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

Received for publication, December 3, 2018, and in revised form, March 28, 2019 Published, Papers in Press, April 4, 2019, DOI 10.1074/jbc.RA118.006947

Youngseob Jung[‡], Hye Guk Ryu[§], Sung Wook Kim[‡], Kyung-Ha Lee[¶], Sohyun Gu[§], Hee Yi[‖], Hyun-Ok Ku[‖], Sung Key Jang[§], and Kyong-Tai Kim[‡§1]

From the [‡]Division of Integrative Biosciences and Biotechnology and [§]Department of Life Sciences, Pohang University of Science and Technology, Pohang, Gyeongbuk 37673, Republic of Korea, the [¶]Division of Cosmetic Science and Technology, Daegu Haany University, Gyeongsan, Gyeongbuk 38610, Republic of Korea, and the [‖]Vet Drugs and Biologics Division, Animal and Plant Quarantine Agency, Gimcheon, Gyeongbuk 39660, Republic of Korea

Edited by Ronald C. Wek

Most living creatures have a circadian rhythm that is generated by a 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 that represents a positive arm of this autoregulatory circadian clock system. Despite the indispensable role of BMAL1 in the 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, quantitative real-time PCR, and real-time bioluminescence experiments, we show that translation of *Bmal1* is negatively regulated by an RNA-binding protein, heterogeneous nuclear ribonucleoprotein Q (hnRNP Q). Interestingly, we found that hnRNP Q rhythmically binds to a specific region of the *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 the 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.

The majority of living organisms have a 24-h 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 of the brain (2), and this central pacemaker synchronizes various peripheral clocks via a combination of neural and humoral signaling (3). Peripheral tissues also harbor their own clock, and most cells, even cultured fibroblasts (4, 5), possess a self-sustained and cell-autonomous circadian rhythm oscillator. This endogenous cellular oscillator is composed of an autoregulatory feedback loop that includes positive elements of the Bmal1 and Clock heterodimer that activates transcription of Period (Per),[2] Cryptochrome (Cry), Rev-erbα, and Rorα. In addition, there is a negative arm of this internal oscillator, a 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 receives much attention because it has critical roles in circadian rhythms as well as various physiological processes. For example, absence of Bmal1 results in immediate and complete impairment of circadian rhythmicity and reduced locomotor activity (6). This reduced activity is explained by a report showing that Bmal1-deficient mice develop joint ankylosis because of ossification in tendons and ligaments of the hind limbs (7). In addition, both glucose metabolism and insulin signaling are abolished in Bmal1 mutant mice, leading to hypoinsulinemia and diabetes (8, 9). It has been reported that embryonic fibroblasts of Bmal1 knockout mice fail to differentiate into adipocytes. Also, Bmal1 overexpression in adipocytes up-regulates lipid synthesis activity (10). Moreover, loss of Bmal1 results in a shortened life span 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 posttranslational regulation. For instance, Rev-

This work was supported by Bio & Medical Technology Development Program Grant 2017M3C7A1023478 and Grant 2019R1A2C2009440 from the National Research Foundation funded by the Korean government (Ministry of Science and ICT, Ministry of Education), the Cooperative Research Program for Agriculture Science and Technology Development (Project PJ01324801) of the Rural Development Administration, and BK21 Plus Grant 10Z20130012243 funded by the Ministry of Education, Korea. The authors declare that they have no conflicts of interest with the contents of this article.

This article contains Figs. S1–S3 and Table S1.

[1] To whom correspondence should be addressed. Tel.: 82-54-279-2297; Fax: 82-54-279-2199; E-mail: ktk@postech.ac.kr.

[2] The abbreviations used are: Per, period; Cry, cryptochrome; hnRNP, heterogeneous nuclear ribonucleoprotein; Fluc, firefly luciferase; qPCR, quantitative PCR; CDS, coding sequence(s); IP, immunoprecipitation; TBS, tris-buffered saline.

erbα and Rorα suppress and enhance Bmal1 transcription, respectively (14–17). Also, Bmal1 protein is known to be phosphorylated by casein kinase 1 ϵ (CK1ϵ) (18), mitogen-activated protein kinase (19), CK2α (20), and glycogen synthase kinase 3 β (GSK3β) (21) to mediate the timekeeping mechanism of the clock system. In addition, it has been reported that SUMOylation of Bmal1 regulates circadian rhythmicity (22). Moreover, ubiquitin protein ligase E3A (UBE3A) has been 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 reports that RNA-binding proteins play a central role in translational modulation (24). We have demonstrated previously that various RNA-binding proteins have an important function in translational control of several clock genes (25–30). Therefore, we hypothesized that a 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. We also show that hnRNP Q binds to a specific region of the Bmal1 mRNA 5′ 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 the 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

