The RNA-binding protein hnRNP Q represses translation of the clock gene Bmal1 in murine cells

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Running Title: hnRNP Q regulates Bmal1 translation

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Keywords: circadian rhythm, translational control, RNA-protein interaction, RNA-binding protein, heterogeneous nuclear ribonucleoprotein (hnRNP), hnRNP Q, brain and muscle ARNT-like 1 (BMAL1), -aryl hydrocarbon receptor nuclear translocator like (ARNTL), post-transcriptional regulation

ABSTRACT

Most living creatures have a circadian rhythm that is generated by precisely regulated transcriptional-translational feedback loop of clock genes. Brain and muscle ARNT-like 1 (BMAL1) is one of the core clock genes and transcription factors, which represents a positive arm of this autoregulatory circadian clock system. Despite the indispensable role of BMAL1 in circadian rhythm, the molecular mechanisms underlying translational control of BMAL1 are largely unknown. Here, using murine NIH-3T3 cells, gene constructs and a variety of biochemical approaches, including RNAi- and luciferase reporter gene-based assays, along with immunoblotting, in vitro transcription, RT-qPCR, and real-time bioluminescence experiments, we show that the translation of Bmal1 is negatively regulated by an RNA-binding protein, heterogeneous nuclear ribonucleoprotein Q (hnRNP Q). Interestingly, we find that hnRNP Q rhythmically binds to a specific region of Bmal1 mRNA 5'UTR and controls its time-dependent expression. Moreover, we demonstrate that knockdown of hnRNP Q modulates BMAL1 protein oscillation amplitude without affecting mRNA rhythmic patterns. Furthermore, hnRNP Q depletion increases mRNA oscillation amplitudes of BMAL1-regulated target genes. Together, our results suggest that hnRNP Q plays a pivotal role in both Bmal1 translation and BMAL1-regulated gene expression.

INTRODUCTION

The majority of living organisms on earth have a 24-hour period circadian clock that is responsible for physiological and behavioral rhythmic phenomena. These rhythmic processes are precisely maintained by transcriptional-
translational feedback loops of several circadian clock genes (1). A master clock of the circadian system is in the hypothalamic suprachiasmatic nucleus (SCN) of the brain (2), and this central pacemaker synchronizes various peripheral clocks via the combination of neural and humoral signaling (3). Peripheral tissues also harbor their own clock, and most cells, even in cultured fibroblasts (4,5), possess self-sustained and cell-autonomous circadian rhythm oscillator. This endogenous cellular oscillator is composed of an autoregulatory feedback loop, which includes positive elements of Bmal1 and Clock heterodimer that activates transcription of Period (Per), Cryptochrome (Cry), Rev-erba, and Rora. In addition, there is a negative arm of this internal oscillator, which is Per and Cry heterodimer that suppresses the function of Bmal1 and Clock complex. Tightly regulated expression of these core clock genes is essential for maintaining the biological rhythm of the organism.

Among the core clock genes, Bmal1 received much attention since it has critical roles in circadian rhythms as well as various physiological processes. For example, the absence of Bmal1 results in immediate and complete impairment of circadian rhythmicity, and shows reduced locomotor activity (6). This reduced activity is later explained by the report that Bmal1-deficient mice develop joint ankylosis due to the ossification in tendons and ligaments of hind limbs (7). In addition, both glucose metabolism and insulin signaling are abolished in Bmal1 mutant mice, leading to hypoinsulinaemia and diabetes (8,9). It is reported that the embryonic fibroblasts of Bmal1 knockout mice fail to differentiate into adipocytes. Also, Bmal1 overexpression in adipocytes upregulates lipid synthesis activity (10). Moreover, the loss of Bmal1 results in shortened life spans and several early aging phenotypes including cataracts, sarcopenia, organ shrinkage, and less subcutaneous fat (11,12). Furthermore, Bmal1 deletion affects the degeneration of synaptic terminals and diminishes cortical connectivity, which promotes neurodegeneration (13).

Given the importance of Bmal1 in circadian timing and other physiological phenomena, the expression of Bmal1 needs to be precisely regulated. Previous studies have described diverse regulatory mechanisms of Bmal1 expression including transcriptional and post-translational regulations. For instance, Rev-erba and Rora suppresses and enhances Bmal1 transcription, respectively (14-17). Also, Bmal1 protein is known to be phosphorylated by casein kinase 1ε (CK1ε) (18), mitogen-activated protein kinase (MAPK) (19), casein kinase 2α (CK2α) (20), and glycogen synthase kinase 3β (GSK3β) (21) to mediate timekeeping mechanism of clock system. In addition, it is reported that SUMOylation of Bmal1 regulates circadian rhythmicity (22). Moreover, ubiquitin protein ligase E3A (UBE3A) is described to interact with and degrade Bmal1 protein (23). However, to date, translational regulation of Bmal1 mRNA remains poorly understood. Therefore, we sought to find the mechanism of Bmal1 protein synthesis. Emerging evidence reported that RNA-binding proteins play a central role in the translational modulation (24). We have previously demonstrated that various RNA-binding proteins have an important function in translational control of several clock genes (25-30). Therefore, we hypothesized that specific RNA-binding protein may have a critical role in Bmal1 mRNA translation. In this study, we determine that an RNA-binding protein hnRNP Q has a critical role in translational control of Bmal1 mRNA. Also, we show that hnRNP Q binds to specific region of Bmal1 mRNA 5’ untranslated region (UTR) and negatively regulates Bmal1 mRNA translation. Moreover, we demonstrate that this translation is controlled by rhythmic interaction between hnRNP Q and Bmal1 mRNA. Furthermore, hnRNP Q-mediated translational repression of Bmal1 increases mRNA oscillation amplitude of Bmal1 target genes, suggesting that this translational control is critical for the circadian clock system.

