Supplemental Information

"CREBH Maintains Circadian Glucose Homeostasis by Regulating Hepatic Glycogenolysis and Gluconeogenesis" by H. Kim, Z. Zheng, P. Walker, G. Kapatos, and K. Zhang.

Material and Methods

Immunoblotting analyses - Total cell lysates were prepared from mouse livers or cultured cells using RIPA cell lysis buffer (1% NP-40; 50mM Tris-HCl, pH 8.0; 150mM NaCl; 5mM NaF; 1mM sodium vanadate; 0.5% sodium deoxycholate; 0.1% SDS) supplemented with protease inhibitor cocktail (Sigma, P2714). Protein concentration of the whole lysates was determined using a Bradford assay (BioRad). Denatured proteins were separated by SDS-PAGE on 8-15% Tris-glycine polyacrylamide gels and transferred to a 0.45-mm PVDF membrane (GE Healthcare). Membrane-bound antibodies were detected using an enhanced chemiluminescence (ECL) detection reagent (GE Healthcare) and Bio-Rad imaging system. Levels of β-actin, tubulin, or GAPDH were determined as loading controls. The signal intensities were determined by Quantity One 4.6.7 (Bio-RadLife Science, CA). A rabbit polyclonal CREBH antibody has been developed in our laboratory and was used to detect the endogenous CREBH protein levels from mouse liver tissue (Kim et al. 2014). The commercially available antibodies were used to detect endogenous protein levels of C/EBPβ, E4BP4, G6PC, FADS2, CPT1α, BDH1, ApoA4 (Santa Cruz Biotech), LXRα (Invitrogen), HNF4α (Invitrogen), SREBP1c (Thermo Scientific), CLOCK (Cell Signaling), BMAL1 (Novus Biologicals), PPARα (Millipore), PCK1 (Sigma), ACC1 (Epitomics), FGF21 (R&D Systems), β-actin (Sigma), tubulin (Sigma), and GAPDH (Sigma), respectively, in mouse liver lysates.

Immunoprecipitation (IP)-Western blot analyses - Endogenous protein-protein interactions between CREBH and E4BP4, PPARα, or CEBPβ in mouse livers across the day-night cycle were determined by IP-Western blot analysis. Approximate 200 μg of liver protein lysates were incubated with 1μg of a rabbit anti-mouse CREBH antibody overnight at 4°C, as indicated in Figure 5A. The rabbit polyclonal CREBH antibody used for pulling down the endogenous CREBH protein was developed in our laboratory (Kim et al. 2014). Protein complexes were immunoprecipited using Dynabeads Protein G (Novex), resolved by SDS-PAGE, and then transferred to PVDF membrane. The assay was followed by Western blot analysis with primary antibodies directed against E4BP4, PPARα, or CEBPβ. The protein interaction signals were visualized by using HRP-conjugated Clean-Blot IP Detection Reagents (Thermo Scientific), which can eliminate detection-interference from both heavy-chain and light-chain IgG fragments of the antibodies used for the initial IP assay. Conjugated HRP was then developed using an enhanced chemiluminescence (ECL) detection reagent (GE Healthcare).

Mouse liver nuclei preparation for ChIP assays – Mouse liver tissues were homogenized using a Teflon pestle in 1:10 (w:v) of ice-cold NP-40 Lysis Buffer supplemented with protease inhibitor cocktail. The liberation of nuclei was monitored by DAPI staining and fluorescence microscopy. To purify the intact nuclei, lysates were then layered over 1M (bottom) and 0.68M (top) of sucrose, and spun at 4620 g for 30 min at 4°C. Following a washing step, nuclear pellets were cross-linked with 1% fresh formaldehyde in PBS for 10 min at room temperature. Cross-linking was terminated by addition of 200mM Tris-HCl (pH 9.4) and 1mM DTT for 10 min and centrifuged at 1160 g for 15 min at 4°C. Nuclear pellets were suspended in SDS lysis buffer containing protease inhibitors, incubated for 10 min on ice, and sonicated in a cold-water bath using chiller circulator-equipped Bioruptor Sonication Device (Diagenode) (Kapatos et al. 2007; Kfoury and Kapatos 2009).

