様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

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科学研究費助成事業　　研究成果報告書

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研究課題名（和文）Translation control of circadian rhythms

研究課題名（英文）Translation control of circadian rhythms

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研究成果の概要（和文）：マウスの肝臓で24時間のリボソームプロファイリングを行い、特定の概日遺伝子のRNAおよびタンパク質発現量と比較した。いくつかのmRNAの上流のオープンリーディングフレーム（uORF）が、下流のコーディング領域でのリボソームの結合を抑制することを発見した。さらに、uORFの数と長さに対するその抑制の程度と、個々の細胞での抑制を調べた。主要な概日遺伝子であるPeriod2のuORFを変異させたマウスでは、オス・メス両方で野生型の同腹仔に比べて有意に睡眠が減少した。これはRNA翻訳の概日リズム制御によりマウスの行動を生理的に変化できることを示唆しており、次世代RNA治療薬としての可能性が示唆される。

研究成果の学術的意義や社会的意義
Defects in circadian rhythms are related to health issues, such as obesity and depression, and understanding how translation is regulated is important for developing new treatments. Our results suggest that non-coding RNA structures alter translation, which has physiological consequences on sleep.

研究成果の概要（英文）：We used a method called ribosome profiling to understand RNA translation in mice liver over a 24-h period, and compared these results to RNA and protein levels for select circadian genes. We discovered that upstream open reading frames (uORFs) in some mRNAs suppress the degree of ribosome binding in the downstream coding region. We explored how the number and length of uORFs affect their degree of downstream repression and examined uORF repression in individual cells. We mutated the uORF in a central circadian gene Period2 and found that both male and female mutant mice had significantly reduced sleep compared to their wild-type littermates. Thus, our research suggests that circadian control of RNA translation can physiologically alter mice behavior and has implications for developing the next generation of RNA therapeutics.

研究分野：Biology at cellular to organismal levels

キーワード：Ribosome profiling　Circadian rhythms　Period2　Translation　uORF　RNA structure　Sleep

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様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９（共通）

１．研究開始当初の背景

Introduction: Life is remarkably adapted to the 24-hour rotational movement of the earth. In mammals, the molecular time-keeping mechanism for circadian rhythms relies primarily on a hierarchical network of transcription activators and repressors in cells and tissues (Mohawk et al., 2012). In the past, circadian clocks have been measured using systems approaches to measure genome-wide changes in RNA levels (Millius and Ueda, 2017), which has resulted in understanding the transcriptional regulatory network, but less is known how translation and post-transcriptional regulation influence biological rhythms.

Although 10% of genes are rhythmic in the liver (Akhtar et al., 2002), de novo transcription is only responsible for a small fraction of this rhythmicity (Koike et al., 2012). Thus, gene expression studies using microarrays and RNA-sequencing may not correlate with translation of the corresponding mRNA nor with protein abundance (Gygi et al., 1999). Proteomic studies have revealed a number of proteins with 24-h rhythms without a corresponding rhythmic RNA transcript (Lim and Allada, 2013; Reddy et al., 2006), which may suggest a role for translation in regulating the clock (Mauvoisin et al., 2014).