RESULTS

hnRNP Q negatively regulates Bmal1 translation

Maintenance of circadian rhythm depends on
oscillation of biological clock gene expression. Post-transcriptional regulations of rhythmic genes are one of the important regulatory mechanisms. In these regulations, RNA-binding proteins have a critical role in governing the life of mRNAs. Although a recent report demonstrated that over 1,500 proteins can interact with RNAs (31), Bmal1 mRNA-binding proteins are poorly understood. In addition, molecular mechanisms in the post-transcriptional regulation of Bmal1 mRNA are still unknown. We, therefore, focused on the post-transcriptional control of Bmal1 mRNA. First, we performed in silico analysis of which RNA-binding proteins could interact with Bmal1 mRNA. As a result, several hnRNPs were expected to bind to Bmal1 mRNA. To identify specific RNA-binding proteins that control Bmal1 expression, we performed RNAi screening by using three different siRNAs targeting hnRNP Q, hnRNP K, or hnRNP I (Fig. S1A). These hnRNPs are known to have a critical role in the post-transcriptional regulations of specific mRNAs (25,26,32-36). Among three siRNAs, only hnRNP Q knockdown caused an increase of Bmal1 protein level (Fig. 1A). To determine whether this result comes from the upregulated Bmal1 mRNA level or stability, we analyzed Bmal1 mRNA level and decay kinetics after hnRNP Q depletion. The result showed that reduction of hnRNP Q does not significantly alter Bmal1 mRNA level and stability (Fig. 1B and C), suggesting that hnRNP Q may inhibit the translation of Bmal1 mRNA.

Because 5’UTR of mRNA has a critical role in the translational regulation (37), Bmal1 5’UTR could be associated with the protein synthesis inhibition mediated by hnRNP Q. By utilizing luciferase reporter system, we sought to verify whether hnRNP Q directs translational repression of Bmal1 via its 5’UTR. The Bmal1 5’UTR sequences were inserted into upstream of firefly luciferase (Fluc) coding sequence of reporter vector. NIH-3T3 cells were transfected with the reporters after hnRNP Q depletion, and its luciferase activities were analyzed. The reduction of hnRNP Q significantly increased Bmal1 5’UTR-harboring reporter activity compared to control reporter (Fig. 1D). To investigate whether the upregulated reporter expression came from translational activation, we analyzed mRNA level of reporters by using qPCR. The result showed unaltered reporter mRNAs level (Fig. S1B), suggesting that hnRNP Q mediates Bmal1 5’UTR when it negatively regulates Bmal1 translation. Next, to further confirm the importance of hnRNP Q in Bmal1 protein synthesis, we performed polysome profiling analysis with or without knockdown of hnRNP Q. The overall ribosome distributions in sucrose gradient were not changed after hnRNP Q depletion (Fig. 1E). This observation was validated by the result where GAPDH mRNA levels were not significantly altered in monosome or polysome fractions after hnRNP Q silencing (Fig. 1F). When hnRNP Q was reduced, however, the distribution of Bmal1 mRNA was shifted from monosome to polysome fraction, indicating an upregulation of Bmal1 mRNA translation (Fig. 1G). These results indicate that hnRNP Q mediates downregulation of Bmal1 protein level by inhibiting Bmal1 mRNA translation through its 5’UTR.

hnRNP Q binds to specific region of Bmal1 mRNA 5’UTR

To determine whether hnRNP Q could bind to Bmal1 5’UTR, we conducted in vitro binding assay using biotin-conjugated Bmal1 5’UTR and cell lysates. While biotinylated Bmal1 5’UTR alone pulled down hnRNP Q protein, this binding affinity was dramatically reduced in combination with a competitor (Fig. 2A), suggesting that it is a specific interaction between Bmal1 5’UTR and hnRNP Q. Moreover, we further confirmed this interaction with GAPDH coding sequences (CDS). Biotin-conjugated GAPDH CDS could not promote hnRNP Q binding, and the interaction between hnRNP Q and Bmal1 5’UTR was not inhibited after the addition of GAPDH CDS as a non-specific competitor (Fig. S2A). Since most RNA-binding proteins interact with stem-loop elements of mRNA, we predicted possible secondary structures of Bmal1 5’UTR by in silico analysis. Assuming that hnRNP Q may bind to specific secondary structures of Bmal1 5’UTR, we generated serially deleted Bmal1 5’UTR constructs that excluded each secondary structures to potentially abolish the binding of
hnRNP Q (Fig. 2B). To discover binding sites of hnRNP Q in Bmal1 5'UTR, we performed in vitro binding assay with biotin-incorporated partially deleted Bmal1 5'UTR. The result demonstrated that hnRNP Q binds to full-length and 182 nucleotides deleted (Δ182) constructs of Bmal1 5'UTR. However, 399 nucleotide truncated (Δ399) mutant showed significantly reduced affinity with hnRNP Q (Fig. 2C), suggesting that binding sites of hnRNP Q resides in the residues 1-399 of the Bmal1 5'UTR. We, then, asked if only 1-182 residues of Bmal1 5'UTR could facilitate hnRNP Q interaction. The result showed that minimal region of 1-182 could promote this binding (Fig. S2B). Next, we wondered whether this binding pattern affects the translational efficiency of Bmal1. To test this possibility, we performed reporter assay with mutant constructs after reduction of hnRNP Q. Interestingly, the result showed an increase of Fluc level in control cells transfected with the Δ399 construct. Moreover, hnRNP Q depletion significantly enhanced Fluc expression in Bmal1 5'UTR full and Δ182 constructs, but this upregulation was not observed in Δ399 construct which previously showed weak binding affinity with hnRNP Q (Fig. 2D). These results suggest that hnRNP Q interacts with residues 1-399 of Bmal1 5'UTR to inhibit translation process.