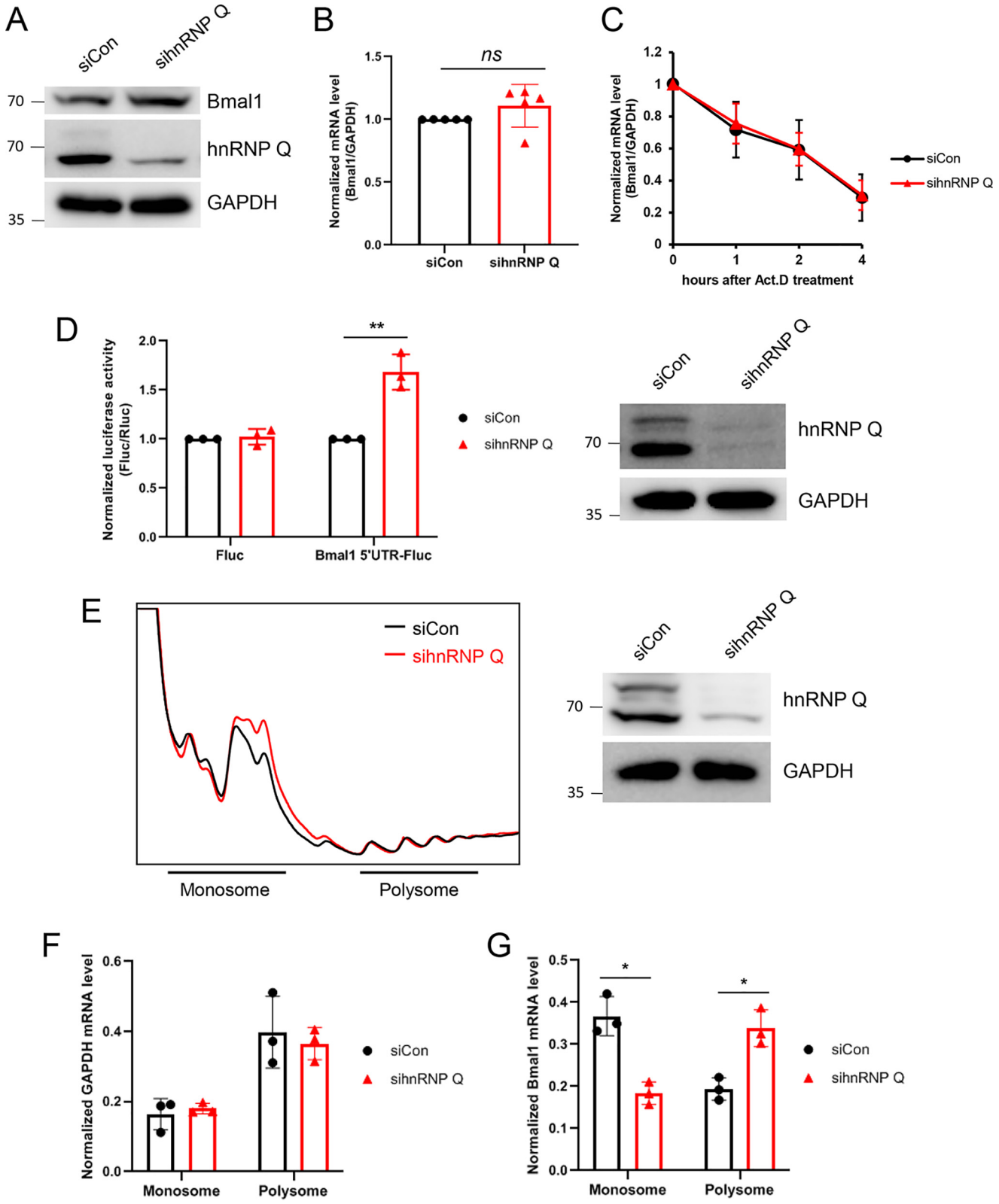
Maintenance of circadian rhythm depends on oscillation of biological clock gene expression. Posttranscriptional regulation of rhythmic genes is one of the important regulatory mechanisms. In this regulation, RNA-binding proteins have a critical role in governing the life of mRNAs. Although a recent report demonstrated that more than 1,500 proteins can interact with RNAs (31), Bmal1 mRNA–binding proteins are poorly understood. In addition, the molecular mechanisms of the posttranscriptional regulation of Bmal1 mRNA are still unknown. We therefore focused on the posttranscriptional control of Bmal1 mRNA. First, we performed *in silico* analysis of which RNA-binding proteins could interact with Bmal1 mRNA. 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. S1*A*). These hnRNPs are known to have a critical role in the posttranscriptional regulation of specific mRNAs (25, 26, 32–36). Of the three siRNAs, only hnRNP Q knockdown caused an increase in the Bmal1 protein level (Fig. 1*A*). To determine whether this result comes from the up-regulated Bmal1 mRNA level or stability, we analyzed the Bmal1 mRNA level and decay kinetics after hnRNP Q depletion. The result showed that reduction of hnRNP Q does not significantly alter the Bmal1 mRNA level and stability (Fig. 1, *B* and *C*), suggesting that hnRNP Q may inhibit the translation of Bmal1 mRNA.

Because the 5′ UTR of mRNA has a critical role in translational regulation (37), the Bmal1 5′ UTR could be associated with protein synthesis inhibition mediated by hnRNP Q. By utilizing a 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 the upstream of firefly luciferase (Fluc) coding sequence of the reporter vector. NIH-3T3 cells were transfected with the reporters after hnRNP Q depletion, and the luciferase activities were analyzed. Reduction of hnRNP Q significantly increased Bmal1 5′ UTR–harboring reporter activity compared with the control reporter (Fig. 1*D*). To investigate whether the up-regulated reporter expression came from translational activation, we analyzed the mRNA level of reporters by using qPCR. The results showed unaltered reporter mRNA levels (Fig. S1*B*), suggesting that hnRNP Q mediates the Bmal1 5′ UTR when it negatively regulates Bmal1 translation. Next, to further confirm the importance of hnRNP Q in Bmal1 protein synthesis, we performed a polysome profiling analysis with or without knockdown of hnRNP Q. The overall ribosome distributions in the sucrose gradient were not changed after hnRNP Q depletion (Fig. 1*E*). This observation was validated by results showing that GAPDH mRNA levels were not significantly altered in monosome or polysome fractions after hnRNP Q silencing (Fig. 1*F*). When hnRNP Q was reduced, however, the distribution of Bmal1 mRNA was shifted from the monosome to the polysome fraction, indicating up-regulation of Bmal1 mRNA translation (Fig. 1*G*). These results indicate that hnRNP Q mediates down-regulation of the Bmal1 protein level by inhibiting Bmal1 mRNA translation through its 5′ UTR.

