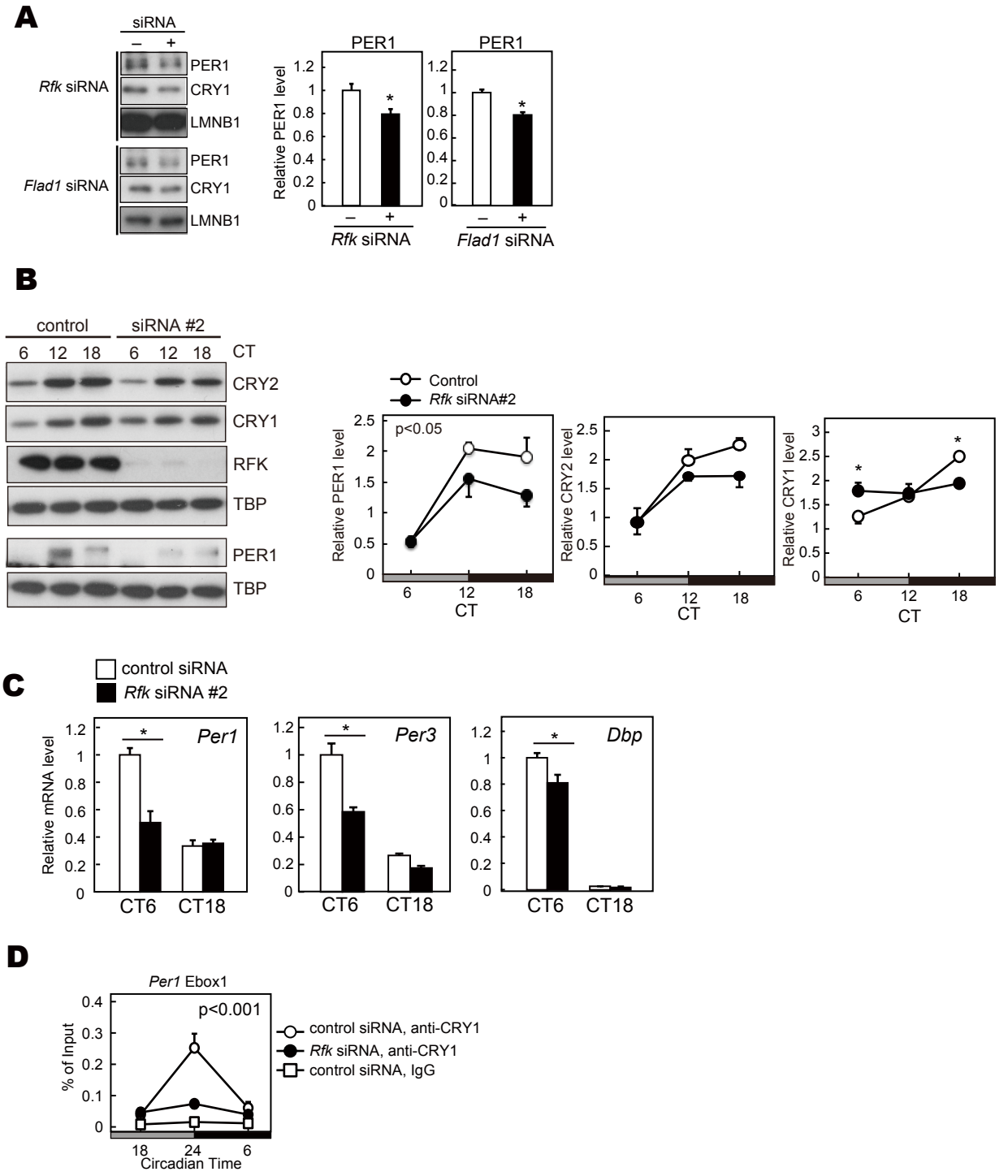
A
siRNA
– +
Rfk siRNA
PER1
CRY1
LMNB1
Flad1 siRNA
PER1
CRY1
LMNB1
PER1
PER1
Relative PER1 level
1.2
1
0.8
0.6
0.4
0.2
0
*
*
– +
Rfk siRNA
– +
Flad1 siRNA
B
control
siRNA #2
6 12 18 6 12 18
CT
CRY2
CRY1
RFK
TBP
PER1
TBP
Control
Rfk siRNA#2
p<0.05
Relative PER1 level
Relative CRY2 level
Relative CRY1 level
2.5
2
1.5
1
0.5
0
3
6 12 18
CT
C
control siRNA
Rfk siRNA #2
Relative mRNA level
Per1
Per3
Dbp
1.2
1
0.8
0.6
0.4
0.2
0
*
CT6 CT18
D
Per1 Ebox1
p<0.001
% of Input
0.4
0.3
0.2
0.1
0
control siRNA, anti-CRY1
Rfk siRNA, anti-CRY1
control siRNA, IgG
18 24 6
Circadian Time

