Cotransfection of 5xMYC-PER2 with untagged CK1δ1 and/ or CK1δ2 shows higher 5xMYC-PER2 phosphorylation (and/or higher stability) when CK1δ2 is present.

**Fig. 4.** CK1δ1 and CK1δ2 cooperate in phosphorylating PER2. (A) CK1δ1 isoform-dependent PER2 FASPS phosphopeptide analysis. Data from technical replicates are provided, side by side. CK1δ peptides were also detected: the first row shows total CK1δ, and the second row corresponds to the CK1δ-specific VASSGLGSLVRH peptide. The 4 × 4 heatmaps show quantification of the FASPS peptide, sorted by number of phosphate moieties detected. The bar on the right is the color legend for each corresponding heatmap. (B) Immunoblotting of hPER2 FASPS phosphorylation by CK1δ isoforms. CK1δ and actin immunoblots are shown as reference and loading control, respectively. Graph on the right shows mean FASPS band intensity ± SEM of n = 4 independent replicate wild-type hPER2 transfections with CK1δ1, CK1δ2, and 1 + 2. *P < 0.05; **P < 0.01. (C) mPER2 was expressed in HEK293 cells in the presence of increasing amounts of vectors (10, 25, 50 ng) encoding CK1δ splice variants as indicated. Forty-eight hours after transfection, lysates were probed with anti-pS659 mPER2, anti-Myc, and anti-tubulin antibodies. (Bottom) The ratio of pS659/total PER2 as a function of CK1δ amounts.
Lack of CK1δ Shortens the Period. We next sought to silence endogenous CK1δ by siRNA in PER2::LUC MEFs. While CK1δ knock-down elongated the circadian period in a manner consistent with previous observations (31), silencing only CK1δ significantly shortened the period (Fig. 5A).

The effect of CK1δ knock-down on endogenous FASPS phosphorylation and PER2 levels was also measured. Knocking down CK1δ in human U-2 OS cells caused an observable decrease in FASPS, despite CK1δ being still present. To ascertain that the band that we detected is truly FASPS-phosphorylated endogenous PER2, as positive controls CK1δ and Per2 were also silenced, leading, as expected, to a decrease in the FASPS band intensity. In addition, diluted lysates of cells transfected with wild-type or S662G mutant hPER2 expression vector, with or without CK1δ expression vectors, confirmed the identity of the endogenous FASPS band (Fig. 5B).

According to our results so far, the consequences of PER2 phosphorylation by CK1δ or CK1ε on PER2 stability should be different: more stable when phosphorylated by CK1δ than by CK1ε due to increased FASPS phosphorylation. We tested this by transfecting hPER2 together with CK1δ or CK1ε and then treating the cells with the translation inhibitor cycloheximide (CHX). PER2 stability was highest without cotransfected CK1ε (Fig. 5C). When comparing CK1δ vs. CK1ε, not only the intensity of phosphorylated PER2 was higher with CK1δ, as observed in Fig. 3F, but the stability of total PER2 was higher in the presence of CK1δ (Fig. 5C), which explains the former observation. Surprisingly, CK1ε did not stabilize transfectant hPER2 as expected. Since the large amounts of transfected vectors may affect normal regulation or PER2 proteins, we sought to investigate the stability of PER2::LUC proteins in stably transfected cells described in Fig. 5E. These control, CK1δ-, and CK1ε-overexpressing PER2::LUC cell lines were synchronized by a dexamethasone shock, and after ~30 h (time of the first PER2 peak), were treated with CHX to assess PER2::LUC stability. In agreement with our hypothesis, while CK1ε destabilized PER2::LUC, CK1δ had a remarkable effect on its stabilization (Fig. 5C).

Finally, we sought to confirm that the long-period phenotype of the −43/−43 mice originates from a more stable PER2 protein due to an increase in CK1δ (2) expression. Primary skin fibroblasts were prepared from adult −43/−43 and wild-type mice, synchronized, and then treated after 30 h with CHX and sampled every hour to measure PER2 stability. Consistent with previous observations, −43/−43 cells had increased CK1δ expression compared with wild type, both wild-type and −43 cells mostly expressing CK1δ (Fig. 5D). In agreement with our hypothesis, PER2 was more stable in −43/−43 cells (Fig. 5D).

Discussion

We have shown that two alternative Ck1δ transcripts are regulated by m6A. When the methylation of Ck1δ 3′-UTR is inhibited or prevented by mutation, the expression of Ck1δ...
isomeric increases, affecting the circadian clock. It is likely, given that Dhp, Fhdx3, and Clock transcripts are also methylated, that global inhibition of mRNA m6A methylation would still affect the clock in ~43–43 animals, as the general disruption of mRNA processing caused by the lack of m6A would still occur. While the loss of ACS5 is embryonic lethal (31), higher expression or increased activity has been linked to cancer progression, migration, and circadian disruption (24). As we demonstrated here, the expression of CK1δ isoforms is kept to a low level by its methylated 3′-UTR. The significance of these observations goes beyond the circadian field, given the manifold functions of CK1δ. Further investigations should reveal what the respective physiological functions of CK1δ isoforms are, and additional discussion on the role and regulation of CK1δs can be found in SI Appendix, Supplemental Discussion.

Materials and Methods

Reagents were from Nacalai Tesque unless stated otherwise.

Animals. All experiments were approved by the animal experimentation committee of Kyoto University. Twelve-week-old C57BL/6 males were maintained at 23 °C ± 1 °C with 50–50% relative humidity, on a 12-h light/12-h dark cycle (lights on 8:00, lights off 20:00), food and water ad libitum. For Fig. 3 A and B, lights were permanently switched off from 8 PM on day 0, and animals were sampled from 8 AM (corresponding to circadian time 0) on day 2.

Mice with a ~43–bp deletion in the 3′-UTR of Cskn1δ by CRISPR-Cas9 were generated with the same method used for similar genetic editing (44) as described in SI Appendix, Supplemental Methods. locomotor activity recording and analysis were obtained from animals housed individually in light-tight, ventilated cages with ad libitum access to food and water. The animals were entrained on a 12-h light (~200 lx fluorescent light)/12-h dark cycle for at least 1 wk to synchronize the circadian clock to the ambient light-dark cycle, before turning lights off for the remainder of the experiment. Locomotor activity was recorded in 5-min bins with passive infrared sensors (PA5 FSR; Omnapath) and analyzed with Clockwork software (Acti-metrics) on MatLab (Mathworks). Free-running period was quantified by χ² periodogram over the entire recording in constant darkness.

In Vitro Experiments. All in vitro experiments are described in SI Appendix, Supplemental Methods.

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