hnRNP Q binds to a specific region of the Bmal1 mRNA 5′ UTR

To determine whether hnRNP Q could bind to the Bmal1 5′ UTR, we conducted an *in vitro* binding assay using the biotin-conjugated Bmal1 5′ UTR and cell lysates. Although the biotinylated Bmal1 5′ UTR alone pulled down hnRNP Q protein, this binding affinity was dramatically reduced in combination with a competitor (Fig. 2*A*), suggesting that it is a specific interaction between the 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 the Bmal1 5′ UTR was not inhibited after addition of the GAPDH CDS as a nonspecific competitor (Fig. S2*A*). Because 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 the Bmal1 5′ UTR, we generated serially deleted Bmal1 5′ UTR constructs that excluded each secondary structure to potentially abolish the binding of hnRNP Q (Fig. 2*B*). To discover binding sites of hnRNP Q in the Bmal1 5′ UTR, we performed an *in vitro* binding assay with a biotin-incorporated, partially deleted Bmal1 5′ UTR. The result demonstrated that hnRNP Q binds to full-length and 182-nt-deleted (Δ182) constructs of the Bmal1 5′ UTR. However, the 399-nt-truncated (Δ399) mutant showed significantly reduced affinity for hnRNP Q (Fig. 2*C*), suggesting that the binding sites of hnRNP Q reside in residues 1–399 of

the Bmal1 5′ UTR. We then asked whether only 1–182 residues of the Bmal1 5′ UTR could facilitate hnRNP Q interaction. The result showed that the minimal region of 1–182 could promote this binding (Fig. S2*B*). Next we wondered whether this binding pattern affects the translational efficiency of Bmal1. To test this possibility, we performed a reporter assay with mutant constructs after reduction of hnRNP Q. Interestingly, the result showed an increase in Fluc level in control cells transfected with the Δ399 construct. Moreover, hnRNP Q depletion significantly enhanced Fluc expression in the Bmal1 5′ UTR full and



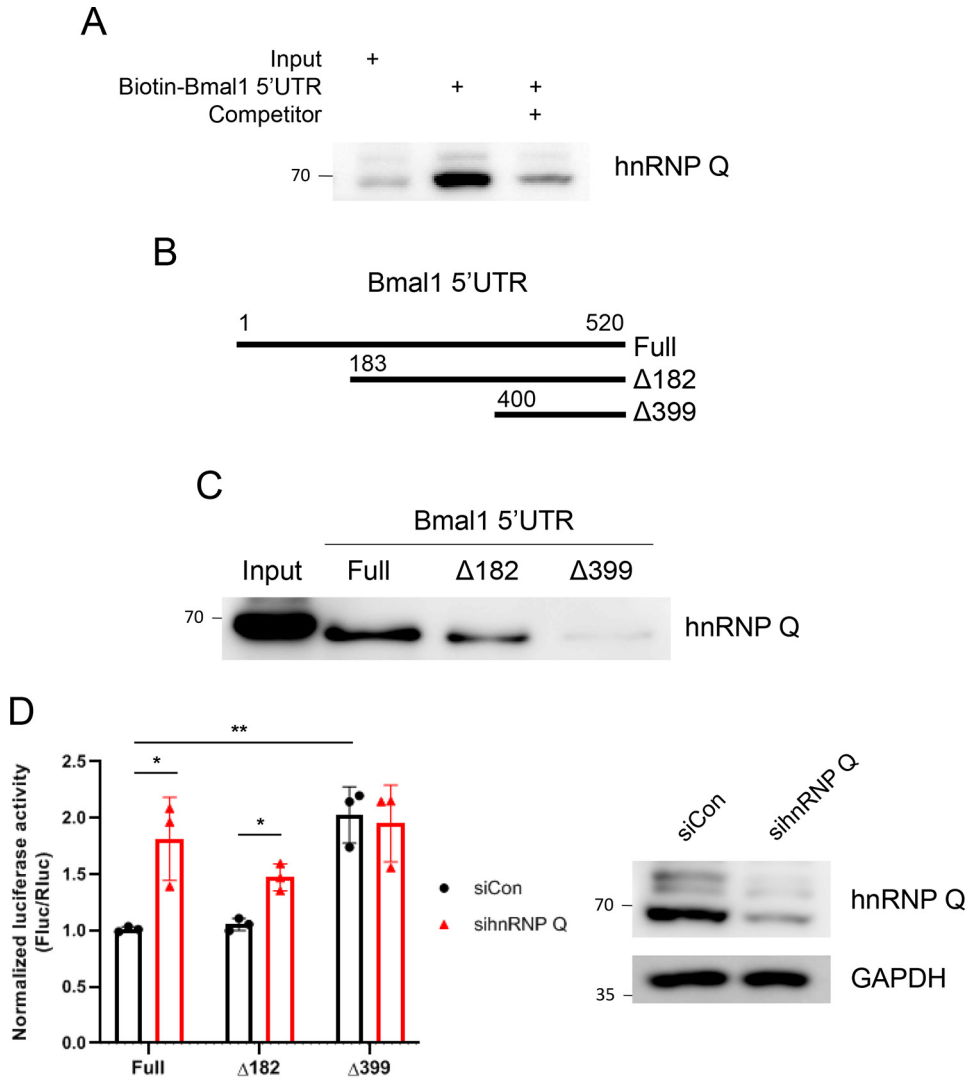


Figure 2. hnRNP Q interacts with the Bmal1 mRNA 5′ UTR. *A*, interaction between hnRNP Q and the Bmal1 mRNA 5′ UTR was determined by an *in vitro* binding assay. The biotin-conjugated Bmal1 5′ UTR was incubated with cytoplasmic cell extracts and subjected to immunoblotting using hnRNP Q antibody. The biotin-unconjugated Bmal1 5′ UTR was used as a competitor. *B*, schematic of serially deleted Bmal1 5′ UTRs. *C*, confirmation of the interaction between hnRNP Q and the Bmal1 5′ UTR. Biotin-conjugated full-length and serially deleted Bmal1 5′ UTRs were incubated with cytoplasmic cell lysate. *D*, translation up-regulation mediated by reduction of hnRNP Q in full-length and the serially deleted Bmal1 5′ UTR reporter. Knockdown of hnRNP Q was confirmed by immunoblotting. Data represent mean ± S.D. ($n = 3$; *, $p < 0.05$; **, $p < 0.001$). *Rluc*, *Renilla* luciferase.