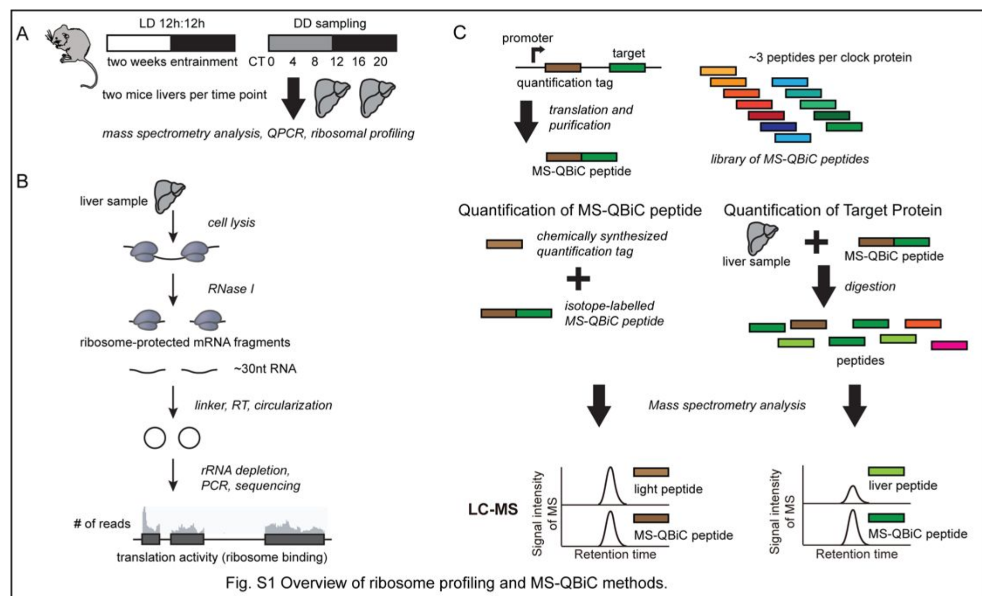

Fig. S1 Overview of ribosome profiling and MS-QBiC methods.

In mouse liver, systems studies of the proteome are unable to detect low-abundant components of the core circadian circuit (Mauvoisin et al., 2014; Robles et al., 2014), unless special care is taken to examine a particular protein on a case-by-case basis (Narumi et al., 2016). Thus, researchers have begun to use next-generation sequencing techniques of ribosome-bound mRNA protected from RNAse degradation (Ingolia et al. 2009) as a proxy for protein abundance and to understand how translation regulation affects protein abundance. Previous studies using ribosomal profiling to measure daily rhythms focused on a cell culture model (Jang et al., 2015) or mouse tissues in light-dark conditions (Castelo-Szekely et al., 2017; Janich et al., 2015), which reflect diurnal gene expression systems that may be influenced by non-circadian time-keeping systems. These studies also examined RNA expression to compare the timing between transcription and translation, but it remains unclear how translated RNA is converted into protein both in terms of efficiency and timing. For example, production of PER and CRY proteins are delayed relative to expression of their mRNA in the liver (Lee et al., 2001) and SCN (Field et al., 2000). This difference in timing may result from a delay in RNA processing before translation, such as export

[1. Research Objectives, Research Method, etc. (continued from the previous page)]

from the nucleus or poly-adenylation, or a delay after translation, such as translation termination, protein folding, or degradation (Honkela et al., 2015; Liu et al., 2016). We used ribosomal profiling to understand RNA translation in liver from circadian entrained mice transferred to constant darkness conditions over a 24-h period compared to absolute protein levels from 20 selected circadian proteins (Fig. S1).

２．研究の目的

Impact: Circadian rhythms are linked to a wide array of biological processes including sleep, metabolism, and proper immune system functioning. Defects in circadian rhythms are related to cancer, depression, and obesity. Therefore, understanding how mRNA translation is modulated throughout the day is crucial for developing new treatments and understanding human health. Importantly, most mRNAs that form the core circuit for circadian rhythms have one or more uORFs like *Period2*. Understanding the uORF regulatory module and how other trans-acting factors modulate translational repression could suggest new avenues for RNA therapy and lead to human studies examining the impact of uORFs in a wide range of disorder

３．研究の方法

Methods: Previously, we developed a mass spectrometry method called MS-based Quantification By isotope-labeled Cell-free products (MS-QBiC) to determine the absolute protein levels of 20 selected circadian proteins in mice liver over a 24-h period (Narumi et al., 2016). The method takes advantage of a reconstituted cell-free protein expression system term the PURE system (Shimizu et al., 2001) to synthesize optimal peptide standards for detection and quantification using SRM-based targeted proteomics analysis. We found delays between the peak level of RNA expression measured by quantitative PCR (qPCR) and the corresponding protein, which suggests a delay in post-transcriptional RNA processing. Here, we investigated the same liver samples by ribosomal profiling to understand the timing and efficiency of translation relative to RNA expression and protein production. We observed more delay between the peak of translation and protein production compared to the peak between transcription and translation. In addition, we found that upstream open reading frames (uORFs) disrupted translation globally, repressed reporter expression in a combinatorial manner, disrupted production of PER2 protein using CRISPR-Cas9 homologous by generating a *Per2* uORF mutant mouse model, and found that mutant mice have significantly reduced sleep compared to their wild-type littermates.

