Supplementary data

hnRNP K supports high-amplitude D site-binding protein (Dbp) mRNA oscillation to sustain circadian rhythms.

This PDF file includes:

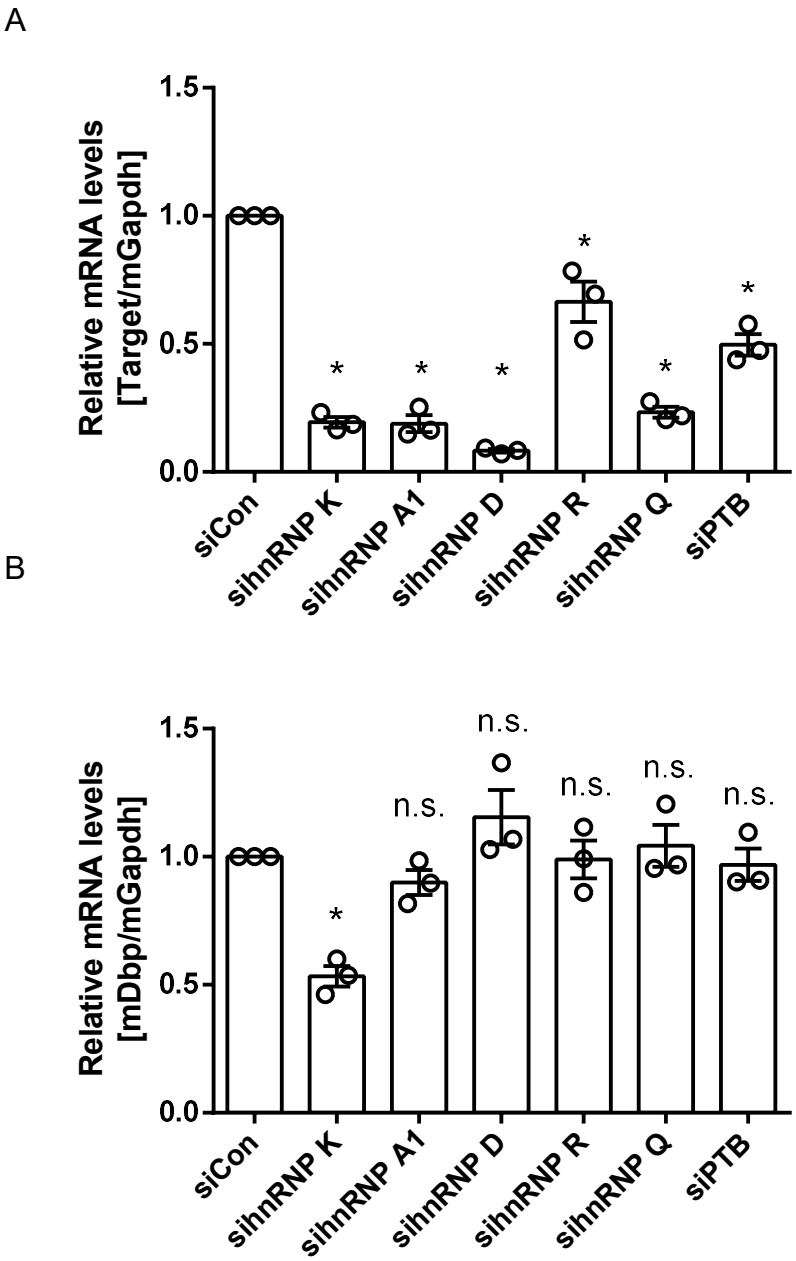
Supplementary Figure S1 to S9
Tables S1 to S2

Supplementary method

Subcellular fractionation

Hypotonic buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl2, DTT 1.0 mM, 0.2 mM PMSF) was added to cells to resuspend them after cell harvest. Then, Lysis buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl2, 1.0 mM DTT, 0.2 mM PMSF, 2.5 % NP-40) was added and the solution was centrifuged at 3500 rpm, 4° C for 4 min. Cytoplasmic fraction was extracted with 5 trials of freezing and thawing of the supernatant of cell lysate followed by centrifugation at 15000 rpm, 4° C, for 20 min. Next, Extraction buffer (20 mM HEPES (pH 7.9), 450 mM NaCl, 1.5 mM MgCl2, 1.0 mM DTT, 0.2 mM PMSF, 0.2 mM EDTA (pH 8.0)) was added to pellet and the solution was centrifuged at 15000 rpm, 4° C, for 20 min after 5 trials of freezing and thawing to extract nuclear fraction. Each fraction was quantified by Bradford assay and was boiled at 95° C for 5 min with sample buffer (40 % Glycerol, 240 mM Tris/HCl pH 6.8, 8 % SDS, 0.04 % bromophenol blue, 5 % beta-mercaptoethanol).