hnRNP Q plays a critical role in Bmal1 protein oscillation

Since Bmal1 plays a major role in the circadian system, we speculated that hnRNP Q-mediated translational control may lead to crucial consequence in circadian rhythmicity. To investigate physiological significance of Bmal1 translational repression mediated by hnRNP Q, we reduced hnRNP Q and synchronized circadian time by dexamethasone treatment to analyze Bmal1 oscillation profile. Silencing of hnRNP Q did not alter the Bmal1 mRNA oscillation profiles (Fig. 3A), however, interestingly, the amplitude of Bmal1 protein oscillation was significantly enhanced by hnRNP Q depletion (Fig. 3, B-D). This implies a critical role of hnRNP Q in Bmal1 protein synthesis. To further confirm this conclusion, we tested polysome profiles with or without hnRNP Q knockdown at certain circadian time points. The overall ribosome profiles were not significantly altered by hnRNP Q knockdown at indicated circadian time points (Fig. S3A). This was confirmed through control GAPDH mRNA (Fig. S3B). However, Bmal1 mRNAs were significantly shifted from monosome to polysome in hnRNP Q depleted condition. Also, as shown above, Bmal1 protein reaches peak level at 12 h after synchronization. In accordance with this, Bmal1 mRNAs were highly associated with polysome fractions with or without hnRNP Q depletion at 12h after synchronization (Fig. S3C). To further examine whether hnRNP Q negatively regulates Bmal1 circadian expression, we monitored Bmal1 rhythmic oscillations in real-time mode using a reporter vector, in which luciferase is fused with the Bmal1 promoter (Bmal1::Luc) (38). We inserted Bmal1 5'UTR at the downstream of the promoter (Bmal1-5'UTR::Luc) to test the function of hnRNP Q in circadian rhythmicity of Bmal1. NIH-3T3 cells were transfected with reporters in the presence or absence of hnRNP Q siRNA, and were synchronized by dexamethasone treatment. We found that hnRNP Q silencing did not affect oscillation pattern in Bmal1::Luc reporter transfected cells (Fig. 3, E-G). On the other hand, the oscillation amplitudes were significantly enhanced in Bmal1+5'UTR::Luc reporter expressing cells under hnRNP Q reduction (Fig. 3, H-J). These results demonstrate that hnRNP Q is a decisive factor in translation as well as circadian oscillation of Bmal1.

Given that protein level of hnRNP Q is relatively constant after synchronization, we wondered how hnRNP Q demonstrates the regulation of oscillatory translation of Bmal1. We postulated that hnRNP Q might rhythmically interact with Bmal1 mRNA to exhibit translational control. To examine our hypothesis, we performed RNA-IP with hnRNP Q antibody after synchronization. The results showed that immunoprecipitated hnRNP Q level and total hnRNP Q protein level remained relatively constant during the experimental time period (Fig. 4A). Also, Bmal1 mRNA was pulled-down by hnRNP Q antibody compared to IgG control (Fig. 4B). Interestingly, co-immunoprecipitated Bmal1
mRNA had its lowest level at 12 h after synchronization (Fig. 4C), which was peak time of Bmal1 protein expression (Fig. 3D). These results suggest that hnRNP Q interacts with Bmal1 mRNA in a time-dependent manner to mediate translational repression.

It is well known that Bmal1 activates transcription of several target genes including Cry, Per, Rev-erbα, and Rora. We asked whether hnRNP Q-induced Bmal1 translational inhibition affects transcriptional activity of those target mRNAs. To test this possibility, we observed mRNA oscillation profiles of Bmal1 target genes with or without hnRNP Q silencing. Remarkably, Cry1, Per1, Rev-erbα, and Rora mRNA oscillation amplitudes were enhanced under hnRNP Q reduction (Fig. 5, A-D). Together, these results suggest that hnRNP Q-mediated translational repression of Bmal1 affects the oscillation patterns of Bmal1 target genes.

DISCUSSION

To date, most molecular studies of circadian rhythmicity have focused on the transcription and translation feedback loops of circadian genes to illustrate their rhythmic expressions. Interestingly, however, a recent study has demonstrated that only 22% of cycling mRNAs are driven by de novo transcription (39), suggesting that post-transcriptional regulations have a major role in circadian system. In line with this report, accumulating evidence show that several clock genes are controlled by post-transcriptional regulations, such as mRNA splicing, decay, and translation (40,41). Especially, we previously demonstrated that RNA-binding proteins play major roles in these regulatory mechanisms. For instance, hnRNP R, hnRNP Q, and hnRNP L cooperatively destabilize arylalkylamine N-acetytransferase (AANAT) mRNA (42). In addition, the IRES-mediated translation of Rev-erbα mRNA is modulated by hnRNP Q and PTB (32). In the present study, we found the novel function of RNA-binding protein hnRNP Q in the translational control of core clock gene Bmal1.

Although our results present several lines of evidence demonstrating the hnRNP Q-mediated translational inhibition of core clock gene Bmal1, we could not specify clear molecular mechanism how hnRNP Q suppresses Bmal1 protein synthesis. Unlike previous studies performed with rat pineal glands (25,28), hnRNP Q protein levels were relatively constant in the mouse fibroblast cell line. The binding between hnRNP Q and Bmal1 mRNA was rhythmic, and this interaction anti-correlated with the Bmal1 protein oscillatory pattern. Mechanisms underlying this rhythmic binding process need to be further investigated. For instance, post-translational modifications on hnRNP Q, including phosphorylation, may influence the affinity of hnRNP Q to Bmal1 mRNA. Although it is not known which kinase could phosphorylate hnRNP Q, it will be interesting to see whether specific kinase has circadian oscillatory activity. Since the secondary structures of mRNA are important in interaction with RNA-binding proteins, the stem-loop structure of Bmal1 mRNA may vary in time-dependent manner that allows different affinity with hnRNP Q. Also, it will be more interesting to see whether hnRNP Q downregulates translation efficiency through a competition with translation initiation factors. Moreover, scanning by preinitiation complex may be stalled in Bmal1 mRNA by strong binding between hnRNP Q and Bmal1 mRNA.

hnRNP Q is known to be ubiquitously expressed in heart, brain, lung, liver, and many organs (43), and currently, hnRNP Q knockout mice is not available. Although generating mutant mice may be technically challenging, it will be valuable to test whether this hnRNP Q-mediated translational repression of Bmal1 could affect circadian rhythm of the organism.