Figure S1

Figure S1. Effects of *Rfk* knockdown on clock protein expression and DNA binding of CRY1, related to Figure 4.

(A) CRY1 and PER1 expression in NIH3T3 cells transfected with *Rfk* or *Flad1* siRNAs (n=3, *: $p<0.05$ by Student's *t*-test). (B) Nuclear protein levels of PER1, CRY1, CRY2 and RFK in DD by *Rfk* siRNA#2. Mice fed riboflavin-free diet for 2 weeks were subjected to tail vein injection of *Rfk* siRNA#2 or control siRNA. Six days after the injection, mice were sacrificed at indicated time points in DD. Data are shown as means ± SEM (n=3, *:$p<0.05$ by two-way ANOVA followed by post-hoc test). (C) mRNA levels of indicated clock genes in *Rfk* knockdown mice. mRNA levels were normalized to *G3pdh*. Data are shown as means ± SEM (n=3, *:$p<0.05$ by two-way ANOVA followed by post-hoc test). (D) ChIP assay of CRY1 protein in *Rfk* knockdown mice. Mice were injected with *Rfk* siRNA and sacrificed in DD at specified time. CRY1 occupancy at the *Per1* promoter containing E-box was analyzed by PCR using specific primers. Data are shown as means ± SEM (n=3, *:$p<0.05$ by two-way ANOVA followed by post-hoc test).