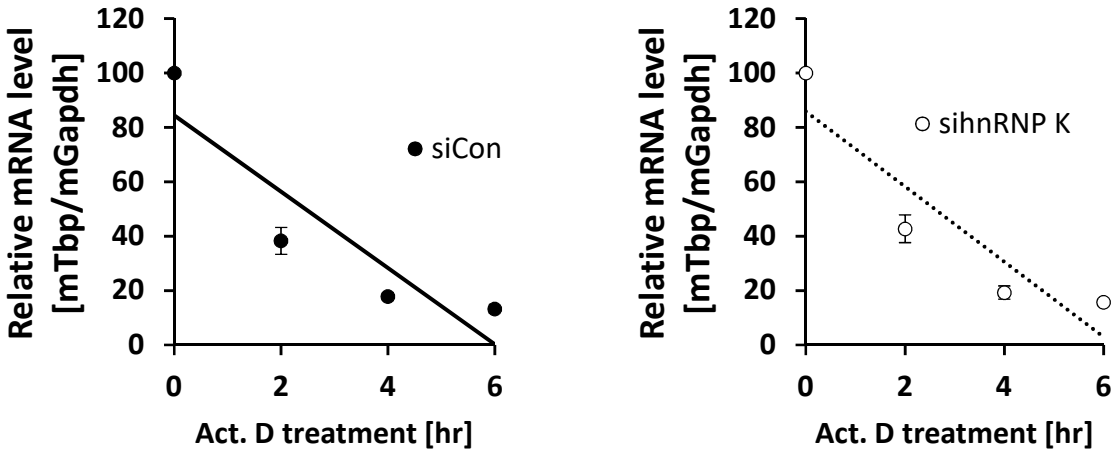
Supplementary Figure S1



Supplementary Figure S1 Knockdown of hnRNP K only triggered a decrease on *Dbp* mRNA expression level.

(A) Each siRNA of hnRNP K, hnRNP A1, hnRNP D, hnRNP R, hnRNP Q, and PTB made its target mRNA level to be decreased (n=3, n.s. : not significant, indicated by asterisks * $p<0.05$ by Unpaired t-test, Error bar : S.E.M.). (B) Only hnRNP K siRNA made a decrease in *Dbp* mRNA expression level (n=3, n.s. : not significant, indicated by asterisks * $p<0.05$ by Unpaired t-test, Error bar : S.E.M.).

Supplementary Figure S2

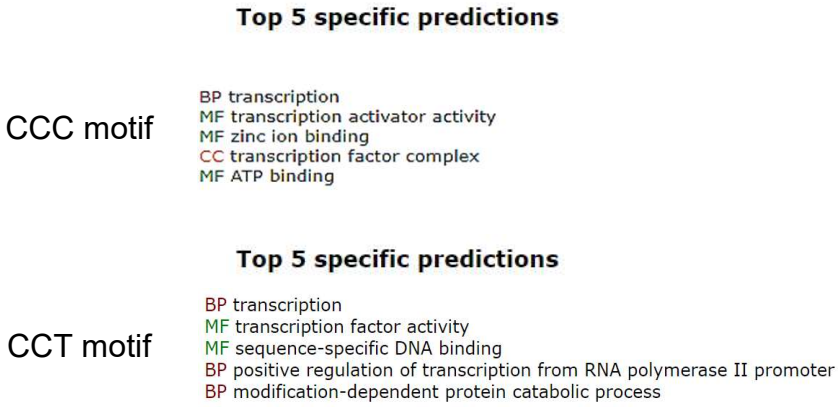


	Half-life (hr)
●siCon	2.5
○sihnRNPK	2.6

Supplementary Figure S2 Knockdown of hnRNP K did not affect mRNA stability of *Tbp.*

Knockdown of hnRNP K did not affect mRNA stability of *Tbp* which was used as a negative control (n=3, Error bar : S.E.M.).

Supplementary Figure S3



Supplementary Figure S3 Analysis of CCC and CCT motif was conducted using GOMo.