Luciferase gene expression reporter analysis - To construct the *Pck1* gene promoter-driven expression reporter plasmid (pGL3-Pck1), the 5′-flanking region from −385 nt to -36 nt of the mouse *Pck1* gene was amplified from mouse cDNA by PCR using the forward primer 5′-ATGGTACCGCAGCCAGCAACATATGAAG-3′ and the reverse primer 5′-ATGAGCTCATAGAAGGGAGGACAGCCCT-3′. PCR products were digested using KpnI and SacI restriction enzymes and cloned into the same sites of the pGL3-basic vector (Promega, USA). The identity of the cloned plasmid was verified by sequence analysis. For the luciferase assay, 1μg of pGL3-Pck1 plasmids and 0.1μg of pGL4.7 plasmids were transiently co-transfected into Hepa1-6 cells using the TransIT-2020 reagent (Mirus Bio, WI). After 24 hour, co-transfected cells were infected with adenovirus expressing GFP (Ade-GFP), activated CREBH (Ade-CREBH), PPARα (Ade-PPARα), E4BP4 (Ade-E4BP4), and/or C/EBPβ (Ade-C/EBPβ), as indicated in Figure 5B-C. Cells were harvested and lysed at 24 hrs after transfection. Luciferase activity was measured using the Dual-Luciferase Assay System (Promega) according to the manufacturer's instructions. The reporter expression readout was presented by normalizing Firefly luciferase activities to Renilla luciferase activities (internal control). Each reporter assay was performed in duplicate.

Histological staining and quantitative analysis of hepatic glycogen - Periodic-acid staining of hepatic glycogen was performed according to the standard protocol (Zhang et al. 2012; Zheng et al. 2013). Briefly, tissue samples were collected from similar liver lobe regions of CREBH-null and WT control mice under the circadian clock and then fixed in 10% formalin. Formalin-fixed, paraffin-embedded liver tissue was sectioned on a cryostat, and sections were deparaffinized, re-hydrated, and oxidized in 0.5% periodic acid solution for 5min. The oxidized tissue sections were incubated in Schiff's reagent (Sigma) for 15 min. Biochemical quantification of hepatic glycogen in the liver tissues of CREBH-null and WT control mice

under the circadian clock was performed using a commercial enzymatic kit (BioAssay Systems, Hayward, CA) (Zhang et al. 2012; Zheng et al. 2013). Approximately 40 mg of liver tissue from similar lobe regions of CREBH null and control mice were homogenized in ice-cold citrate buffer (0.1M, pH 4.2). Homogenates were immediately subjected to glycogen measurement using the glycogen assay kit following the manufacturer's instruction. Levels of hepatic glycogen were presented after normalization to liver mass.

Supplemental figure legends

S-Figure 1. (**A**) Quantification of PYGL, PCK1, and G6PC protein levels in the livers of CREBH-null and WT control mice under feeding or after 6-, 12- and 24-hour fasting. Fold changes of protein levels are shown by comparing to that of the mouse under the feeding condition. Each bar donates mean ± SEM. Levels of β-actin were determined as loading controls. (**B**) Quantification of PYGL, PCK1, and G6PC protein levels in the livers of mice over-expressing GFP or activated CREBH. Fold changes of protein levels are shown by comparing to that of one of the control mice over-expressing GFP. Each bar donates mean ± SEM (n=3 mice per group). Levels of β-actin were determined as loading controls.

S-Figure 2. Expression levels of Gsk3β, Ugp2, Phka2, Gbe, and Agl mRNAs in the livers of CREBH-null and WT control mice under feeding or fasting for 6, 12, and 24 hours. Expression levels of mRNAs were determined by qRT-PCR. Fold changes of mRNA levels are shown by comparing to that of one of the wild-type control mice under the feeding condition. Each bar denotes mean ± SEM (n = 3-4 mice per time point). * $p < 0.05$; ** $p < 0.01$..

S-Figure 3. Rhythmic expression of GYS2 in CREBH-null and WT control mouse livers under the circadian clock. (**A**) Expression levels of the Gys2 mRNA in the livers of CREBH-null and WT mice under the circadian clock. Expression levels of mRNAs were determined by qRT-PCR. Fold changes of mRNA levels are shown by comparing to that of one of the wild-type control mice at the starting circadian time point. Each bar denotes mean ± SEM (n = 3-5 mice per group per time point). (**B**) Western blot analysis of rhythmic levels of GYS2 protein in CREBH-null and WT mouse livers collected every 4 hours in a 24-hour circadian period. Pooled liver protein lysates from 3-5 mice per time point per genotype group were used. Levels of β-actin were determined as the loading controls.