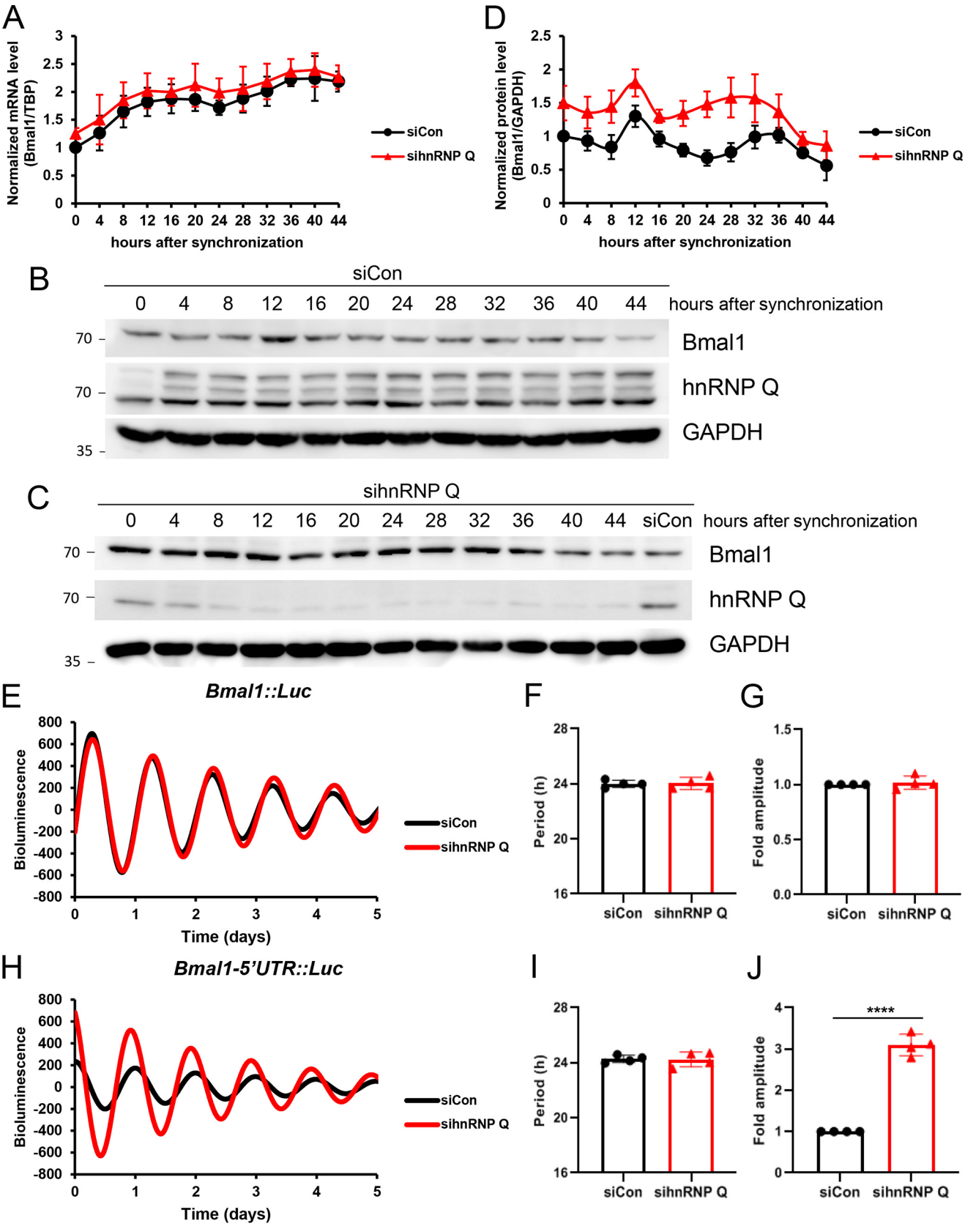
Δ182 constructs, but this up-regulation was not observed in the Δ399 construct, which previously showed weak binding affinity for hnRNP Q (Fig. 2*D*). These results suggest that hnRNP Q interacts with residues 1–399 of the Bmal1 5′ UTR to inhibit the translation process.

hnRNP Q plays a critical role in Bmal1 protein oscillation

Because Bmal1 plays a major role in the circadian system, we speculated that hnRNP Q–mediated translational control may lead to crucial consequences for circadian rhythmicity. To investigate the physiological significance of Bmal1 translational repression mediated by hnRNP Q, we reduced hnRNP Q and synchronized circadian time by dexamethasone treatment to analyze the Bmal1 oscillation profile. Silencing of hnRNP Q did not alter the Bmal1 mRNA oscillation profiles (Fig. 3*A*); 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 syn-