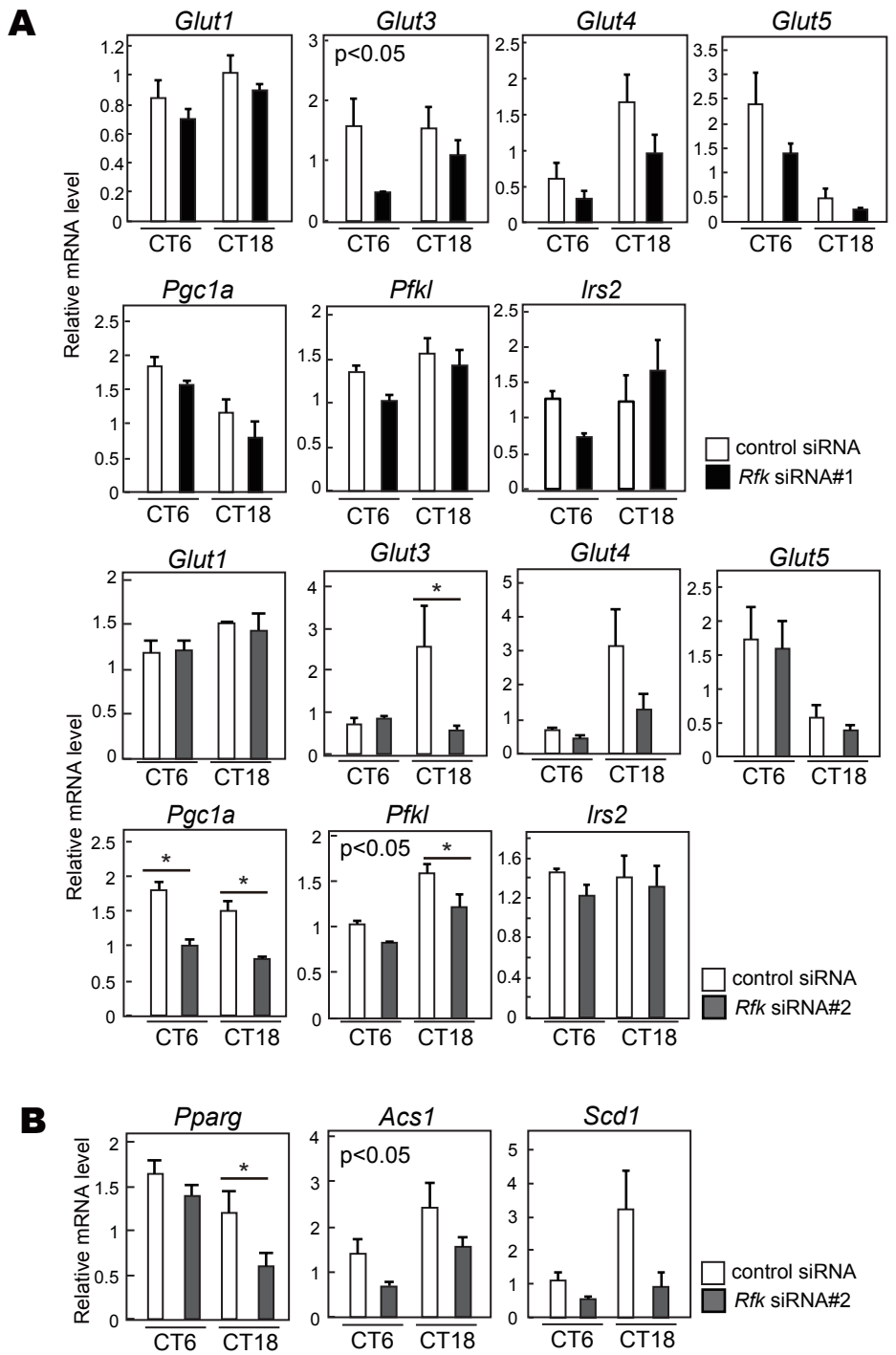
A
Glut1
Glut3
Glut4
Glut5
p<0.05
Relative mRNA level
CT6
CT18
Pgc1a
Pfkl
Irs2
control siRNA
Rfk siRNA#1
Glut1
Glut3
Glut4
Glut5
Relative mRNA level
Pgc1a
Pfkl
Irs2
p<0.05
control siRNA
Rfk siRNA#2
B
Pparg
Acs1
Scd1
p<0.05
Relative mRNA level
control siRNA
Rfk siRNA#2

Figure S2

Figure S2. Expression of clock-controlled genes in *Rfk* knockdown mouse, related to Figure 5.

(A) (B) mRNA levels of indicated genes in *Rfk* knockdown mouse. Mice fed riboflavin-free diet for 2 weeks were subjected to tail vein injection of *Rfk* siRNA#1 or control siRNA. Six days after the injection, mice were sacrificed at indicated time points in DD. mRNA levels were normalized to *G3pdh*. Data are shown as means ± SEM (n=3, *:$p<0.05$ by two-way ANOVA followed by post-hoc test). *Glut*; *Glucose transporter*, *Pgc1a*; *Papprg coactivator 1a*, *Pfkl*; *Phosphofructokinase*, *Irs2*; *Insulin receptor substrate2*. *Pparg; Peroxisome proliferator activated receptor gamma*, *Acs1; Acetyl-coenzyme A synthetase 1, Scd1; Stearoyl-coenzyme A desaturase 1*.