４．研究成果

Results: An experimental workflow used to analyze ribosome-protected mRNA fragments from liver samples previously examined by MS-QBiC (Narumi et al., 2016) is shown (Fig. S1). Briefly, mice were entrained to a 12-h light/12-h dark cycle for 2 wk, transferred to constant darkness for 24 h, and liver samples from two mice were collected and analyzed at circadian times (CT0, CT4, CT8, CT12, CT16, and CT24) according to (Ingolia et al., 2012). We prepared ribosome profiling libraries and sequenced ~70 million reads per sample, which yield 25-45 million reads mapped to mRNA. Ribosome-protected fragments primarily aligned to the coding region and 5' untranslated region (5' UTR) of mRNA with few reads mapping to the 3' untranslated region (3'UTR) (Fig. S2a). Alignment of CDS-mapping reads according to (Ingolia et al., 2011) based on the footprint length revealed reading frame periodicity. Reads were of the expected size, mapped with a high percentage to mRNA, and were correlated between samples.

From ~14,000 well-translated transcripts with average of mapped read density of at least one across the coding region, we identified rhythms in ribosome-protected read fragments using the JTK_CYCLE algorithm

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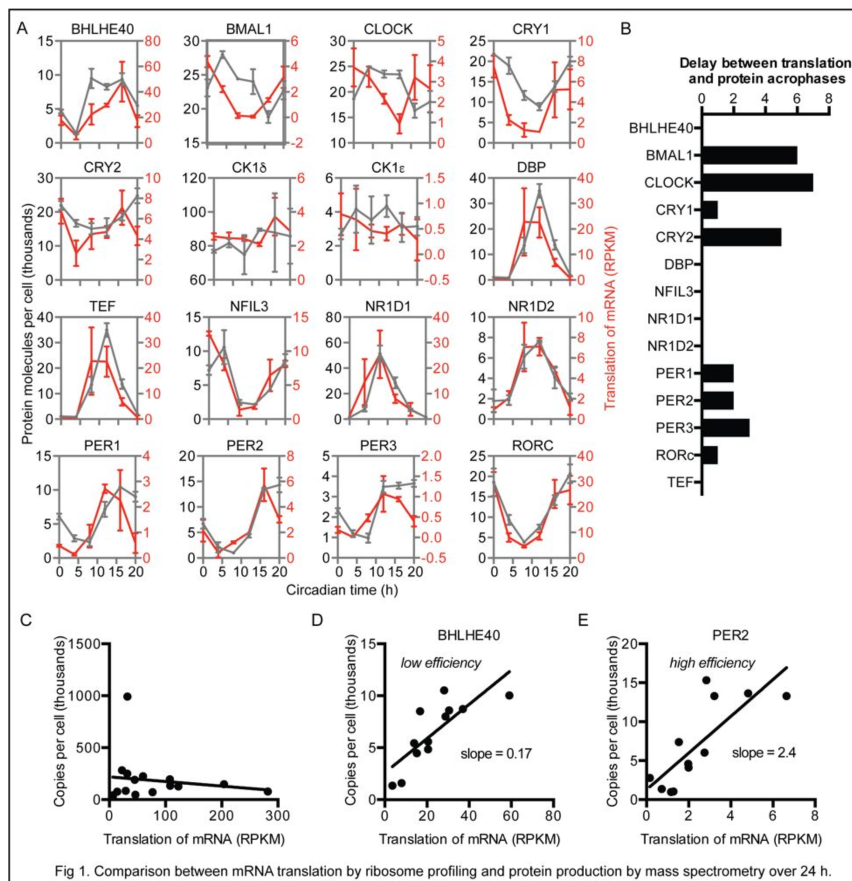

Fig 1. Comparison between mRNA translation by ribosome profiling and protein production by mass spectrometry over 24 h.

(Hughes et al., 2010) and found 2952 rhythmic transcripts with an adjusted p-value < 0.05, which included well-known circadian transcripts such as *Bmal1, Per1, Per2, Clock,* and *Cry1*.

We compared the timing of translation as measured by ribosome profiling reads to that of absolute protein molecules per cell for 16 selected core circadian proteins as determined by (Narumi et al., 2016). There was broad agreement in the timing of translation compared to that of protein levels (Fig. 1A), but for several circadian proteins, such as BMAL1 and CLOCK, there was a significant delay between translation and protein acrophases (Fig. 1B). We examined the average number of ribosome profiling reads over a 24-h period and the number of protein molecules to determine if ribosome profiling could provide a rough snapshot of the average protein levels over the course a day, but found remarkably little correlation (Fig. 1C). Thus, depending on the protein, ribosome profiling reads may serve as a proxy for quantitative proteomics at individual time points but post-transcriptional and post-translation mechanisms may delay protein production (Kojima et al., 2011).