S-Figure 4. Potential CREBH- and PPARα- binding sequences in the promoter region of mouse Pck1, Pygl, and G6pc genes. The binding motifs are highlighted. The complementary sequence is presented if the binding motif locates in the negative strand. The *Pck1* gene promoter oligo probe sequences used for EMSA analysis were listed. The probe contains the integrated CREBH-PPARα binding motif in the *Pck1* gene promoter region. The complimentary oligo sequence to the CREBH-PPARα motif was shown.

S-Figure 5. Quantification of PYGL, PCK1, and G6PC protein levels in the pooled livers of CREBH-null and WT control mice under the circadian clock (n=3 mice/genotype/time point). CREBH protein signal intensity, determined by Western blot densitometry, was normalized to that of GAPDH. Fold changes of protein levels are shown by comparing to that of at 6 pm.

S-Table 1. Primer sequences used for real-time qPCR, ChIP-qPCR, and EMSA in this study

References

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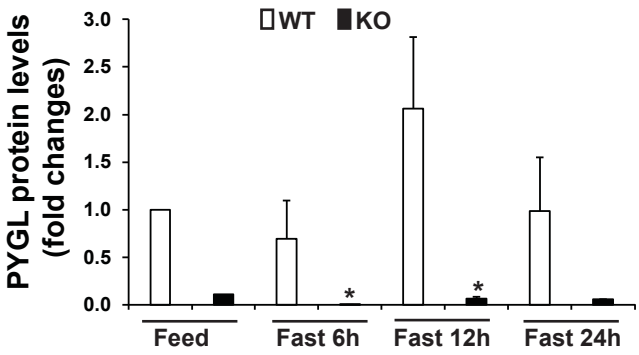
A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KO
PYGL protein levels (fold changes)
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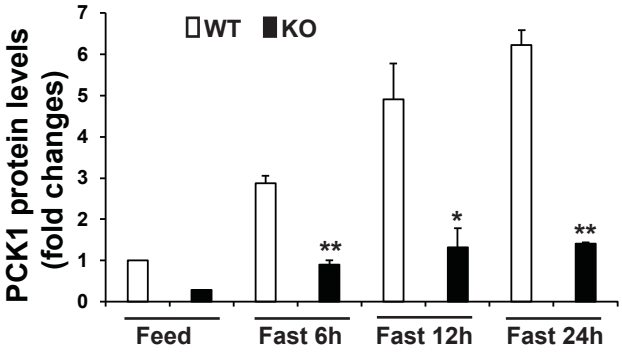
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Feed
Fast 6h
Fast 12h
Fast 24h


WT
KO
PCK1 protein levels (fold changes)
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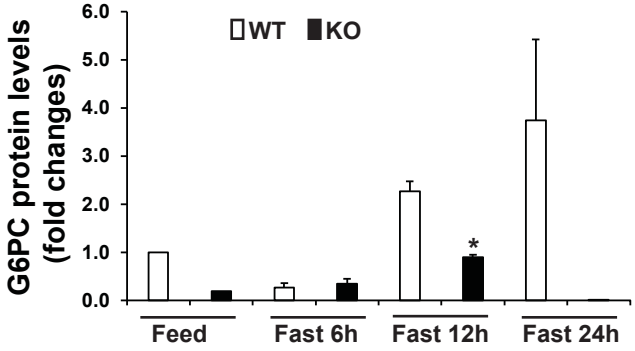
6
5
4
3
2
1
0
**
*
**
Feed
Fast 6h
Fast 12h
Fast 24h


WT
KO
G6PC protein levels (fold changes)
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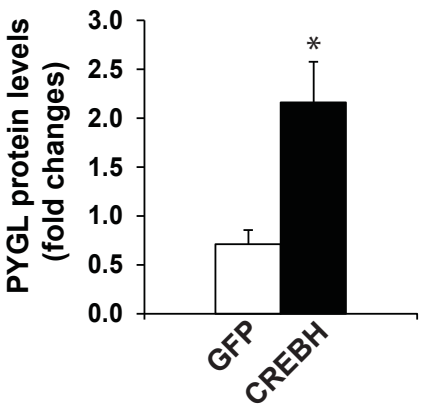
5.0
4.0
3.0
2.0
1.0
0.0
*
Feed
Fast 6h
Fast 12h
Fast 24h