It is well known that Bmal1 is a main component of circadian clock system and works as a transcription factor alongside of a protein, Clock. Interestingly, as expected, hnRNP Q knockdown enhanced mRNA oscillation of Bmal1 target genes. Further investigations will be needed whether this result comes from increased Bmal1 and Clock heterodimer transcription activity. In addition, we could not exclude the possibilities that other RNA-binding proteins could interact with Bmal1 mRNA to regulate its
protein synthesis. When and how Bmal1 expression is controlled by RNA-binding protein repertoire remains to be determined. Moreover, tissue-specific hnRNP Q knockout mice will be instrumental to understand the function of hnRNP Q in the overall circadian system.

In conclusion, our results showed the novel function of hnRNP Q in translational repression of Bmal1 that may be an important regulatory stage in circadian clock system. Given the importance of Bmal1, this study may help to understand rhythmic translational regulation and give insights to precisely controlled circadian clock system.

**EXPERIMENTAL PROCEDURES**

**DNA constructs**

To construct reporter plasmids, full length and serially-deleted mouse Bmal1 5’UTRs were amplified from full-length mouse Bmal1 cDNA (NM_007489.4). These PCR products were digested with HindIII and SmaI, and inserted upstream of Fluc coding sequence. The PCR products were also digested with EcoRI and XbaI and then subcloned into the pSK vector for *in vitro* binding assays. For real-time bioluminescence luciferase reporter, Bmal1::Luc plasmid (38) and Bmal1 5’UTR plasmid were used as a template. To construct Bmal1-5’UTR::Luc plasmid, the first round of PCR, forward primer (5’-AAGAGCTCAGAGTCCGCAACGCAGTGCCCTCAGCG-3’) and reverse primer (5’-AGGCCCGGGCCCCCGCCACCAATCGCTGT-3’) of Bmal1 promoter region, and forward primer (5’-GTGGGCGGGGCGCCGGCTGGGCGGCGG GG-3’) and reverse primer (5’-AACCCTAGTGAAAGGAAGGTGGTCTGCAA GGGACTCTAACT-3’) of Bmal1 5’UTR were used to create two fragments. The two PCR products were mixed together for a second round of PCR. The final product of Bmal1 promoter and 5’UTR sequences were digested with SacI and XhoI, and inserted into pGL3 basic plasmid.

**Cell culture and drug treatment**

NIH-3T3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Hyclone) containing 10 % fetal bovine serum (FBS, Hyclone), 100 units/ml penicillin, and 100 μg of streptomycin (Welgene) and maintained in a humidified atmosphere of 5% CO2 at 37°C. To synchronize the circadian oscillation, NIH-3T3 cells were treated with 100 nM dexamethasone. After 2 h, the medium was replaced with complete medium, and cells were harvested at the indicated time points for further experiments. To block the transcription or translation, NIH-3T3 cells were treated with actinomycin D (5 mg/ml) or cycloheximide (100 μg/ml), respectively.

**Transfection and RNA interference**

Reporter plasmid expression or siRNA transfection for transient knockdown in cell lines were performed using Lipofectamine 2000 (Invitrogen) or Neon Transfection system (Invitrogen) according to manufacturer’s recommendations. Cells were harvested 24 h after transfection for subsequent experiments. Small interfering RNA (siRNA) targeting hnRNP Q was used as previously described (29).

** Luciferase assay**

NIH-3T3 cells were transfected with luciferase reporters and harvested 24 h post-transfection. Cells were lysed with reporter lysis buffer, Renilla and firefly luciferase activities were analyzed using Dual-luciferase reporter assay system (Promega) according to the manufacturer’s instructions. Firefly luciferase activities were normalized by Renilla luciferase activities.

**Western Blot**

NIH-3T3 cells were lysed with RIPA buffer containing protease inhibitor tablet (Thermo Scientific), and protein samples were resolved by SDS-PAGE, transferred to nitrocellulose...
membranes (Pall Corporation) and immunoblotted with specific antibodies. Blocking was performed with 5% non-fat dry milk in Tris-buffered saline and 0.1% Tween-20 (TBST) for 0.5-1 h. Membranes were incubated with primary antibodies in blocking solution overnight at 4°C. Primary antibodies used in this study are as follows: anti-Bmal1 (ab3350, abcam), anti-hnRNP Q (ab10687, abcam), anti-hnRNP K (ab70492, abcam), anti-hnRNP I (homemade), anti-GAPDH (A300-641A, Bethyl). After several washes with TBST, the membranes were incubated with secondary horse radish peroxidase (HRP)-conjugated mouse (Thermo Scientific) or rabbit (Promega) secondary antibodies for 1-2 h, and they were visualized with SUPEX ECL reagent (Neuronex) and a LAS-4000 system (FUJIFILM), according to the manufacturer’s instructions. Acquired images were further analyzed with the Image Gauge program (FUJIFILM).