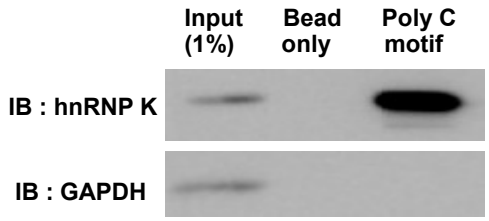
Analysis was conducted to identify the motifs of hnRNP K bound promoter genes within proximal promoter region (~1kb). We were able to extract the motifs which are involved in transcription by using GOMo. MF; Molecular Function, BP; Biological process, CC; Cellular component

Supplementary Figure S4

A

NP_001288274.1	**Mass:** 48480	**Score:** 145	**Expect:** 1e-09	**Matches:** 12	heterogeneous nuclear ribonucleoprotein K isoform 4 [Mus musculus]
NP_001288273.1	**Mass:** 48532	**Score:** 145	**Expect:** 1e-09	**Matches:** 12	heterogeneous nuclear ribonucleoprotein K isoform 3 [Mus musculus]
EAW62680.1	**Mass:** 48774	**Score:** 145	**Expect:** 1e-09	**Matches:** 12	heterogeneous nuclear ribonucleoprotein K, isoform CRA_f [Homo sapiens]
EAW62676.1	**Mass:** 51050	**Score:** 144	**Expect:** 1.3e-09	**Matches:** 12	heterogeneous nuclear ribonucleoprotein K, isoform CRA_b [Homo sapiens]
BAD92799.1	**Mass:** 48774	**Score:** 144	**Expect:** 1.3e-09	**Matches:** 12	heterogeneous nuclear ribonucleoprotein K isoform a variant, partial [Homo sapiens]
EAW62678.1	**Mass:** 51187	**Score:** 144	**Expect:** 1.3e-09	**Matches:** 12	heterogeneous nuclear ribonucleoprotein K, isoform CRA_d [Homo sapiens]
NP_079555.1	**Mass:** 50944	**Score:** 144	**Expect:** 1.3e-09	**Matches:** 12	heterogeneous nuclear ribonucleoprotein K isoform 2 [Mus musculus]
EAW62679.1	**Mass:** 51239	**Score:** 143	**Expect:** 1.6e-09	**Matches:** 12	heterogeneous nuclear ribonucleoprotein K, isoform CRA_e [Homo sapiens]
NP_112553.1	**Mass:** 50996	**Score:** 143	**Expect:** 1.6e-09	**Matches:** 12	heterogeneous nuclear ribonucleoprotein K isoform a [Homo sapiens]

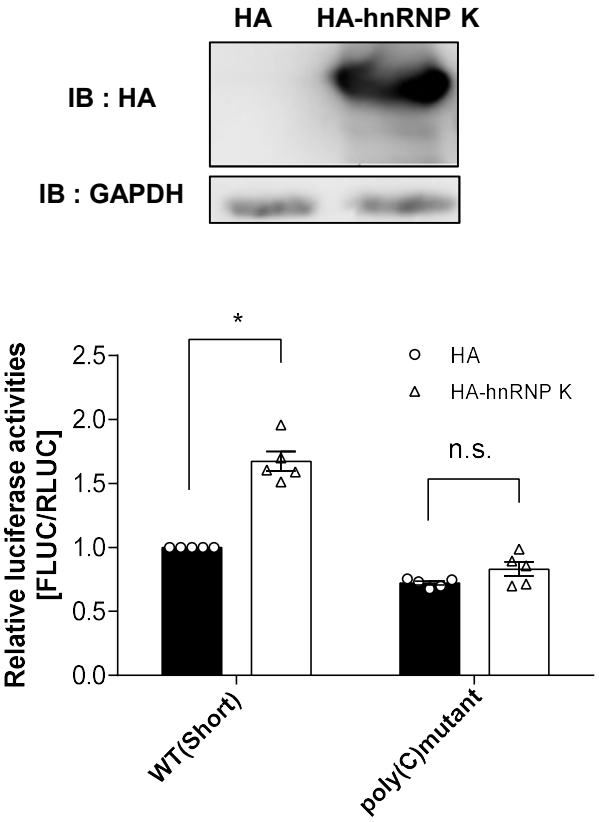
B



Supplementary Figure S4 hnRNP K bound to the poly(C) motif.

(A) Result of Mass spectrometry described that hnRNP K was a major binding factor to the poly(C) motif of *Dbp* promoter region. Protein scores greater than 68 are significant ($p<0.05$). (B) Detection of immunoblotting showed that hnRNP K, which was known as a regulator of transcription, was bound to the poly(C) motif.