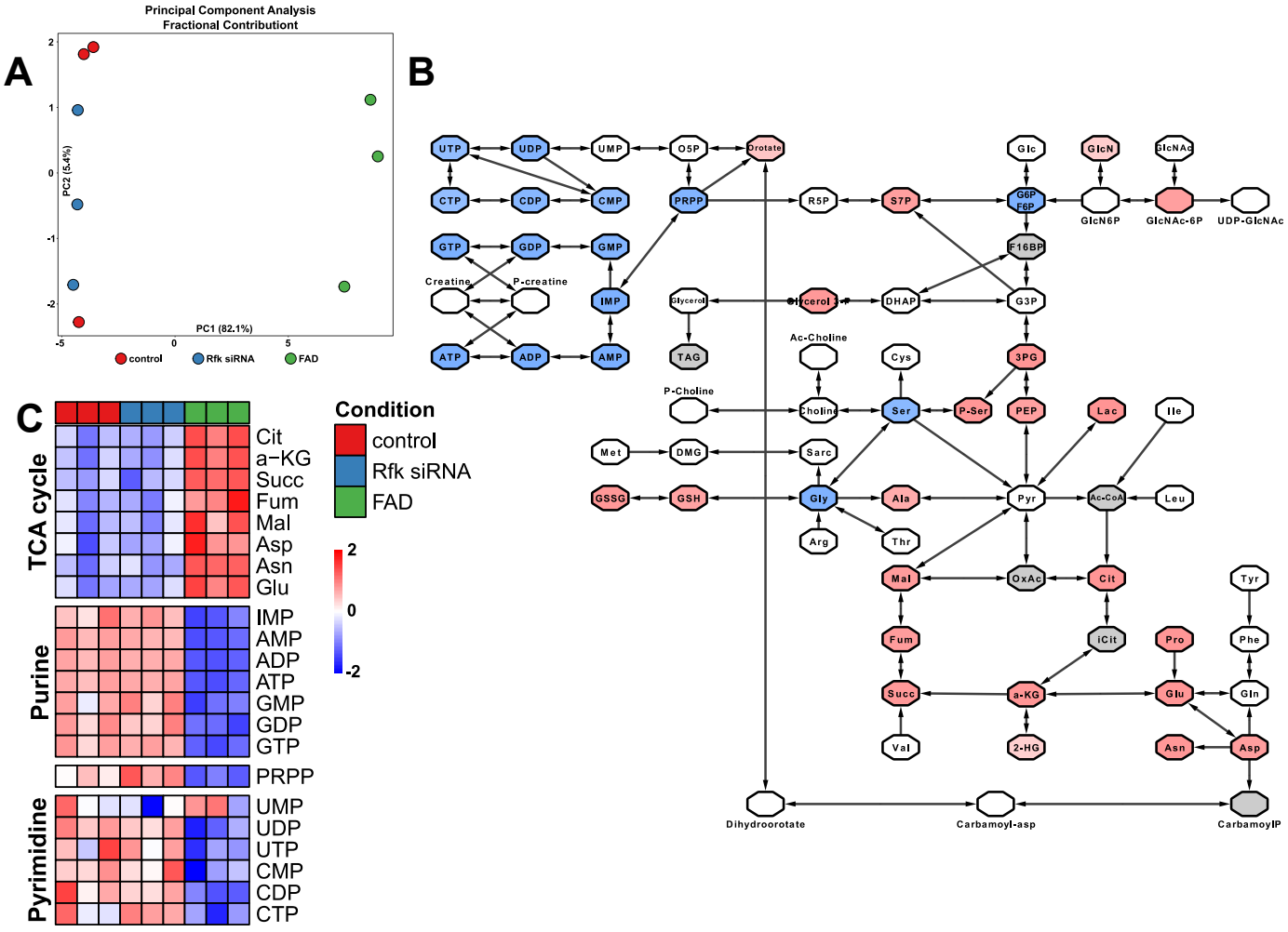
A
Principal Component Analysis
Fractional Contributiont
PC1 (82.1%)
PC2 (5.4%)
control
Rfk siRNA
FAD
B
UTP
UDP
UMP
O5P
Orotate
CTP
CDP
CMP
PRPP
R5P
S7P
Glc
GlcN
GlcNAc
G6P
F6P
GlcN6P
GlcNAc-6P
UDP-GlcNAc
GTP
GDP
GMP
F16BP
Creatine
P-creatine
IMP
Glycerol
Glycerol-3-P
DHAP
G3P
ATP
ADP
AMP
TAG
Ac-Choline
Cys
3PG
P-Choline
Choline
Ser
P-Ser
PEP
Lac
Ile
Met
DMG
Sarc
GSSG
GSH
Gly
Ala
Pyr
Ac-CoA
Leu
Arg
Thr
Mal
OxAc
Cit
Tyr
Fum
iCit
Pro
Phe
Succ
a-KG
Glu
Gln
Val
2-HG
Asn
Asp
Dihydroorotate
Carbamoyl-asp
CarbamoylP
C
Condition
control
Rfk siRNA
FAD
2
0
-2
TCA cycle
Cit
a−KG
Succ
Fum
Mal
Asp
Asn
Glu
Purine
IMP
AMP
ADP
ATP
GMP
GDP
GTP
PRPP
Pyrimidine
UMP
UDP
UTP
CMP
CDP
CTP
D
Relative amounts of Currency metabolites
ADP ***
ADP/ATP
AMP *
AMP/ATP *
ATP
Creatine
Creatine/P−Creatine *
Creatinine
GSH
GSH/GSSG **
GSSG ***
NAD+
NADH **
NADP+ **
P−Creatine
Relative Amounts
Conditions
control
Rfk siRNA
FAD