**Polysome profile analysis**

NIH-3T3 cells were transfected with control siRNA or hnRNP Q targeting siRNA, and treated with cycloheximide (100 μg/ml) for 30 min on ice and harvested. Cell extracts were subjected to sucrose density gradient analysis as previously described (27). Briefly, cells were lysed in polysome lysis buffer (300 mM KCl, 5 mM MgCl₂, 10 mM HEPES (pH 7.4), 0.5% NP-40, 5 mM DTT and 100 μg/ml CHX). Cell lysates were loaded on 15–45% sucrose gradients in polysome buffer (300 mM KCl, 5 mM MgCl₂ and 10 mM HEPES (pH7.4)) and centrifuged at 32,000 rpm in a SW41Ti rotor at 4 °C for 3 h. The gradients were collected using a Brandel gradient density fractionator and analyzed by an Econo UV monitor (Bio-Rad). Total RNAs in each fraction were extracted using TRI-Solution (Bio Science Technology). RNA levels were quantified by quantitative PCR.

**In vitro transcription, in vitro binding assay, and RNA-immunoprecipitation**

pSK vectors containing Bmal1 5’UTR or GAPDH CDS sequences were linearized with XbaI and in vitro transcribed in the presence of biotin-UTP using T7 RNA polymerase (Promega) for in vitro binding assay. Cytoplasmic lysates were prepared from NIH-3T3 cells using hypoionic 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. Cytoplasmic fraction of NIH-3T3 cells was incubated with in vitro transcribed biotin-labeled mRNA and subjected to streptavidin-agarose beads (Thermo Scientific). Bead-bound proteins were pull-downed and analyzed by SDS-PAGE followed by immunoblotting. For RNA-immunoprecipitation (RNA-IP), NIH-3T3 cells were lysed with RNA-IP buffer (10 mM HEPES (pH 7.5), 100 mM KCl, 5 mM MgCl₂, 0.1% NP-40, protease inhibitor, and RNase inhibitor). Mouse normal IgG or hnRNP Q antibody was incubated with NIH-3T3 cell lysates at 4°C overnight and then incubated with Protein G beads (Thermo Scientific) at 4°C for 4 h. We washed the beads 3 times with RNA-IP buffer and isolated RNA using TRI-Solution (Bio Science Technology). RNA levels were quantified by quantitative PCR.

**RNA isolation and quantitative PCR**

Total RNAs were extracted from NIH-3T3 cells using TRI-Solution (Bio Science Technology) following manufacturer’s instructions. Isolated RNAs were reverse transcribed with ImProm-II™ Reverse Transcription System (Promega) according to the manufacturer’s recommendations. RNA levels were detected by quantitative PCR using StepOnePlus Real-Time System (Applied Biosystems) with FastStart Universal SYBR Green Master (Roche) according to manufacturer’s instructions. Specific primer pairs used are shown in Table S1.

**Real-time bioluminescence measurement**

NIH-3T3 cells were plated in 35 mm dishes, and allowed to grow 80% of confluence. The Bmal1::Luc (38) or Bmal1-5’UTR::Luc reporters were co-transfected with control siRNA or
hnRNP Q targeting siRNA to NIH-3T3 cells using Lipofectamine 2000 (Invitrogen) according to manufacturers’ recommendations. Cells were synchronized with 100 nM dexamethasone treatment for 2 hours, and medium was changed to 1 mM luciferin (Promega) containing phenol-red free DMEM (Hyclone). 35 mm dishes were then transferred to LumiCycle 32 (Actimetrics) kept in the 37°C incubator, and photons were measured for 6 days. Lumicycle analysis software (Actimetrics) was used for lumicycle data analysis.

**Statistical analysis**

All quantitative data are presented as the mean ± standard deviation (SD). Statistical analyses were performed using Student’s t-test, one-way analysis of variance (ANOVA) or two-way ANOVA with post hoc Tukey’s multiple comparison test. P values of less than 0.05 were considered statistically significant. GraphPad Prism 8 was used to perform statistical analysis and to generate figures.

**ACKNOWLEDGEMENTS**

This work was supported by the Bio & Medical Technology Development program (2017M3C7A1023478) and grant (2019R1A2C2009440) of the National Research Foundation (NRF) funded by the Korean government (MSIT, MOE), the Cooperative Research Program for Agriculture Science and Technology Development (project No. PJ01324801) of the Rural Development Administration, and the BK21 Plus (10Z20130012243) funded by the Ministry of Education, Korea.

**CONFLICT OF INTEREST**

The authors declare that they have no conflicts of interest with the contents of this article.
REFERENCES

1. Dibner, C., Schibler, U., and Albrecht, U. (2010) The mammalian circadian timing system: organization and coordination of central and peripheral clocks. *Annu Rev Physiol* **72**, 517-549

2. Moore, R. Y. (1997) Circadian rhythms: basic neurobiology and clinical applications. *Annu Rev Med* **48**, 253-266

3. Schibler, U., and Sassone-Corsi, P. (2002) A web of circadian pacemakers. *Cell* **111**, 919-922

4. Balsalobre, A., Damiola, F., and Schibler, U. (1998) A serum shock induces circadian gene expression in mammalian tissue culture cells. *Cell* **93**, 929-937

5. Nagoshi, E., Saini, C., Bauer, C., Laroche, T., Naef, F., and Schibler, U. (2004) Circadian gene expression in individual fibroblasts: cell-autonomous and self-sustained oscillators pass time to daughter cells. *Cell* **119**, 693-705

6. Bunger, M. K., Wilsbacher, L. D., Moran, S. M., Clendenin, C., Radcliffe, L. A., Hogenesch, J. B., Simon, M. C., Takahashi, J. S., and Bradfield, C. A. (2000) Mop3 is an essential component of the master circadian pacemaker in mammals. *Cell* **103**, 1009-1017

7. Bunger, M. K., Walisser, J. A., Sullivan, R., Manley, P. A., Moran, S. M., Kalscheur, V. L., Colman, R. J., and Bradfield, C. A. (2005) Progressive arthropathy in mice with a targeted disruption of the Mop3/Bmal-1 locus. *Genesis* **41**, 122-132