Next, we investigated the efficiency of protein production based on ribosome profiling reads. For some proteins, such as BHLHE40, a large amount of translation resulted in a moderate amount of protein (Fig. 1D) whereas for other proteins, such as PER2, a much smaller amount of translation produced the same amount of protein as BHLHE40 (Fig. 1E). We examined how the timing and amount of RNA transcription as measured by

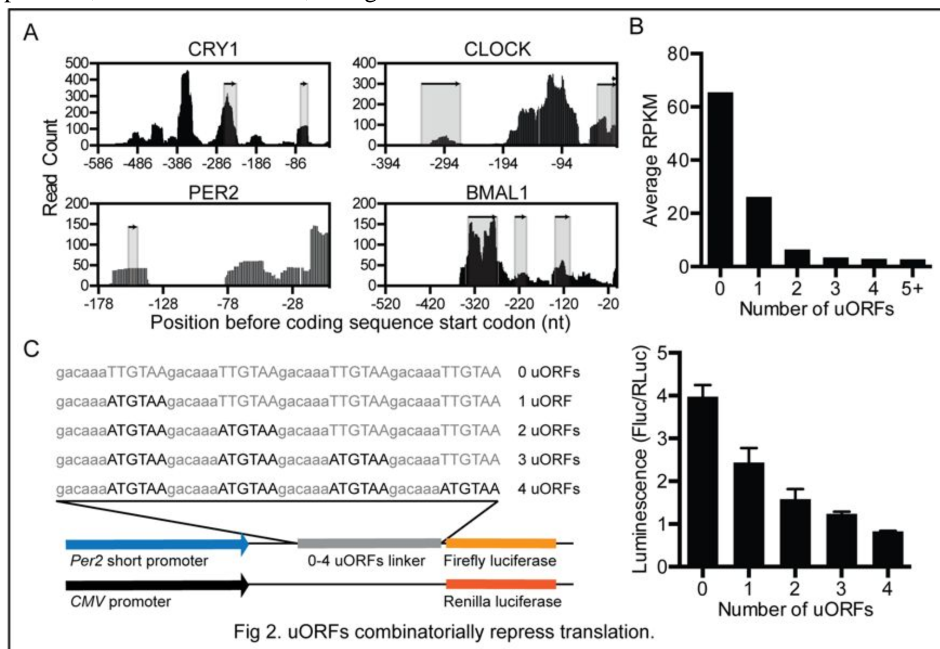

Fig 2. uORFs combinatorially repress translation.

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qPCR (Narumi et al., 2016) related to the ribosome profiling reads and found a tight correlation between transcription and translation as observed in other ribosome profiling studies of circadian transcripts (Jang et al., 2015; Janich et al., 2015).

Similar to previous reports (Jang et al., 2015; Janich et al., 2015), we observed a correlation between ribosome occupancy and upstream open reading frames (uORFs). In particular, for circadian transcripts, such as *Cry1* and *Bmal1*, there appeared to be increased ribosome binding in uORF regions (Fig. 2A). mRNAs with increased numbers of uORFs had lower levels of ribosome occupancy in the downstream coding region (Fig. 2B), whereas the length of the uORF and the distance of the uORF to the start codon did not have a significant impact on ribosome binding in the downstream coding region. To investigate whether uORFs were sufficient to suppress translation in a combinatorial manner, we created a luciferase reporter vector with multiple synthetic uORFs. Predictably, increasing the number of uORFs reduced luminescence from the reporter (Fig. 2C). Thus, uORF number, but not their length or distance to the start codon, are sufficient to post-transcriptionally repress translation.