Figure 1. hnRNP Q suppresses Bmal1 translation. *A*, representative image of Western blot analysis after siRNA transfection to NIH-3T3 cells. Knockdown of hnRNP Q and up-regulation of the Bmal1 protein level were confirmed by comparison with a control siRNA–treated sample. *B*, the endogenous mRNA level of Bmal1 was determined after knockdown of hnRNP Q by qPCR. The Bmal1 mRNA level was normalized by the GAPDH mRNA level. Data represent mean ± S.D. ($n = 5$). *ns*, not significant. *C*, mRNA degradation kinetics of endogenous Bmal1. NIH-3T3 cells were treated with actinomycin D (*Act.D*) in the presence or absence of hnRNP Q siRNA and harvested at the indicated times. The amount of Bmal1 mRNA was normalized by the GAPDH mRNA level. Data represent mean ± S.D. ($n = 3$). *D*, control or Bmal1 5′ UTRs harboring firefly luciferase reporter activity were analyzed in the presence of hnRNP Q siRNA. Fluc activity was normalized by *Renilla* luciferase (*Rluc*) activity. Knockdown of hnRNP Q was confirmed by immunoblotting. Data represent mean ± S.D. ($n = 3$; **, $p < 0.01$). *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 immunoblotting. *F* and *G*, total RNAs were isolated from monosome and polysome fractions in sucrose gradient. GAPDH (*F*) and Bmal1 (*G*) mRNA levels were examined by qPCR. Data present mean ± S.D. ($n = 3$; *, $p < 0.05$).

thesis. 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 the indicated circadian time points (Fig. S3*A*). This was confirmed through control GAPDH mRNA (Fig. S3*B*). However, Bmal1 mRNAs



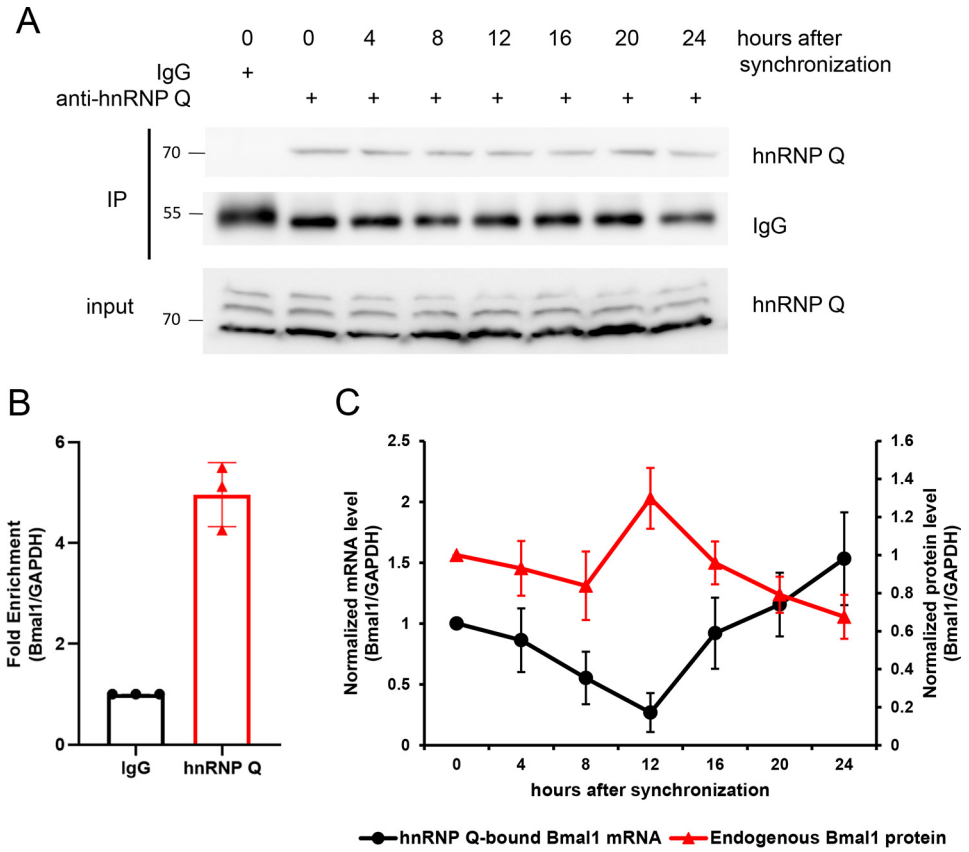


Figure 4. Rhythmic interaction between hnRNP Q and Bmal1 mRNA. *A*, NIH-3T3 cells were synchronized and harvested at the indicated times. Cytosolic fractions were prepared for IP with hnRNP Q antibody or normal IgG as a control. *B*, cytosolic extracts from *A* were used for RNA-IP with IgG and hnRNP 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 ± S.D. ($n = 3$). *C*, the co-immunoprecipitated mRNAs with hnRNP Q antibody shown in *A* were measured by qPCR. The amount of Bmal1 mRNA was normalized by the GAPDH mRNA level (*black line*). Data represent mean ± S.D. ($n = 3$). The endogenous Bmal1 protein oscillation levels presented in Fig. 3*D* are also shown (*red line*).

were significantly shifted from monosome to polysome under hnRNP Q–depleted conditions. Also, as shown above, Bmal1 protein reaches peak level 12 h after synchronization. In accordance with this, Bmal1 mRNAs were highly associated with polysome fractions with or without hnRNP Q depletion 12 h after synchronization (Fig. S3*C*). 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 was fused with the Bmal1 promoter (*Bmal1::Luc*) (38). We inserted the Bmal1 5′ UTR 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 synchronized by dexamethasone treatment. We found that hnRNP Q silencing did not affect the 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 the translation as well as circadian oscillation of Bmal1.

