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Crebh, A Novel Liver Clock Keeper For Energy Metabolism

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CREBH, A NOVEL LIVER CLOCK KEEPER FOR ENERGY METABOLISM

by

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DEDICATION

This work is dedicated to my parents and my husband Daochun Sun for their continuous support and understanding during the years of my education, and to my lovely little daughter Amber for her joyful and peaceful smiles to encourage me to complete this dissertation. I could not achieve my goal without them.

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ACC1, Acetyl-CoA Carboxylase 1

Acot4, Acyl-CoA Thioesterase 4

Apoa4, apolipoprotein A-IV

ApoC2, Apolipoprotein C-II

APR, acute phase response

BDH1, 3-Hydroxybutyrate Dehydrogenase 1

BMAL1, Aryl hydrocarbon receptor nuclear translocator-like

bZIP, basic leucine zipper

CB1R, cannabinoid 1 receptor

CCG, clock-controlled genes

ChIP, chromatin immunoprecipitation

CLOCK, circadian locomotor output cycles kaput

CPT1α, carnitine palmitoyltransferase 1A

CREB3L3, cyclic-AMP-response-element-binding protein 3-like 3 (as known as CREBH)

CREBH, cyclic-AMP-response-element-binding protein, hepatic specific

CRP, C-reactive protein

CT, circadian time

DBP, D-element binding protein

DD, constant darkness

Dgat2, diacylglycerol O-acyltransferase 2

Dhcr24, 24-Dehydrocholesterol Reductase

E4BP4, nuclear factor interleukin-3-regulated protein

ECL, enhanced chemiluminescence

Elovl6, ELOVL fatty acid elongase 6

ER, endoplasmic reticulum

ERAD, ER-associated degradation

FA, fatty acids

FADS2, fatty acid desaturase 2

FGF21, fibroblast growth factor 21

G6pc, glucose-6-phosphatase

Gys2, glycogen synthase 2

Hmgcl, 3-hydroxymethyl-3-methylglutaryl-CoA lyase

HPLC, high-performance liquid chromatography

IACUC, Institutional Animal Care and Use Committee

Lcat, Lecithin-Cholesterol Acyltransferase

LD, 12 hour light:12 hour dark

LXRα, liver X receptor α

NASH, non-alcoholic steatohepatitis

NFIL3, interleukin 3-regulated nuclear factor (also known as E4BP4)

NR1D1, nuclear receptor-subfamily 1, group D, member 1 (also known as REV-ERBα)

PAS, Periodic acid-Schiff

Pck1, phosphoenolpyruvate carboxykinase 1

PGC-1α, PPARγ coactivator-1α

PPARα, peroxisome proliferator-activated receptor α

qRT-PCR, Quantitative real-time PCR

RXR, retinoid X receptor

S1P, site-1 protease

S2P, site-2 protease

SAP, serum amyloid P-component

SCN, suprachiasmatic nuclei

SNP, single nucleotide polymorphism

SREBPs, sterol regulatory element-binding proteins

TG, triglyceride

VCO2, carbon dioxide production

VO2, Oxygen consumption

WT, wild-type

KO, knockout

CHAPTER I: CREBH, a Liver Transcription Factor and More

Introduction

Circadian rhythms are biological processes that exhibit endogenous oscillations over a 24-hour light-dark cycle and are entrainable by internal and external stimuli. Circadian rhythms are generated at the level of gene transcription by a network of clock-controlled genes (CCGs) that form an autoregulatory feedback loop. Genes that are directly regulated by the CLOCK/BMAL1 core circadian transcription complex are referred as first-order CCGs (Hughes, DiTacchio et al. 2009). The CLOCK/BMAL1 heterodimer drives circadian expression of many other transcription factors, thereby extending and enhancing other circadian regulatory functions. Local rhythms in peripheral organs, such as the liver, are synchronized by master clock oscillators located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus (Reppert and Weaver 2001). Circadian oscillators in peripheral organs respond differently to entraining signals and control different physiological outputs.