Figure S3

Figure S3. Metabolomics analysis of *RFK* knockdown cells and FAD-treated cells, related to Figure 5. Cells were treated with 100 μM FAD for 24 hours after siRNA. All cells were isotope labeled with 13C6 D-glucose for metabolomics analysis. (A) Principal component analysis of metabolomics data. FAD treatment had a large effect on metabolism. (B) Simplified metabolism map showing affected pathways. Metabolites labeled with red showed induced synthesis from isotope labeled glucose, while those labeled with blue showed reduction. (C) Heat map for metabolites related to TCA cycle and nucleotide synthesis. (D) Relative amounts of currency metabolites. Data were shown as means ± SEM (n=3, *:p<0.05, **:p<0.01, ***:p<0.0001 by one-way ANOVA).

SUPPELEMENTAL EXPERIMENTAL PROCEDURES

Plasmids construction

DNA constructs used for transfections are as follows (Hirano et al., 2016b): hCRY2-WT-Myc-His/pcDNA3.1, FLAG-hCRY2-WT/p3×FLAG-CMV-10, FLAG-hCRY2-A260T/p3×FLAG-CMV-10, FLAG-hFBXL3/p3×FLAG-CMV-10, HA-hFBXL3/pCMV-tag2B. For knockdown of human *Rfk, CRY1, CRY2* and mouse *Rfk* and *Flad1*, Hs_*RFK,* Hs_*CRY1,* Hs_*CRY2,* Mm_*Rfk* and Mm_*Flad1* FlexiTube siRNA (QIAGEN) and control siRNA (QIAGEN) were purchased. shRNA constructs of mouse *Rfk* were created by inserting shRNA target sequences into pSilencer3.1-H1-puro. The target sequences were: *Rfk* shRNA#1 5'-ATAGATGTAATATGTTGATTAA-3', *Rfk* shRNA#2 5'-ACAGGCACTTAAACATAATTTA-3', *Rfk* shRNA#3 5'-AACTTTGATTCTTTAGAGTCA-3', negative control shRNA 5'-AATTCTCCGAACGTGTCACGT-3'.

Chromatin immunoprecipitation assay (ChIP)

ChIP assay was performed as previously described (Yoshitane et al., 2014). Briefly, brain nuclear extracts were incubated in 1% formaldehyde for the cross-linking at RT for 15 min and the reactions were subsequently quenched by adding glycine (final 0.125 M). DNA was physically fragmented by sonication (25 sec pulse and 25 sec rest at power 30% was repeated 6 times). Antibody reaction and DNA purification were performed using the EZ-Magna ChIP A/G assay kit (EMD Millipore). ChIP signal was determined by real-time PCR using specific primers listed below (Koike et al., 2012).

ChIP-m*Per1*-E-box-fw:5' AGCCAGCCTGCACGTGTTCC 3'

ChIP-m*Per1*-E-box-rv:5' CAGAGACAACCCCGCCCTGC 3'

Metabolomics analysis

HepG2 cells were transfected with *RFK* siRNA or control siRNA. Twenty-four hours after the transfection, cells were treated with FAD as well as labeled with D-glucose isotope (U-13C6) (Cambridge Isotope Laboratories, Inc., CLM-1396-PK) for 24 hours. Cells were rinsed with cold 150 mM NH_4AcO (pH7.3) and collected with 1ml 80% MeOH, followed with addition of 5 nmol Norvaline (Sigma aldrich). Cell extracts were centrifuged at 14 krpm for 5 min. Extraction was repeated by adding 200 µl 80% MeOH to precipitate. Combined supernatant was dried down using SpeedVac. Metabolites were analyzed by Q Extractive mass spectrometry coupled to a UltiMate 3000 HPLC chromatography systems (Thermo Fisher Scientific).