Given that the 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 an hnRNP Q antibody after synchronization. The results showed that the immunoprecipitated hnRNP Q level and total hnRNP Q protein level remained relatively constant during the experimental time period (Fig. 4*A*). Also, Bmal1 mRNA was pulled down by the hnRNP Q antibody compared with the IgG control (Fig. 4*B*). Interestingly, co-immunoprecipitated Bmal1 mRNA had its

Figure 3. Bmal1 oscillatory profiles under hnRNP Q silencing. *A*, the endogenous Bmal1 mRNA oscillation profile was measured by qPCR analysis after synchronization with or without hnRNP Q knockdown. The Bmal1 mRNA level was normalized by the TATA-box-binding protein (TBP) mRNA level. Data represent mean ± S.D. ($n = 6$). *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* and *C* was plotted. The intensity at 0 h of the siCon group was arbitrarily set to 1. Data represent mean ± S.D. ($n = 6$). *E*, real-time bioluminescence oscillation recording in synchronized NIH-3T3 cells expressing the *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 ± S.D. ($n = 4$). *H*, real-time bioluminescence rhythms of the *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 ± S.D. ($n = 4$; ****, $p < 0.0001$).

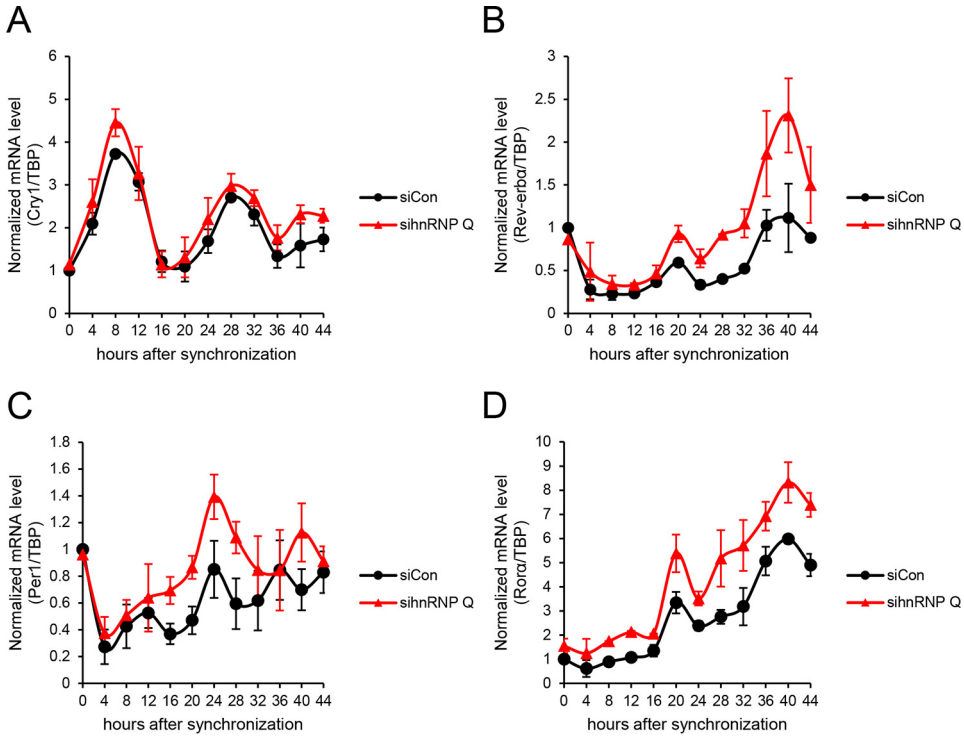


Figure 5. mRNA oscillation profiles of Bmal1 target genes. *A–D*, NIH-3T3 cells were synchronized and harvested at the indicated times. Total mRNAs were purified and subjected to qPCR analysis with specific primers for Cry1 (*A*), Rev-erbα (*B*), Per1 (*C*), and Rorα (*D*). These mRNA levels were normalized by the TATA-box-binding protein (TBP) mRNA levels. Data represent mean ± S.D. ($n = 3$).

lowest level 12 h after synchronization (Fig. 4*C*), which was the peak time of Bmal1 protein expression (Fig. 3*D*). 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 the transcription of several target genes, including Cry, Per, Rev-erbα, and Rorα. We asked whether hnRNP Q–induced Bmal1 translational inhibition affects the transcriptional activity of those target mRNAs. To test this possibility, we observed the mRNA oscillation profiles of Bmal1 target genes with or without hnRNP Q silencing. Remarkably, Cry1, Per1, Rev-erbα, and Rorα 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 expression. Interestingly, however, a recent study has demonstrated that only 22% of cycling mRNAs are driven by *de novo* transcription (39), suggesting that posttranscriptional regulation has a major role in the circadian system. In line with this report, accumulating evidence shows that several clock genes are controlled by posttranscriptional regulation, such as mRNA splicing, decay, and translation (40, 41). We demonstrated previously that RNA-binding proteins play major roles in these regulatory mechanisms. For instance, hnRNP R, hnRNP Q, and hnRNP L cooperatively destabilize arylalkylamine *N*-acetyltransferase mRNA (42). In addition, the internal ribosome entry site (IRES)-mediated translation of Rev-erbα mRNA is modulated by hnRNP Q and polypyrimidine tract– binding protein (32). In this study, we found a novel function of the RNA-binding protein hnRNP Q in translational control of the core clock gene Bmal1.

Although our results present several lines of evidence demonstrating hnRNP Q–mediated translational inhibition of the core clock gene Bmal1, we could not specify a clear molecular mechanism of 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. The mechanisms underlying this rhythmic binding process need to be investigated further. For instance, posttranslational modifications of hnRNP Q, including phosphorylation, may influence the affinity of hnRNP Q for Bmal1 mRNA. Although it is not known which kinase can phosphorylate hnRNP Q, it will be interesting to see whether a specific kinase has circadian oscillatory activity. Because the secondary structures of mRNA are important in interaction with RNA-binding proteins, the stem–loop structure of Bmal1 mRNA may vary in a time-dependent manner that allows a different affinity for hnRNP Q. Also, it will be more interesting to see whether hnRNP Q down-regulates translation efficiency through 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 the heart, brain, lung, liver, and many other organs (43), and currently, hnRNP Q knockout mice are 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 the circadian rhythm of the organism.