8. Rudic, R. D., McNamara, P., Curtis, A. M., Boston, R. C., Panda, S., Hogenesch, J. B., and Fitzgerald, G. A. (2004) BMAL1 and CLOCK, two essential components of the circadian clock, are involved in glucose homeostasis. *PLoS Biol* **2**, e377

9. Marcheva, B., Ramsey, K. M., Buhr, E. D., Kobayashi, Y., Su, H., Ko, C. H., Ivanova, G., Omura, C., Mo, S., Vitaterna, M. H., Lopez, J. P., Philipson, L. H., Bradfield, C. A., Crosby, S. D., JeBailey, L., et al. (2010) Disruption of the clock components CLOCK and BMAL1 leads to hypoinsulinemia and diabetes. *Nature* **466**, 627-631

10. Shimba, S., Ishii, N., Ohta, Y., Ohno, T., Watabe, Y., Hayashi, M., Wada, T., Aoyagi, T., and Tezuka, M. (2005) Brain and muscle Arnt-like protein-1 (BMAL1), a component of the molecular clock, regulates adipogenesis. *Proc Natl Acad Sci USA* **102**, 12071-12076

11. Kondratov, R. V., Kondratova, A. A., Gorbacheva, V. Y., Vykhovanets, O. V., and Antoch, M. P. (2006) Early aging and age-related pathologies in mice deficient in BMAL1, the core component of the circadian clock. *Genes Dev* **20**, 1868-1873

12. Yang, G., Chen, L., Grant, G. R., Paschos, G., Song, W. L., Musiek, E. S., Lee, V., McLoughlin, S. C., Grosser, T., Cotsarelis, G., and FitzGerald, G. A. (2016) Timing of expression of the core clock gene Bmal1 influences its effects on aging and survival. *Sci Transl Med* **8**, 324ra316

13. Musiek, E. S., Lim, M. M., Yang, G., Bauer, A. Q., Qi, L., Lee, Y., Roh, J. H., Ortiz-Gonzalez, X., Dearborn, J. T., Culver, J. P., Herzog, E. D., Hogenesch, J. B., Wozniak, D. F., Dikranian, K., Giasson, B. I., et al. (2013) Circadian clock proteins regulate neuronal redox homeostasis and neurodegeneration. *J Clin Invest* **123**, 5389-5400

14. Preitner, N., Damiola, F., Lopez-Molina, L., Zakany, J., Duboule, D., Albrecht, U., and Schibler, U. (2002) The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell* **110**, 251-260

15. Ueda, H. R., Chen, W., Adachi, A., Wakamatsu, H., Hayashi, S., Takasugi, T., Nagano, M., Nakahama, K., Suzuki, Y., Sugano, S., Iino, M., Shigeyoshi, Y., and Hashimoto, S. (2002) A transcription factor response element for gene expression during circadian night. *Nature* **418**, 534-539

16. Akashi, M., and Takumi, T. (2005) The orphan nuclear receptor RORalpha regulates circadian transcription of the mammalian core-clock Bmal1. *Nat Struct Mol Biol* **12**, 441-448

17. Guillamond, F., Dardente, H., Giguere, V., and Cermakian, N. (2005) Differential control of Bmal1 circadian transcription by REV-ERB and ROR nuclear receptors. *J Biol Rhythms* **20**, 391-403

18. Eide, E. J., Vielhaber, E. L., Hinz, W. A., and Virshup, D. M. (2002) The circadian regulatory proteins BMAL1 and cryptochromes are substrates of casein kinase I epsilon. *J Biol Chem* **277**, 5389-5400
19. Sanada, K., Okano, T., and Fukada, Y. (2002) Mitogen-activated protein kinase phosphorylates and negatively regulates basic helix-loop-helix-PAS transcription factor BMAL1. *J Biol Chem* **277**, 267-271

20. Tamaru, T., Hirayama, J., Isojima, Y., Nagai, K., Norioka, S., Takamatsu, K., and Sassone-Corsi, P. (2009) CK2alpha phosphorylates BMAL1 to regulate the mammalian clock. *Nat Struct Mol Biol* **16**, 446-448

21. Sahar, S., Zocchi, L., Kinoshita, C., Borrelli, E., and Sassone-Corsi, P. (2010) Regulation of BMAL1 protein stability and circadian function by GSK3beta-mediated phosphorylation. *PLoS One* **5**, e8561

22. Cardone, L., Hirayama, J., Giordano, F., Tamaru, T., Palvimo, J. J., and Sassone-Corsi, P. (2005) Circadian clock control by SUMOylation of BMAL1. *Science* **309**, 1390-1394

23. Gossan, N. C., Zhang, F., Guo, B., Jin, D., Yoshitane, H., Yao, A., Glossop, N., Zhang, Y. Q., Fukada, Y., and Meng, Q. J. (2014) The E3 ubiquitin ligase UBE3A is an integral component of the molecular circadian clock through regulating the BMAL1 transcription factor. *Nucleic Acids Res* **42**, 5765-5775

24. Harvey, R. F., Smith, T. S., Mulroney, T., Queiroz, R. M. L., Pizzinga, M., Dezi, V., Villenueva, E., Ramakrishna, M., Lilley, K. S., and Willis, A. E. (2018) Trans-acting translational regulatory RNA binding proteins. *Wiley Interdiscip Rev RNA* **9**, e1465

25. Lim, I., Jung, Y., Kim, D. Y., and Kim, K. T. (2016) HnRNP Q Has a Suppressive Role in the Translation of Mouse Cryptochrome1 mRNA and hnRNP Q modulates the circadian oscillation of mouse Rev-erb alpha via IRES-mediated translation. *Nucleic Acids Res* **42**, 3590-3606