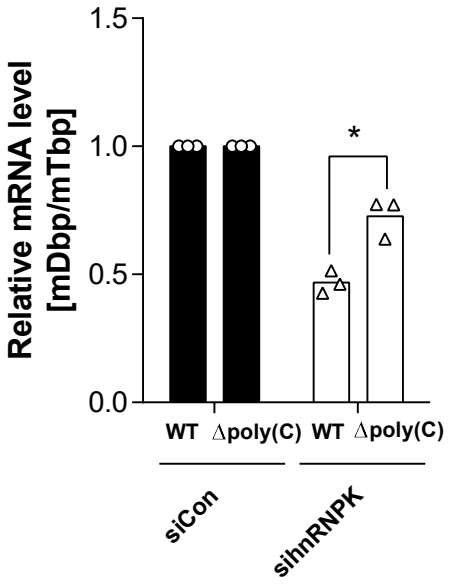
Supplementary Figure S5



Supplementary Figure S5 Overexpression of hnRNP K increased a WT(Short) promoter activity.

Overexpression of hnRNP K increased a WT(Short) promoter activity, whereas the promoter activity of the mutant was not altered (n=5, n.s. : not significant, indicated by asterisks; * $p<0.05$ by One-way ANOVA).

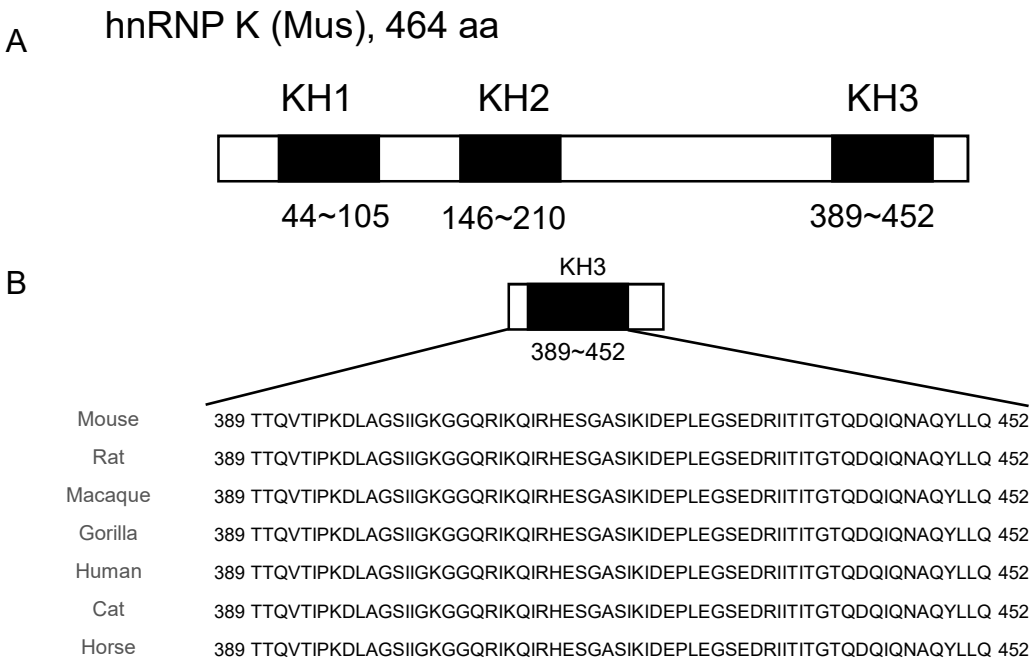
Supplementary Figure S6



Supplementary Figure S6 A significant decrease on the endogenous Dbp mRNA expression was shown by the knockdown of hnRNP K when the poly(C) motif was intact .

A significant decrease on the endogenous Dbp mRNA expression was shown by the knockdown of hnRNP K when the poly(C) motif was intact (n=3, n.s. : not significant, indicated by * $p<0.05$ by Two way ANOVA followed by Sidak's multiple comparisons test, Error bar: S.E.M.).

