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論文題目	The role of methyl cycle and N^6-methyladenosine in the regulation of biological clock		

（論文内容の要旨）

The methyl cycle is a universally conserved metabolic pathway operating in prokaryotes and eukaryotes. In this pathway, S-adenosylmethionine (SAM) is used as the methyl donor co-substrate by many methyltransferases that add methyl groups on various biomolecules such as nucleic acids and histones, which is the foundation of epigenetic regulations of spatiotemporal gene expression. The methyl cycle is indispensable for key cellular functions, and its disruption causes a variety of diseases. Previously, our lab reported that inhibition of the methyl cycle in mammalian cells leads to the lengthening of circadian clock period, suggesting that the methyl cycle may be a central regulator of gene expression and metabolism. Since the methyl cycle and the circadian clock are conserved from bacteria to humans, I decided to investigate whether the regulation of the circadian clock by the methyl cycle is also conserved across evolution. Moreover, I also further characterized the N^6-adenosine methylation (m6A) of mRNA as a putative link between the methyl cycle and the clock in mammals.

In the first chapter, I investigated the conservation of the methyl cycle as a regulator of the circadian clock across species. This study, spanning from cyanobacteria to humans, revealed an extraordinary evolutionary conservation of the link between the methyl cycle and the circadian clock. Moreover, the methyl cycle was also shown to regulate the somite segmentation clock, which is also a transcription-translation negative feedback loop (TTFL)-based timing mechanism orchestrating embryonic development in vertebrates. As mentioned above, the methyl cycle regulates the epigenome and epitranscriptome through the methylation on DNA, RNA and histone. In order to gain insights into the mechanisms involved, the circadian clock in cyanobacteria treated with a specific DNA methylation inhibitor was measured, but no significant effect was detected on the period, while a global direct methyltransferases (MTases) inhibitor and known mRNA cap methylation inhibitor, had a more pronounced effect on the period compared to the inhibition of the methyl cycle by 3-Deazaneplanocin A (DZnep). I propose the hypothesis that RNA methyltransferases, which are the oldest MTases, are major conserved mediators between the methyl cycle and the biological clock. Supporting this notion, previous results also revealed that specific inhibition of m6A in mRNA was sufficient to elicit period lengthening in mammals. It is therefore likely that RNA methylation, especially m6A, at least partially contributes to the period lengthening observed across species.

Based on these findings, in Chapter 2 I then focused on uncovering the mechanisms linking m6A and the circadian clock in mammals. The m6A is a prevalent and well-studied RNA modification in mammalian cells that has been implicated to be critical in various biological processes. Three types of enzymes are responsible for maintaining the balance of m6A modification: the “writers”, the “erasers” and the “readers”, the latter being the effectors of epitranscriptomic regulation. The present study mainly focuses on the cytoplasmic reader YTHDF2, which is the most well-studied m6A reader among the other homologues YTHDF1 and 3. Here, I found that *Ythdf2* knock-down (KD) by RNAi leads to an increased circadian period in mouse cells, and *Ythdf2* knock-out (KO) by CRISPR-CAS9 in mouse embryonic fibroblasts further confirmed these

results. I observed that the stability of major TTFL-clock transcripts increased after *Ythdf2* deletion. Next-generation mRNA sequencing revealed that YTHDF2 targets *Per2*, *Cipc* and *Rora* mRNAs, coding for essential clock components. I also observed that YTHDF2 localization is dynamic and, under heat shock, relocated mainly from a diffuse cytoplasmic distribution to stress granules. This discovery might provide a clue to further clarify the role of YTHDF2 in the regulation of the circadian clock. Overall, YTHDF2 was confirmed as an important modulator of circadian rhythms.

In Chapter 3, my study delved deeper into how m6A regulates the expression of Casein Kinase 1 Delta (CK1δ), a critical kinase that controls circadian rhythms notably via phosphorylating PER2. Studying CK1δ would help understand the link between m6A and the circadian clock and potentially find a new therapeutic strategy for Familial Advanced Sleep Phase Syndrome (FASPS), a circadian sleep disorder originating from a deficiency in PER2 phosphorylation by CK1δ. Interestingly, two alternatively spliced isoforms of *Ck1δ* were discovered, *Ck1δ1* and *Ck1δ2*, both bearing a highly m6A-methylated site in the 3'-UTR. Using *in vitro*-transcribed mRNA coupled with *in vitro* translation assays, I revealed that m6A is an intrinsic negative regulator of *Ck1δ* translation and stability. Since the *Ck1δ* transcripts are methylated in the 3'-UTR, close to the STOP codon, I further investigated the overall role of the 3'-UTR of *Ck1δ* transcripts and revealed that it acts as a limiting factor for *Ck1δ1* and *Ck1δ2* translation: deletion of the 3'-UTR leads to increased translation of *Ck1δ1* and *Ck1δ2*.

In conclusion, these studies revealed and clarified the role of the methyl cycle in the regulation of the biological clock, demonstrating the extraordinary evolutionary conservation of the link between the methyl cycle and the circadian clock. Focusing on m6A methylation as a potential effector, I identified clock targets of the key m6A reader YTHDF2 that mediates the epitranscriptomic regulation of circadian rhythms. These new insights may lay the foundation in pursuit of novel therapeutics to treat diseases related to biological rhythms disorders.

（続紙 ２ ）

（論文審査の結果の要旨）

メチルサイクルは、地球上のほぼすべての生命に普遍的に保存された代謝経路である。この経路では、S-アデノシルメチオニン（SAM）がメチル基ドナー補助基質となり、遺伝子発現の時空間的制御を司るエピジェネティックあるいはエピトランスクリプトーム制御の基盤となるDNA、RNA、ヒストンや転写因子の化学修飾基としてメチル基が提供される。哺乳類細胞を用いた検討から、メチルサイクルの阻害は概日時計の周期延長につながることが示されていたが、哺乳類以外の生物種での検討がなされていなかった。このような中、YE Shiqi氏は本論文の第一章において、メチルサイクルによる概日時計の調節が、藍藻から、植物、線虫、昆虫、非哺乳類脊椎動物全般に保存されることを明らかにした。さらに、第二章・第三章では、このメチルサイクルと概日時計の分子的接点に関する詳細な検討が時遺伝子のmRNAのN6-アデノシンメチル化（m6A）を中心になされた。第二章ではとくに、m6A修飾RNA結合タンパク質YTHDF2を介した時計遺伝子のmRNA安定性および翻訳効率調節に関する知見がもたらされ、第三章ではmRNAの3’非翻訳領域が高度にm6A化される時計タンパク質キナーゼCK1Dにおけるm6A修飾の役割が試験管内翻訳反応を用いて明らかにされた。したがって、本論文は、メチルサイクルを介した概日時計機構の進化保存性およびその背後の分子メカニズムに迫る重要な知見をもらしたといえる。よって、本論文は博士（薬科学）の学位論文として価値あるものと認める。また、2019年8月22日、論文内容とそれに関連した事項について試問を行った結果、合格と認めた。

なお、本論文は、京都大学学位規程第14条第2項に該当するものと判断し、公表に際しては、当分の間、当該論文の全文に代えてその内容を要約したものとすることを認める。

要旨公表可能日：　　年　　月　　日以降