26. Ostareck, D. H., Ostareck-Lederer, A., Wilm, M., Thiele, B. J., Mann, M., and Hentze, M. W. (1997) mRNA silencing in erythroid differentiation: hnRNP K and hnRNP E1 regulate 15-lipoxygenase translation from the 3’ end. *Cell* **89**, 597-606

27. Bomszyt, K., Denisenko, O., and Ostrowski, J. (2004) hnRNP K: one protein multiple processes. *Bioessays* **26**, 629-638

28. Kaminski, A., Hunt, S. L., Patton, J. G., and Jackson, R. J. (1995) Direct evidence that polypyrimidine tract binding protein (PTB) is essential for internal initiation of translation of encephalomyocarditis virus RNA. *RNA* **1**, 924-938

29. Galban, S., Kuwano, Y., Pullmann, R., Jr., Martindale, J. L., Kim, H. H., Lal, A., Abdelmohsen, K., Yang, X., Dang, Y., Liu, J. O., Lewis, S. M., Holcik, M., and Gorospe, M. (2008) RNA-binding proteins HuR and PTB promote the translation of hypoxia-inducible factor 1alpha. *Mol Cell Biol* **28**, 93-107

30. Hinnebusch, A. G., Ivanov, I. P., and Sonenberg, N. (2016) Translational control by 5’-
untranslated regions of eukaryotic mRNAs. Science 352, 1413-1416

38. Welsh, D. K., Yoo, S. H., Liu, A. C., Takahashi, J. S., and Kay, S. A. (2004) Bioluminescence imaging of individual fibroblasts reveals persistent, independently phased circadian rhythms of clock gene expression. Curr Biol 14, 2289-2295

39. Koike, N., Yoo, S. H., Huang, H. C., Kumar, V., Lee, C., Kim, T. K., and Takahashi, J. S. (2012) Transcriptional architecture and chromatin landscape of the core circadian clock in mammals. Science 338, 349-354

40. Kojima, S., Shingle, D. L., and Green, C. B. (2011) Post-transcriptional control of circadian rhythms. J Cell Sci 124, 311-320

41. Lim, C., and Allada, R. (2013) Emerging roles for post-transcriptional regulation in circadian clocks. Nat Neurosci 16, 1544-1550

42. Kim, T. D., Kim, J. S., Kim, J. H., Myung, J., Chae, H. D., Woo, K. C., Jang, S. K., Koh, D. S., and Kim, K. T. (2005) Rhythmic serotonin N-acetyltransferase mRNA degradation is essential for the maintenance of its circadian oscillation. Mol Cell Biol 25, 3232-3246

43. Lau, P. P., Chang, B. H., and Chan, L. (2001) Two-hybrid cloning identifies an RNA-binding protein, GRY-RBP, as a component of apobec-1 editosome. Biochem Biophys Res Commun 282, 977-983
FIGURE LEGENDS

Figure 1. hnRNP Q suppresses Bmal1 translation. A, Representative image of Western blot analysis after siRNAs transfection to NIH-3T3 cells. Knockdown of hnRNP Q and upregulation of Bmal1 protein level were confirmed by comparing them to control siRNA treated sample. B, Endogenous mRNA level of Bmal1 was determined after knockdown of hnRNP Q by qPCR. Bmal1 mRNA level was normalized by GAPDH mRNA level. Data represent mean±SD (n=5). C, mRNA degradation kinetics of endogenous Bmal1. NIH-3T3 cells were treated with actinomycin D in the presence or absence of hnRNP Q siRNA and harvested at indicated times. The amount of Bmal1 mRNA was normalized by GAPDH mRNA level. Data represent mean±SD (n=3). D, Control or Bmal1 5’UTR harboring firefly luciferase reporters activity were analyzed in the presence of hnRNP Q siRNA. Fluc activity was normalized by Rluc activity. Knockdown of hnRNP Q was confirmed by immunoblot. Data represent mean±SD (n=3, **P<0.01). E, NIH-3T3 cells were treated with control siRNA or hnRNP Q targeting siRNA, and ribosomal distribution was evaluated by sucrose gradient analysis in cell lysates. Knockdown of hnRNP Q was confirmed by immunoblot. F and G, Total RNAs were isolated from monosome and polysome fractions in sucrose gradient. GAPDH (F) and Bmal1 (G) mRNA level were examined by qPCR. Data present mean±SD (n=3, *P<0.05)

Figure 2. hnRNP Q interacts with Bmal1 mRNA 5’UTR. A, Interaction between hnRNP Q and Bmal1 mRNA 5’UTR was determined by in vitro binding assay. Biotin-conjugated Bmal1 5’UTR was incubated with cytoplasmic cell extracts and subjected to immunoblotting using hnRNP Q antibody. Biotin-unconjugated Bmal1 5’UTR was used as competitor. B, Schematic diagrams of serially deleted Bmal1 5’UTR are shown. C, Confirmation of the interaction between hnRNP Q and Bmal1 5’UTR. Biotin-conjugated full length and serially deleted Bmal1 5’UTR were incubated with cytoplasmic cell lysate. D, Translation upregulation mediated by reduction of hnRNP Q in full length and serially deleted Bmal1 5’UTR reporter is shown. Knockdown of hnRNP Q was confirmed by immunoblot. Data represent mean±SD (n=3, *P<0.05, **P<0.001).