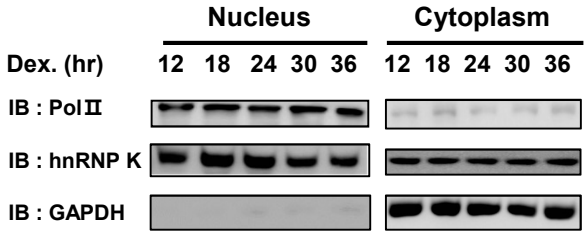
Supplementary Figure S7



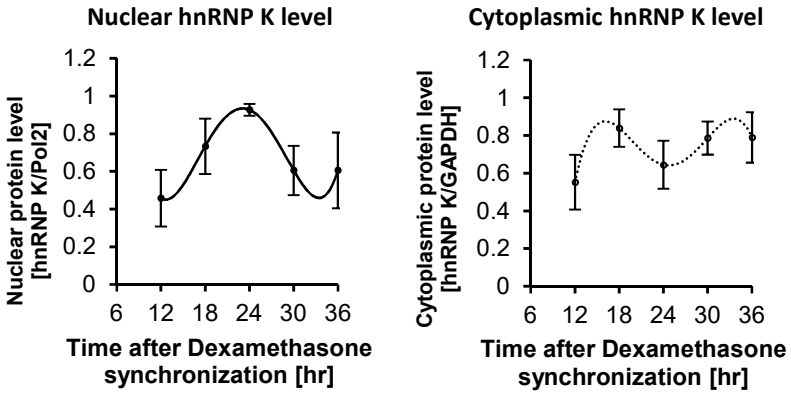
Supplementary Figure S7 KH3 domain of hnRNP K was highly conserved among eutherian mammals. (A) hnRNP K consists of KH domains of KH1, KH2 and KH3 which are able to have nucleic acid binding. (B) The KH3 domain was known for single strand DNA binding domain. We aligned KH3 domains among eutherian mammals, which was shown to have a perfect matching by Ensemble database (http://www.ensembl.org).

Supplementary Figure S8

A



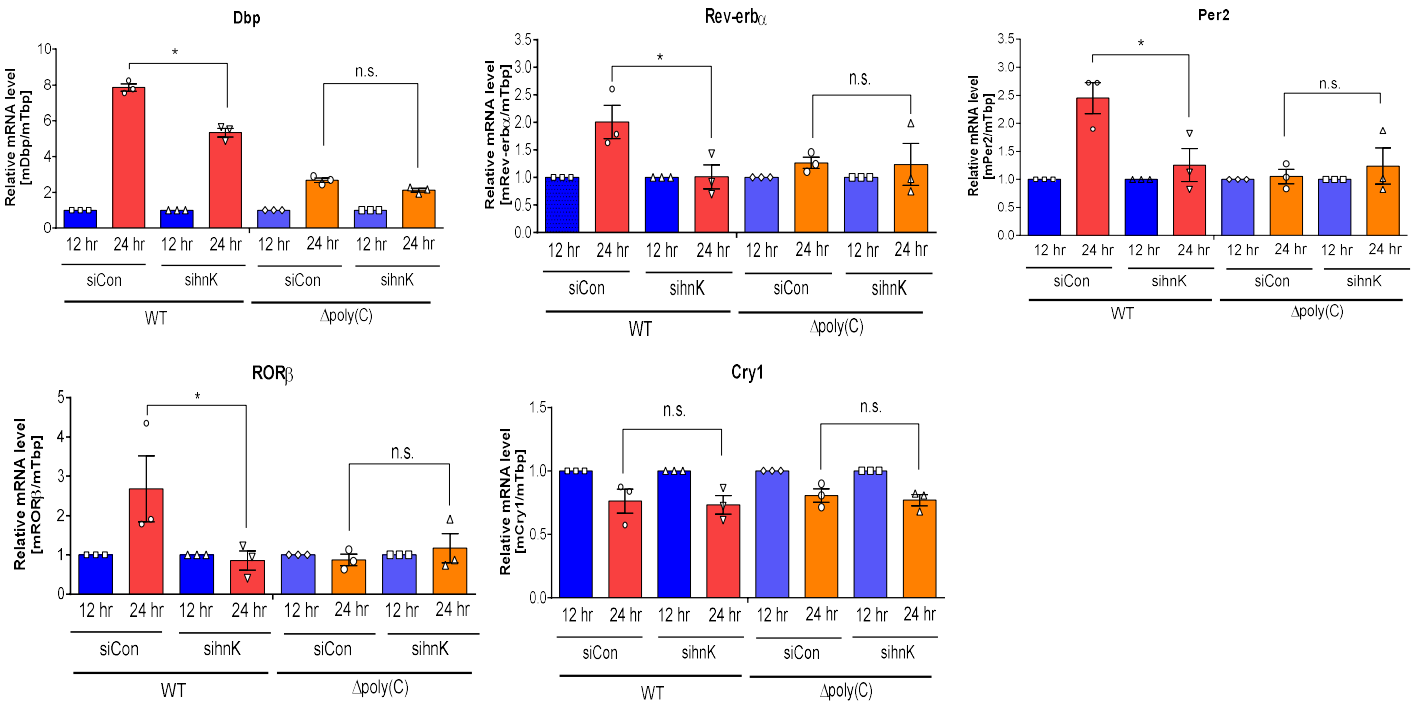
B



Supplementary Figure S8 The cycling of the nuclear hnRNP K protein could be a major contributing factor of rhythmic binding of hnRNP K.