Table S1 Primer sequence used for real-time PCR analysis, related to Figure 4.

primer name	primer sequence 5'-3'
mPer1-fw	CAGGCTAACCAGGAATATTACCAGC
mPer1-rv	CACAGCCACAGAGAAGGTGTCCTGG
mPer2-fw	ATGCTCGCCATCCACAAGA
mPer2-rv	GCGGAATCGAATGGGAGAAT
mCry1-fw	CCCAGGCTTTTCAAGGAATGGAACA
mCry1-rv	TCTCATCATGGTCATCAGACAGAGG
mCry2-fw	GGGACTCTGTCTATTGGCATCTG
mCry2-rv	GTCACTCTAGCCCGCTTGGT
mDbp-fw	AATGACCTTTGAACCTGATCCCGCT
mDbp-rv	GCTCCAGTACTTCTCATCCTTCTGT
mReverba-fw	GGGCACAAGCAACATTACCA
mReverba-rv	CACGTCCCCACACACCTTAC
mPer3-fw	CCGCCCCTACAGTCAGAAAG
mPer3-rv	GCCCCACGTGCTTAAATCCT
mG6pc-fw	TCGGAGACTGGTTCAACCTC
mG6pc-rv	AGGTGACAGGGAACTGCTTTAT
mPck1-fw	AAGCATTCAACGCCAGGTTC
mPck1-rv	GGGCGAGTCTGTCAGTTCAAT
mPparg-fw	CACAATGCCATCAGGTTTGG
mPparg-rv	GCTGGTCGATATCACTGGAGATC
mScd1-fw	TGCCCCTGCGGATCTT
mScd1-rv	GCCCATTCGTACACGTCATT
mAcs1-fw	GCTGCCGACGGGATCAG
mAcs1-rv	TCCAGACACATTGAGCATGTCAT
mPfkl-fw	TGCAGCCTACAATCTGCTCC
mPfkl-rv	GTCAAGTGTGCGTAGTTCTGA
mPgc1α-fw	AGCCGTGACCACTGACAACGAG
mPgc1α-rv	AGCCGTGACCACTGACAACGAG
mIrs2-fw	GGAGAACCCAGACCCTAAGCTACT
mIrs2-rv	GATGCCTTTGAGGCCTTCAC
mGlut1-fw	CGTGCTTATG GGTTTCTCCAAA
mGlut1-rv	GACACCTCCCCCACATACATG
mGlut3-fw	TGAAGCCATGA GCTTTGTCTGT
mGlut3-rv	GCCCTGGCT GAAGAGTTCAG
mGlut4-fw	CAACTGGACCTGTAACTTCATCGT
mGlut4-rv	ACGGCAAATAGAAGGAAGACGTA
mGlut5-fw	CCAATATGGGTACAACGTAGCTG
mGlut5-rv	GCGTCAAGGTGAAGGACTCAATA
mG3pdh-fw	ACGGGAAGCTCACTGGCATGGCCTT
mG3pdh-rv	CATGAGGTCCACCACCCTGTTGCTG
mTbp-fw	ACCTAAAGACCATTGCACTTCG
mTbp-rv	GCTCTCTTATTCTCATGATGACTGC
mRfk-fw	TGGAAACACACATCATCCATACC
mRfk-rv	CACCTTGAATTGCAGAAATAAGTGAC
mFlad1-fw	CCTCTTCCATGCAGCCGTG
mFlad1-rv	CGGCTTCTAACACCTGGAGATTATAC

SUPPELEMENTAL REFERENCES

Hirano, A., Shi, G., Jones, C.R., Lipzen, A., Pennacchio, L.A., Xu, Y., Hallows, W.C., McMahon, T., Yamazaki, M., Ptáček, L.J., et al. (2016). A Cryptochrome 2 mutation yields advanced sleep phase in humans. eLife *5*, e16695.

Yoshitane, H., Ozaki, H., Terajima, H., Du, N.H., Suzuki, Y., Fujimori, T., Kosaka, N., Shimba, S., Sugano, S., Takagi, T., et al. (2014). CLOCK-Controlled Polyphonic Regulation of Circadian Rhythms through Canonical and Noncanonical E-Boxes. Molecular and Cellular Biology *34*, 1776–1787.