Figure 3. Bmal1 oscillatory profiles under hnRNP Q silencing. A, Endogenous Bmal1 mRNA oscillation profile was measured by qPCR analysis after synchronization with or without hnRNP Q knockdown. Data represent mean±SD (n=6). B and C, Representative Western blot of Bmal1 protein oscillation after synchronization in the presence of control siRNA (B) or hnRNP Q targeting siRNA (C). D, The normalized relative expression profile of Bmal1 protein in (B), (C) was plotted. The intensities at 0 hour of siCon group were arbitrarily set to 1. Data represent mean±SD (n=6). E, Real-time bioluminescence oscillations recording in synchronized NIH-3T3 cells expressing Bmal1::Luc reporter plasmid with (red line) or without (black line) hnRNP Q silencing. F and G, Period (F) and relative amplitude (G) values of Bmal1::Luc oscillations. Data represent mean±SD (n=4). H, Real-time bioluminescence rhythms of Bmal1-5’UTR::Luc reporter in synchronized NIH-3T3 cells with (red line) or without (black line) hnRNP Q depletion. I and J, Period (I) and relative amplitude (J) values of Bmal1-5’UTR::Luc oscillations. Data represent mean±SD (n=4, ****P<0.0001).

Figure 4. Rhythmic interaction between hnRNP Q and Bmal1 mRNA. A, NIH-3T3 cells were synchronized and harvested at indicated times. Cytosolic fractions were prepared to perform immunoprecipitation (IP) with hnRNP Q antibody or normal IgG as a control. B, Cytosolic extracts from (A) were used for RNA-IP with IgG and hnRNP Q antibody. RNA abundance in IP samples was determined by qPCR. Bmal1 mRNA levels were normalized by the amount of GAPDH mRNA. Data represent mean±SD (n=3). C, The co-immunoprecipitated mRNAs with hnRNP Q antibody shown in (A) were measured by qPCR. The amount of Bmal1 mRNA was normalized by GAPDH mRNA level (black line). Data represent mean±SD (n=3). Endogenous Bmal1 protein oscillation levels presented in (Fig. 3D) are also shown (red line).
Figure 5. mRNA oscillation profiles of Bmal1 target genes. A-D, NIH-3T3 cells were synchronized and harvested at indicated times. Total mRNAs were purified and subjected to qPCR analysis with specific primers for Cry1 (A), Rev-erba (B), Per1 (C), and Rorα (D). Data represent mean±SD (n=3).
Figure 1

hnRNP Q regulates Bmal1 translation

(A) Western blot analysis of Bmal1, hnRNP Q, and GAPDH.

(B) Normalized mRNA level (Bmal1/GAPDH) in siCon and sihnRNP Q groups. ns indicates no significant difference.

(C) Graph showing normalized mRNA level (Bmal1/GAPDH) over time after Act.D treatment.

(D) Bar graph showing normalized luciferase activity (Fluc/RLuc) for Fluc and Bmal1 5’UTR-Fluc.

(E) Graph showing Monosome and Polysome levels of hnRNP Q and GAPDH.

(F) Bar graph showing normalized GAPDH mRNA level in Monosome and Polysome for siCon and sihnRNP Q.

(G) Bar graph showing normalized Bmal1 mRNA level in Monosome and Polysome for siCon and sihnRNP Q.
Figure 2

hnRNP Q regulates Bmal1 translation

A

Input
Biotin-Bmal1 5'UTR
Competitor

+ + +

hnRNP Q

B

Bmal1 5'UTR

1 520
183 Δ182
400 Δ399

C

Bmal1 5'UTR

Input
Full Δ182 Δ399

hnRNP Q

D

Normalized luciferase activity (Fluc/RLuc)

siCon sihnRNP Q

GAPDH

hnRNP Q
hnRNP Q regulates Bmal1 translation

Figure 3

A

Normalized mRNA level (Bmal1/TBP) vs. hours after synchronization

B

Western blot images for Bmal1, hnRNP Q, and GAPDH under siCon and sihnRNP Q conditions.

C

Western blot images for Bmal1, hnRNP Q, and GAPDH under sihnRNP Q conditions.

D

Normalized protein level (Bmal1/GAPDH) vs. hours after synchronization

E

Bioluminescence of Bmal1::Luc under siCon and sihnRNP Q conditions.

F

Graph showing period (h) for bioluminescence.

G

Graph showing fold amplitude for bioluminescence.

H

Bioluminescence of Bmal1-5'UTR::Luc under siCon and sihnRNP Q conditions.

I

Graph showing period (h) for bioluminescence.

J

Graph showing fold amplitude for bioluminescence.
Figure 4

**A**

|                  | 0   | 0   | 4   | 8   | 12  | 16  | 20  | 24  |
|------------------|-----|-----|-----|-----|-----|-----|-----|-----|
| IgG              | +   | +   | +   | +   | +   | +   | +   | +   |
| anti-hnRNP Q     |     |     |     |     |     |     |     |     |

**B**

Fold Enrichment (Bmal1/GAPDH)

| Sample  | IgG | hnRNP Q |
|---------|-----|---------|
| Enrichment | 2   | 4.5     |

**C**

Normalized mRNA level (Bmal1/GAPDH)

| Time (hours) | 0   | 4   | 8   | 12  | 16  | 20  | 24  |
|--------------|-----|-----|-----|-----|-----|-----|-----|
| Bmal1 mRNA   | 1.0 | 1.5 | 2.0 | 1.5 | 1.0 | 0.5 | 0.2 |

Normalized protein level (Bmal1/GAPDH)

| Time (hours) | 0   | 4   | 8   | 12  | 16  | 20  | 24  |
|--------------|-----|-----|-----|-----|-----|-----|-----|
| Bmal1 Protein| 1.6 | 1.4 | 1.2 | 1.0 | 0.8 | 0.6 | 0.4 |

- **hnRNP Q-bound Bmal1 mRNA**
- **Endogenous Bmal1 protein**
Figure 5

hnRNP Q regulates Bmal1 translation
The RNA-binding protein hnRNP Q represses translation of the clock gene Bmal1 in murine cells

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J. Biol. Chem. published online April 4, 2019

Access the most updated version of this article at doi: 10.1074/jbc.RA118.006947

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