B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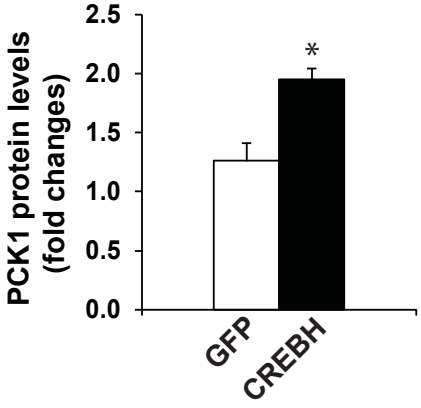
2.0
1.5
1.0
0.5
0.0
*
GFP
CREBH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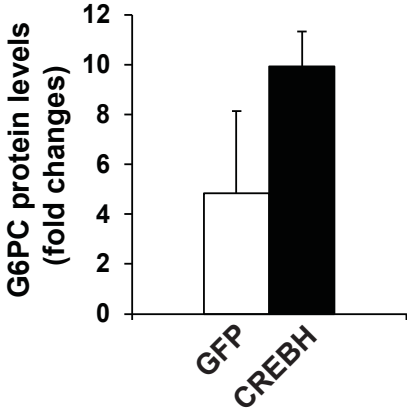
2.0
1.5
1.0
0.5
0.0
*
GFP
CREBH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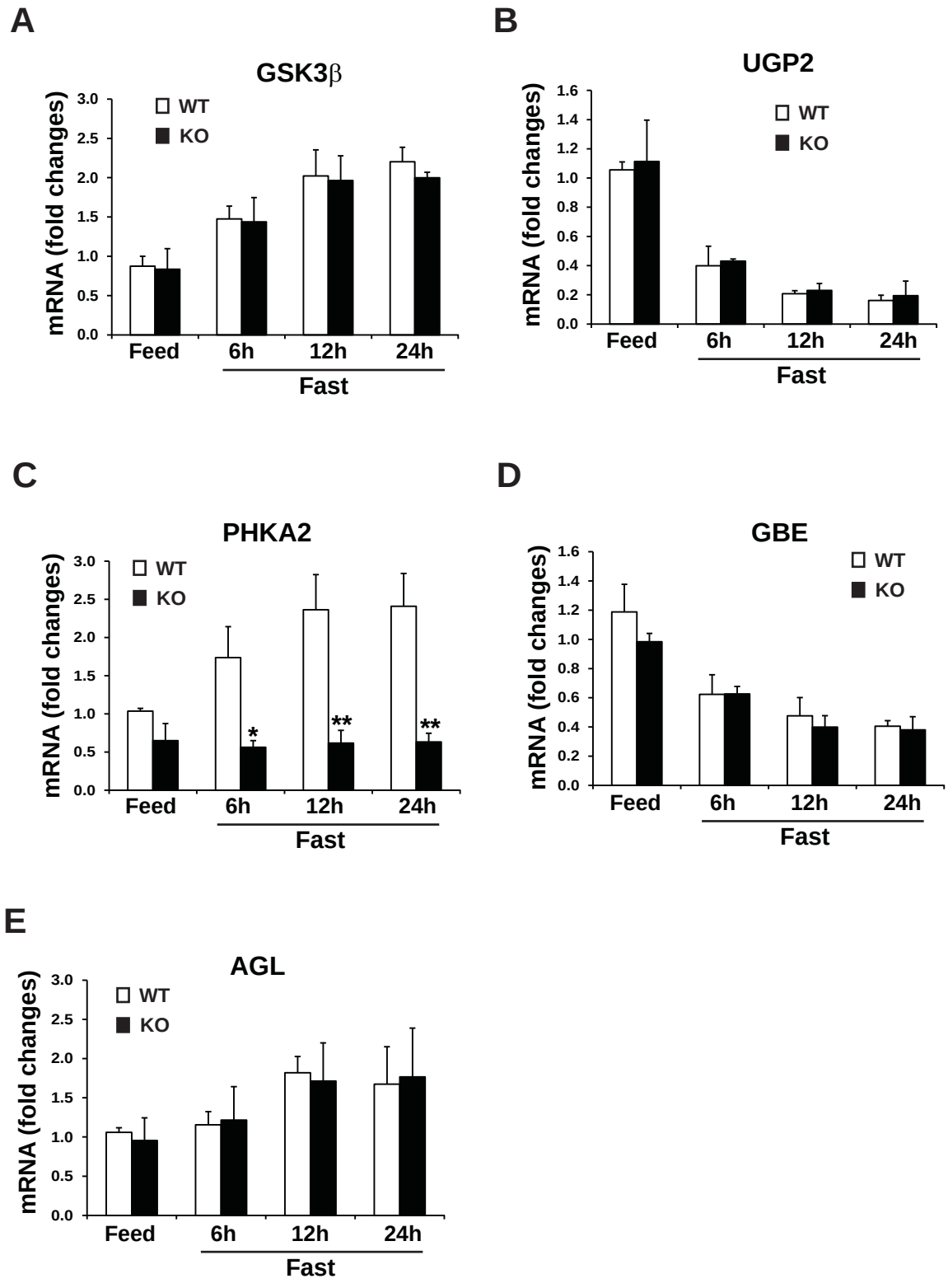
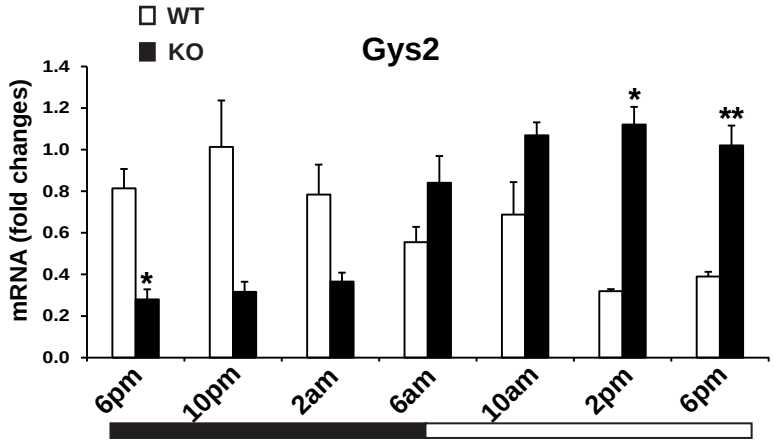
10
8
6
4
2
0
GFP
CREBH