(A) After triggering the circadian rhythm by dexamethasone synchronization, the levels of nuclear hnRNP K and cytoplasmic hnRNP K were measured after the subcellular fractionation. Pol2 was used as a nuclear marker gene and GAPDH was used as a cytoplasmic marker gene. (B) The nuclear protein levels and cytoplasmic protein levels of hnRNP K were quantified (n=5, Error bar: S.E.M.).

Supplementary Figure S9



Supplementary Figure S9 The mRNA levels of *Dbp, Rev-erbα, Per2*, and *RORβ* at peak time were significantly lowered during hnRNP K knockdown only when the poly(C) motif within *Dbp* promoter was intact. *Cry1* was not affected (n=3, n.s. : not significant, indicated by asterisks; * $p<0.05$ by One way ANOVA followed by Tukey's multiple comparisons test, Error bar: S.E.M.).

Table S1. Specific primers for qPCR

Mouse pre-Dbp	F	5’-GCT CTG AGA ACG CAG ACC TC-3’
	R	5’-AGG TCA TTA GCA CCT CCA CG-3’
Mouse Rorβ	F	5‘-GGC AGA CCC ACA CCT ACG A-3'
	R	5‘-CAG AGC CTC CCT GGA CTT G-3'
Mouse Per2	F	5’-GCC TTC AGA CTC ATG ATG ACA GA-3’
	R	5’-TTT GTG TGC GTC AGC TTT GG-3’
Mouse Rev-erbα	F	5’-CTG GAG GGC TGC AGT ATA GC-3’
	R	5’-GTC CAG GGT CGT CAT GTC TT-3’
Mouse Cry1	F	5’-TCC GCT GCG TCT ATA TCC TC-3’
	R	5’-ATT TCC CAG GCT TTT CAA GG-3’
Mouse Dbp	F	5’-AAT GAC CTT TGA ACC TGA TCC CGC T-3’
	R	5’-GCT CCA GTA CTT CTC ATC CTT CTG T-3’
Mouse Nfil3	F	5’-ATG AGG GTG TAG TGG GCA AG-3’
	R	5’-GTT CAC TTC CGG AAC CTT CA-3’
Mouse Tef	F	5’-ATG TCG TCT CAG GTA GCA GG-3’
	R	5’-AGA TTT TGG GGA GGG TGA GG-3’
Mouse Hlf	F	5’-CAT CCT GAA GAC GCA TTT A-3’
	R	5’-ATA AGG TGG GTC CCA AG-3’
Mouse Tbp	F	5'-GCA GCC TCA GTA CAG CAA TC-3'
	R	5'-CTG CGG TAC AAT TCC AGA GC-3'
Mouse Gapdh	F	5’-AAA TGG TGA AGG TCG GTG TG-3’
	R	5'-TGA AGG GGT CGT TGA TGG-3‘
Mouse hnRNPK	F	5’-GCC ATT ATC CTC TGC TTC TCC-3’
	R	5’-GGC TTG CTC CTC TTC CAT ATC-3’
Mouse Eif4e	F	5’-CTG CAT TCC TGG CTG TCT TG-3’
	R	5’-CTC ATC AGC CAC CTT CAT GC-3’

Table S2. List of ChIP-qPCR primers

RB1	F	5'-GGA AAG CAT TTT GGC ATA AAA A-3'
	R	5'-CTA GGA GGC TTG CAA ATG GT-3'
DBP_R1	F	5'-CCT CCT TCT CCA CGT CCT GAT-3'
	R	5'-GGT GAG AAA GGA CAA GGG ATG T-3'
DBP_R2	F	5'-CCT CAT TGG CCC GAA GTG-3'
	R	5'-GGC GGG ACA CTG ACC TAT AT-3'
DBP_R3	F	5'-ATG CTC ACA CGG TGC AGA CA-3'
	R	5'-CTG CTC AGG CAC ATT CCT CAT-3'
Gapdh	F	5'-GAT TAC GGG ATG GGT CTG AA-3'
	R	5'-GCT GCA CCT CTG GTA ACT CC-3'