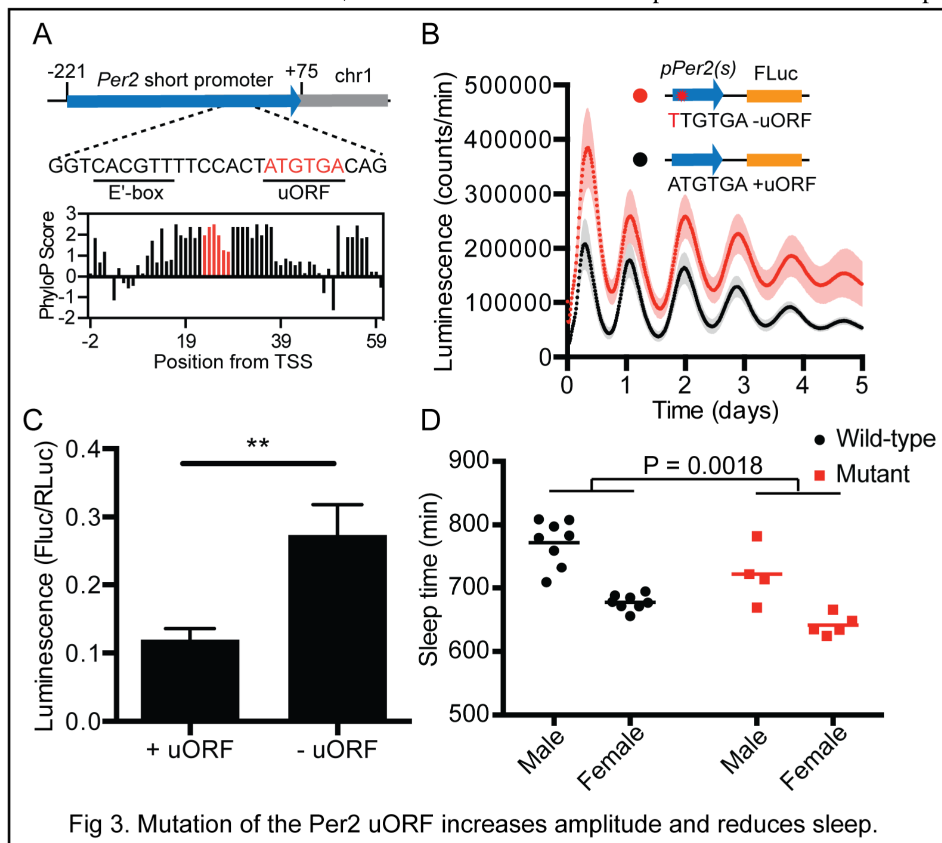

Fig 3. Mutation of the Per2 uORF increases amplitude and reduces sleep.

Next we focused on the uORF in the circadian transcript *Per2* because it's evolutionarily conserved and short (Fig. 3A), which eliminates the effects of translated peptides on the regulation of *Per2*. Mutation of the uORF increased the amplitude of expression without affecting the phase or period (Fig. 3B, C), and this increase in amplitude was not affected by the amount of transfected plasmid, inclusion of the full-length Per2 5'UTR, or addition of PER2 protein. Finally, we created a mouse line with a mutation in the *Per2* uORF and found significantly reduced sleep in male and female mutant mice compared to their wild-type littermates (Fig. 3D). Thus, uORFs can alter the translational amplitude of protein production, which can have effects on sleep and circadian rhythms.

５．主な発表論文等

〔雑誌論文〕　計2件（うち査読付論文　2件／うち国際共著　2件／うちオープンアクセス　2件）

１．著者名 Millius A, Ode KL, Ueda HR.	４．巻 eCollection 2019
２．論文標題 A period without PER: understanding 24-hour rhythms without classic transcription and translation feedback loops.	５．発行年 2019年
３．雑誌名 F1000Res.	６．最初と最後の頁 Rev-499
掲載論文のDOI（デジタルオブジェクト識別子） 10.12688/f1000research.18158.1	査読の有無 有
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１．著者名 Millius A, Ode KL, Ueda HR.	４．巻 Rev-499
２．論文標題 A period without PER: understanding 24-hour rhythms without classic transcription and translation feedback loops.	５．発行年 2019年
３．雑誌名 F1000 Research	６．最初と最後の頁 eCollection
掲載論文のDOI（デジタルオブジェクト識別子） 10.12688/f1000research.18158.1	査読の有無 有
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〔学会発表〕　計3件（うち招待講演　0件／うち国際学会　3件）

１．発表者名 Arthur Millius
２．発表標題 Understanding Circadian Translation and Upstream Open Reading Frames
３．学会等名 14th Annual Meeting of the Oligonucleotide Therapeutics Society（国際学会）
４．発表年 2018年

１．発表者名 Arthur Millius
２．発表標題 Understanding Circadian Translation and Upstream Open Reading Frames
３．学会等名 15th Annual Scientific Meeting of the Australasian Chronobiology Society（国際学会）
４．発表年 2018年

１．発表者名 Arthur Millius
２．発表標題 Circadian ribosomal profiling and analysis of upstream open reading frames (uORFs)
３．学会等名 Society for Research on Biological Rhythms, Amelia Island, Florida（国際学会）
４．発表年 2018年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関