A
GSK3β
mRNA (fold changes)
WT
KO
Feed
6h
12h
24h
Fast
B
UGP2
C
PHKA2
*
**
**
D
GBE
E
AGL

A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KO
Gys2
mRNA (fold changes)
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1.2
1.0
0.8
0.6
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0.2
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*
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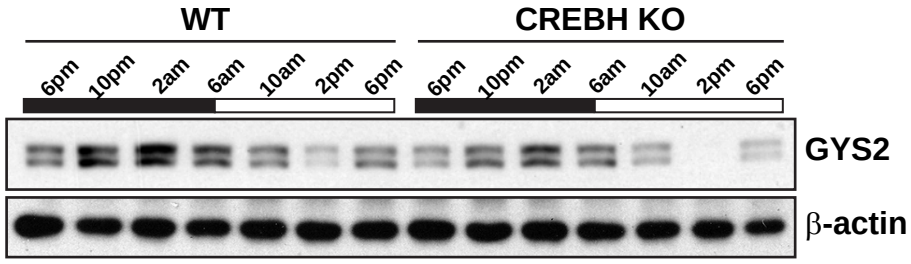
6pm
10pm
2am
6am
10am
2pm
6pm

B


WT
CREBH KO
6pm
10pm
2am
6am
10am
2pm
6pm
GYS2
β-actin

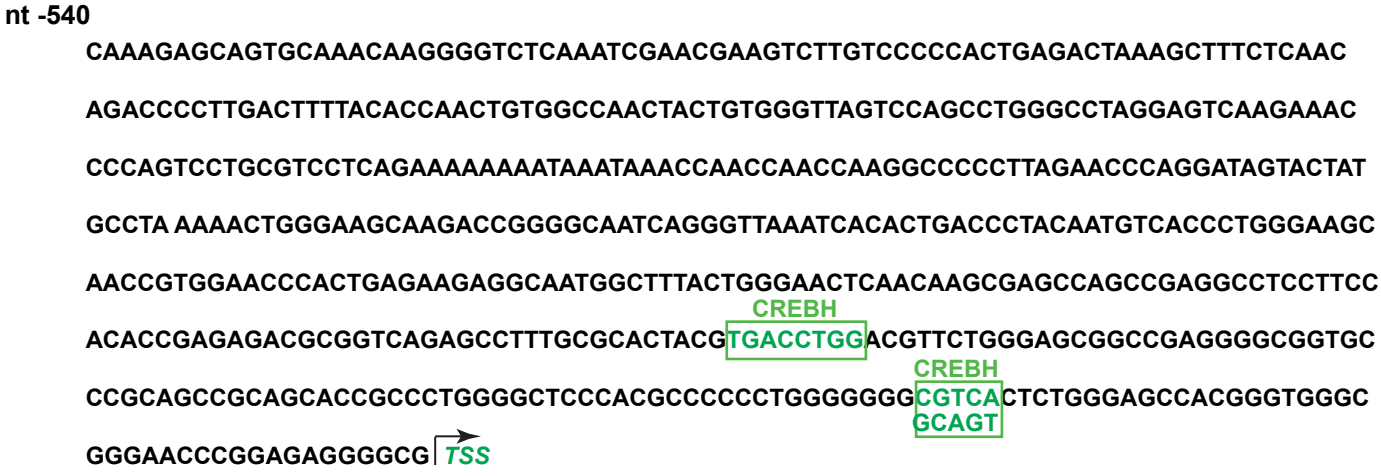
A

Transcription factor binding elements in mouse *Pck1* gene promoter region

mPCK1 EMSA-F-1 : 5'-Biotin-CCTTCTCATGACCTTTGGCCGTGGGAGTGACACCTCACAG-3'
mPCK1 EMSA-R-1 : 5'-Biotin-CTGTGAGGTGTCACTCCCACGGCCAAAGGTCATGAGAAGG-3'

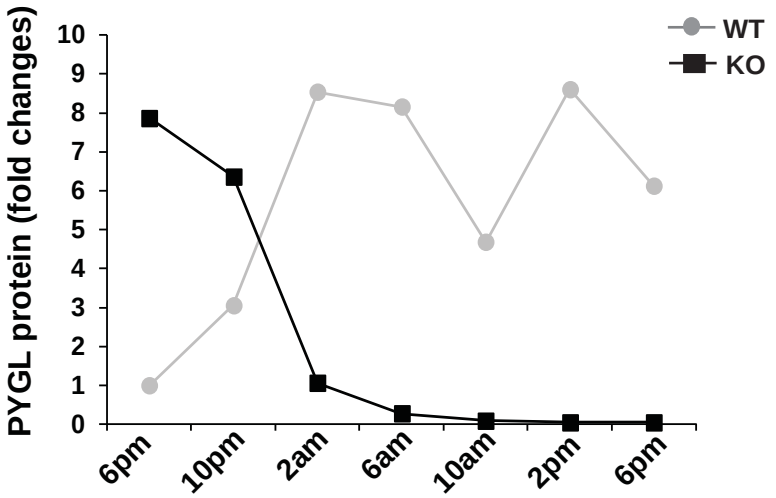
B

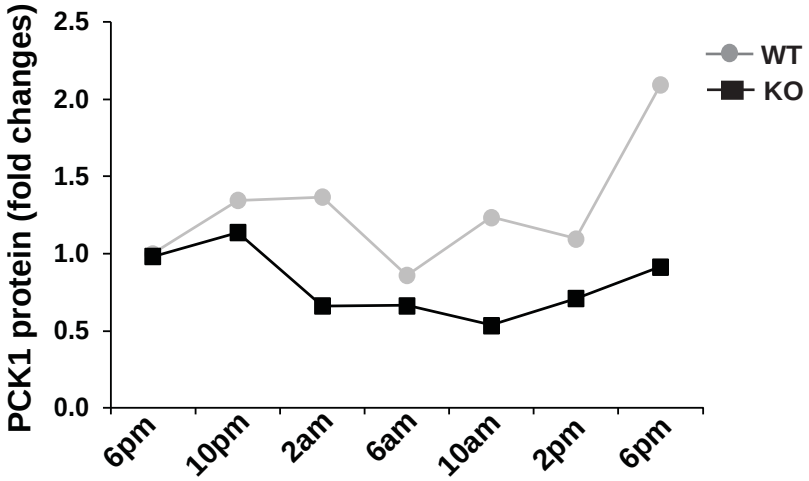
Transcription factor binding elements in mouse *Pygl* gene promoter region