It is well known that Bmal1 is a main component of the circadian clock system and works as a transcription factor alongside the protein Clock. Interestingly, as expected, hnRNP Q knockdown enhanced the mRNA oscillation of Bmal1 target genes. Further investigation will be needed to find out whether this result comes from increased Bmal1 and Clock heterodimer transcription activity. In addition, we could not exclude the possibility that other RNA-binding proteins can interact with Bmal1 mRNA to regulate its protein synthesis. When and how Bmal1 expression is controlled by the 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 show a 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 us to understand rhythmic translational regulation and give insights into the 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 complementary DNA (NM_007489.4). These PCR products were digested with HindIII and SmaI and inserted upstream of the 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 the real-time bioluminescence luciferase reporter, the *Bmal1::Luc* plasmid (38) and Bmal1 5′ UTR plasmid were used as a template. To construct the *Bmal1–5′UTR::Luc* plasmid, in the first round of PCR, the forward primer (5′-AAGAGCTCGCAGAGTCCGCAACGCAGTGGCCTCAGCG-3′) and reverse primer (5′-AGGCCCGGCCCCCCGCCCACCAATCGCTGT-3′) of the Bmal1 promoter region and forward primer (5′-GTGGGCGGGGGGCCGGGCCTGGGCCGGCGG-3′) and reverse primer (5′-AACTCGAGTGGAAGGAAGGTGCTTGCAAGGGACTCTAACT-3′) of the 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 the Bmal1 promoter and 5′ UTR sequences were digested with SacI and XhoI and inserted into the pGL3 basic plasmid.

Cell culture and drug treatment

NIH-3T3 cells were cultured in DMEM (Hyclone) containing 10% FBS (Hyclone), 100 units/ml penicillin, and 100 μg of streptomycin (Welgene) and maintained in a humidified atmosphere of 5% CO_2 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 transcription or translation, NIH-3T3 cells were treated with actinomycin D (5 mg/ml) or cycloheximide (100 μg/ml), respectively.

Transfection and RNAi

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

Luciferase assay

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

Western blotting

NIH-3T3 cells were lysed with radioimmune precipitation assay buffer containing a protease inhibitor tablet (Thermo Scientific), and protein samples were resolved by SDS-PAGE, transferred to nitrocellulose membranes (Pall Corp.), and immunoblotted with specific antibodies. Blocking was performed with 5% nonfat dry milk in TBS and 0.1% Tween 20 (TBST) for 0.5–1 h. Membranes were incubated with primary antibodies in blocking solution overnight at 4 °C. The primary antibodies used in this study were as follows: anti-Bmal1 (ab3350, Abcam), anti-hnRNP Q (ab10687, Abcam), anti-hnRNP K (ab70492, Abcam), anti-hnRNP I (homemade), and anti-GAPDH (A300-641A, Bethyl). After several washes with TBST, the membranes were incubated with secondary HRP-conjugated mouse (Thermo Scientific) or rabbit (Promega) secondary antibodies for 1–2 h and then 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, treated with cycloheximide (100 μg/ml) for 30 min on ice, and harvested. Cell extracts were subjected to sucrose density gradient analysis as described previously (27). Briefly, cells were lysed in polysome lysis buffer (300 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES (pH 7.4), 0.5% NP-40, 5 mM DTT, and 100 μg/ml cycloheximide). Cell lysates were loaded on 15–45% sucrose gradients in polysome buffer (300 mM KCl, 5 mM $MgCl_2$, 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 the Bmal1 5′ UTR or GAPDH CDS were linearized with Xba1 and *in vitro* transcribed in the presence of biotin-UTP using T7 RNA polymerase (Promega) for the *in vitro* binding assay. 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 $MgCl_2$, 1 mM DTT, 0.2 mM PMSF, and 2.5% NP-40. The 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 pulled down and analyzed by SDS-PAGE followed by immunoblotting. For RNA-IP, NIH-3T3 cells were lysed with RNA-IP buffer (10 mM HEPES (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, 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. The beads were washed three times with RNA-IP buffer, and RNA was isolated 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 the manufacturer's instructions. Isolated RNAs were reverse-transcribed with the ImProm-II™ reverse transcription system (Promega) according to the manufacturer's recommendations. RNA levels were detected by quantitative PCR using the StepOnePlus real-time system (Applied Biosystems) with FastStart Universal SYBR Green Master (Roche) according to the manufacturer's instructions. Specific primer pairs used are shown in Table S1.

Real-time bioluminescence measurements

NIH-3T3 cells were plated in 35-mm dishes and allowed to grow to 80% 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 manufacturer's recommendations. Cells were synchronized with 100 nM dexamethasone for 2 h, and the 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 ± S.D. Statistical analyses were performed using Student's *t* test, one-way analysis of variance, or two-way analysis of variance with post hoc Tukey's multiple comparisons test. *p* values of less than 0.05 were considered statistically significant. GraphPad Prism 8 was used to perform statistical analyses and to generate figures.

Author contributions—Y. J., K.-H. L., and K.-T. K. conceptualization; Y. J. data curation; Y. J. and S. G. formal analysis; Y. J. and K.-T. K. validation; Y. J. and H. G. R. investigation; Y. J., H. G. R., S. W. K., H. Y., and H.-O. K. methodology; Y. J. writing-original draft; Y. J., H. G. R., S. W. K., K.-H. L., and K.-T. K. writing-review and editing; K.-H. L., S. K. J., and K.-T. K. supervision; K.-T. K. funding acquisition; K.-T. K. project administration.