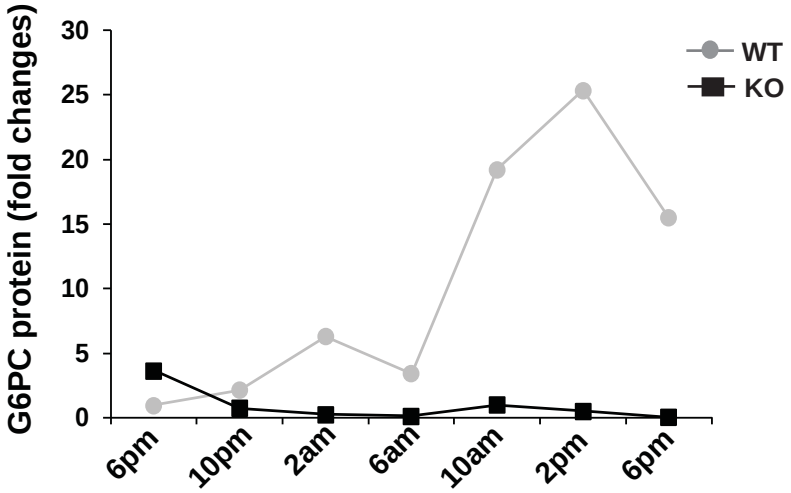


C

Transcription factor binding elements in mouse *G6pc* gene promoter region


PYGL protein (fold changes)
10
9
8
7
6
5
4
3
2
1
0
6pm
10pm
2am
6am
10am
2pm
6pm
WT
KO


PCK1 protein (fold changes)
2.5
2.0
1.5
1.0
0.5
0.0
6pm
10pm
2am
6am
10am
2pm
6pm
WT
KO


G6PC protein (fold changes)
30
25
20
15
10
5
0
6pm
10pm
2am
6am
10am
2pm
6pm
WT
KO

S-Table 1

Group	Target region	Organism	Primer name	5'- Sequence -3'
ChIP-qPCR	Pygl promotor	Mus	msPygl-F	ACT GGG AAC TCA ACA AGC GA
			msPygl-R	CTT GCT GCT TTT GAG ACG CT
	Pck1 promotor	Mus	msPck1-F	CACCTAGTGAGGTAACACAC
			msPck1-R	TCATATGTTGCTGGCTGCAC
	G6P promotor	Mus	msG6pc-F	TACGTAAATCACCCTGAACATG
			msG6pc-R	CAAGGCACAGACTGATAGCA
Gene expression qPCR	Pygl	Mus	msPygl-F	CCT ATG GCT ACG GCA TTC GT
			msPygl-R	TCT CCC AAG GGT TTC CAT GC
	Pck1	Mus	msPck1-F	CTC AGC TGC ATA ACG GTC TG
			msPck1-R	CTT CAG CTT GCG GAT GAC AC
	G6pc	Mus	msG6P-F	CTGTCACCTGTGAGACCGGA
			msG6P-R	AGATGACGTTCAAACACCGGAA
	Acc1	Mus	msAcc1-F	CAGTAACCTGGTGAAGCTGGA
			msAcc1-R	GCCAGACATGCTGGATCTCAT
	Comt	Mus	msComt-1F	GTGTCAGAGCCCGTGTCCG
			msComt-1R	AGGACTCTCTCATCCCCTCGT
	Gys2	Mus	msGys2-F	CCAGCTTGACAAGTTCGACA
			msGys2-R	ATCAGGCTTCCTCTTCAGCA
	Gsk3b	Mus	msGsk3b-F	TCCATTCCTTTGGAATCTGC
			msGsk3b-R	CAATTCAGCCAACACACACAGC
	Ugp2	Mus	msUgp2-F	ACCTGGGATACCTGCCGTG
			MsUgp2-R	CCTGCTCACCCCTTCCTTC
	Phka2	Mus	msPhka2-F	TTATGGGATGTGGGAGCGTG
			msPhka2-R	TGACTGGCAGTGCTCAACTT
	Gbe1	Mus	msGbe1-F	ACACCAGGGAAGTTCAAAATTGTAC
			msGbe1-R	GTGTTGTGGTCCAGTCTCTGATG
	Agl	Mus	msAgl-F	TTGAGGTCGGGAGACTGGAT
			MsAgl-R	GCGTCAAAGTAGCAGGGGAT

Mouse Pck1 gene promoter oligo probes for EMSA

mPCK1 EMSA-F-1 : 5'-Biotin-CCTTCTCATGACCTTTGGCCGTGGGAGTGACACCTCACAG-3'
mPCK1 EMSA-R-1 : 5'-Biotin-CTGTGAGGTGTCACTCCCACGGCCAAAGGTCATGAGAAGG-3'