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 CrossRef Medline
2. Moore, R. Y. (1997) Circadian rhythms: basic neurobiology and clinical applications. *Annu. Rev. Med.* **48,** 253–266 CrossRef Medline
3. Schibler, U., and Sassone-Corsi, P. (2002) A web of circadian pacemakers. *Cell* **111,** 919–922 CrossRef Medline
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 CrossRef Medline
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 CrossRef Medline
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 CrossRef Medline
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 CrossRef Medline
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 CrossRef Medline
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 hypoinsulinaemia and diabetes. *Nature* **466,** 627–631 CrossRef Medline
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. U.S.A.* **102,** 12071–12076 CrossRef Medline
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 CrossRef Medline
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,** 324ra16 CrossRef Medline
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 CrossRef Medline
14. Preitner, N., Damiola, F., Lopez-Molina, L., Zakany, J., Duboule, D., Albrecht, U., and Schibler, U. (2002) The orphan nuclear receptor REV-ERBα controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell* **110,** 251–260 CrossRef Medline
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 CrossRef Medline
16. Akashi, M., and Takumi, T. (2005) The orphan nuclear receptor RORα regulates circadian transcription of the mammalian core-clock Bmal1. *Nat. Struct. Mol. Biol.* **12,** 441–448 CrossRef Medline
17. Guillaumond, F., Dardente, H., Giguère, V., and Cermakian, N. (2005) Differential control of Bmal1 circadian transcription by REV-ERB and ROR nuclear receptors. *J. Biol. Rhythms* **20,** 391–403 CrossRef Medline
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 Iε. *J. Biol. Chem.* **277,** 17248–17254 CrossRef Medline
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 CrossRef Medline
20. Tamaru, T., Hirayama, J., Isojima, Y., Nagai, K., Norioka, S., Takamatsu, K., and Sassone-Corsi, P. (2009) CK2α phosphorylates BMAL1 to regulate the mammalian clock. *Nat. Struct. Mol. Biol.* **16,** 446–448 CrossRef Medline
21. Sahar, S., Zocchi, L., Kinoshita, C., Borrelli, E., and Sassone-Corsi, P. (2010) Regulation of BMAL1 protein stability and circadian function by GSK3β-mediated phosphorylation. *PLoS ONE* **5,** e8561 CrossRef Medline
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 CrossRef Medline
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 CrossRef Medline
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 CrossRef Medline
25. Kim, T. D., Woo, K. C., Cho, S., Ha, D. C., Jang, S. K., and Kim, K. T. (2007) Rhythmic control of AANAT translation by hnRNP Q in circadian melatonin production. *Genes Dev.* **21,** 797–810 CrossRef Medline
26. Lee, K. H., Woo, K. C., Kim, D. Y., Kim, T. D., Shin, J., Park, S. M., Jang, S. K., and Kim, K. T. (2012) Rhythmic interaction between Period1 mRNA and hnRNP Q leads to circadian time-dependent translation. *Mol. Cell Biol.* **32,** 717–728 CrossRef Medline
27. Lee, K. H., Kim, S. H., Kim, H. J., Kim, W., Lee, H. R., Jung, Y., Choi, J. H., Hong, K. Y., Jang, S. K., and Kim, K. T. (2014) AUF1 contributes to cryptochrome1 mRNA degradation and rhythmic translation. *Nucleic Acids Res.* **42,** 3590–3606 CrossRef Medline
28. Lee, H. R., Kim, T. D., Kim, H. J., Jung, Y., Lee, D., Lee, K. H., Kim, D. Y., Woo, K. C., and Kim, K. T. (2015) Heterogeneous ribonucleoprotein R regulates arylalkylamine *N*-acetyltransferase synthesis via internal ribosomal entry site-mediated translation in a circadian manner. *J. Pineal Res.* **59,** 518–529 CrossRef Medline
29. Lim, I., Jung, Y., Kim, D. Y., and Kim, K. T. (2016) HnRNP Q Has a Suppressive role in the translation of mouse cryptochrome1. *PLoS ONE* **11,** e0159018 CrossRef Medline
30. Kim, H. J., Lee, H. R., Seo, J. Y., Ryu, H. G., Lee, K. H., Kim, D. Y., and Kim, K. T. (2017) Heterogeneous nuclear ribonucleoprotein A1 regulates rhythmic synthesis of mouse Nfil3 protein via IRES-mediated translation. *Sci. Rep.* **7,** 42882 CrossRef Medline
31. Gerstberger, S., Hafner, M., and Tuschl, T. (2014) A census of human RNA-binding proteins. *Nat. Rev. Genet.* **15,** 829–845 CrossRef Medline
32. Kim, D. Y., Woo, K. C., Lee, K. H., Kim, T. D., and Kim, K. T. (2010) hnRNP Q and PTB modulate the circadian oscillation of mouse Rev-erb α via IRES-mediated translation. *Nucleic Acids Res.* **38,** 7068–7078 CrossRef Medline
33. 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 CrossRef Medline
34. Bomsztyk, K., Denisenko, O., and Ostrowski, J. (2004) hnRNP K: one protein multiple processes. *Bioessays* **26,** 629–638 CrossRef Medline
35. 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 Medline
36. Galbán, 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 1α. *Mol. Cell Biol.* **28,** 93–107 CrossRef Medline
37. Hinnebusch, A. G., Ivanov, I. P., and Sonenberg, N. (2016) Translational control by 5′-untranslated regions of eukaryotic mRNAs. *Science* **352,** 1413–1416 CrossRef Medline
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 CrossRef Medline
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 CrossRef Medline
40. Kojima, S., Shingle, D. L., and Green, C. B. (2011) Post-transcriptional control of circadian rhythms. *J. Cell Sci.* **124,** 311–320 CrossRef Medline
41. Lim, C., and Allada, R. (2013) Emerging roles for post-transcriptional regulation in circadian clocks. *Nat. Neurosci.* **16,** 1544–1550 CrossRef Medline
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 CrossRef Medline
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 CrossRef Medline