Dysregulation of circadian rhythm is closely associated with the development of human metabolic disease, such as obesity and type-2 diabetes. The intimate and reciprocal interaction between the circadian clock system and fundamental metabolic pathways has demonstrated by many studies (Green, Takahashi et al. 2008; Bass and Takahashi 2010; Feng and Lazar 2012; Hatori, Vollmers et al. 2012). Survey of nuclear receptor mRNA profiles in metabolic tissues suggested that approximately half of the known nuclear receptors or transcriptional regulators exhibit rhythmic expression (Yang, Downes et al. 2006). BMAL1-binding sites are associated with carbohydrate and lipid

metabolism (Rey, Cesbron et al. 2011) has been revealed by genome-wide and phase-specific DNA-binding rhythms for the core circadian transcriptional oscillators. In the liver, nuclear receptors or transcription factors are inducible by metabolites or hormones, and therefore, may serve as direct links between metabolic pathways and the circadian control of gene expression. For example, the nuclear receptor PPARα, which binds fatty acid ligands, and the core clock regulator BMAL1 reciprocally regulate each other to provide a feedback loop that integrates lipid metabolic processes to circadian oscillations (Inoue, Shinoda et al. 2005; Oishi, Shirai et al. 2005; Canaple, Rambaud et al. 2006). The clock-controlled nuclear receptors REV-ERBs are key regulators of circadian lipid biosynthesis in the liver, and ablation of REV-ERBs causes hepatic steatosis through de-repression of lipogenesis (Feng, Liu et al. 2011; Bugge, Feng et al. 2012). PPARγ coactivator-1α (PGC-1α) also serves a link between the clock and energy metabolism, as PGC-1α varies rhythmically and has been shown to stimulate expression of Bmal1 and nuclear receptors of the ROR family (Liu, Li et al. 2007). Furthermore, recent publications implicate that the circadian clock synchronizes distinct signaling pathways, which play important roles in circadian metabolism at the translational or post-transcriptional level (Cretenet, Le Clech et al. 2010; Mauvoisin, Wang et al. 2014). For example, the circadian clock synchronizes the rhythmic activation of the primary endoplasmic reticulum (ER) stress sensor IRE1α (Cretenet, Le Clech et al. 2010). The disruption of rhythmic activation of the IRE1α pathway which leads to impaired lipid metabolism through aberrant activation of the sterol-regulated SREBP transcription factors is related to circadian clock malfunction.

We recently reported that an ER-tethered, liver-enriched transcription factor, named CREBH, regulates energy homeostasis under metabolic stress. The expression

and activation of CREBH in the liver are influenced by a variety of inflammatory and metabolic signals, such as the pro-inflammatory cytokine TNFα, saturated fatty acids, insulin, fasting stress, and atherogenic high-fat diets (Zhang, Shen et al. 2006; Zhang, Wang et al. 2012). Activated CREBH serves as a multifaceted activator of transcription that induces expression of the genes involved in hepatic acute-phase response, fatty acid (FA) oxidation, lipolysis, lipogenesis, and gluconeogenesis (Lee, Chanda et al. 2010; Lee, Giannikopoulos et al. 2011; Zhang, Wang et al. 2012; Kim, Mendez et al. 2014). Non-alcoholic steatohepatitis (NASH) and hypertriglyceridemia when fed an atherogenic high-fat diet are profoundly developed in CREBH-null mice (Zhang, Wang et al. 2012). In humans, patients with hypertriglyceridemia exhibit a high-rate of functional mutations of the CREBH gene (Lee, Giannikopoulos et al. 2011). More recently, we demonstrated that CREBH and PPARα function as binary transcriptional activators to regulate production of fibroblast growth factor 21 (FGF21), a hepatic hormone that regulates whole body energy homeostasis under metabolic stress (Kim, Mendez et al. 2014).

In this study, we demonstrate that CREBH is an organ-specific, diurnal regulator of energy metabolism (Figure 1). CREBH plays an indispensable role in maintaining glucose and lipid homeostasis under circadian control by regulating expression of the genes involved in bi-directional metabolic pathways of both energy storage and utilization. Loss-of-function of CREBH in mice leads to impaired rhythmic profiles of lipid and glucose, hyper-locomotion, and time-shifted feeding behavior. Our finding that CREBH functions as a liver metabolic circadian oscillator therefore has important implications in the understanding of the molecular basis of circadian metabolism and the prevention and treatment of metabolic disorders.

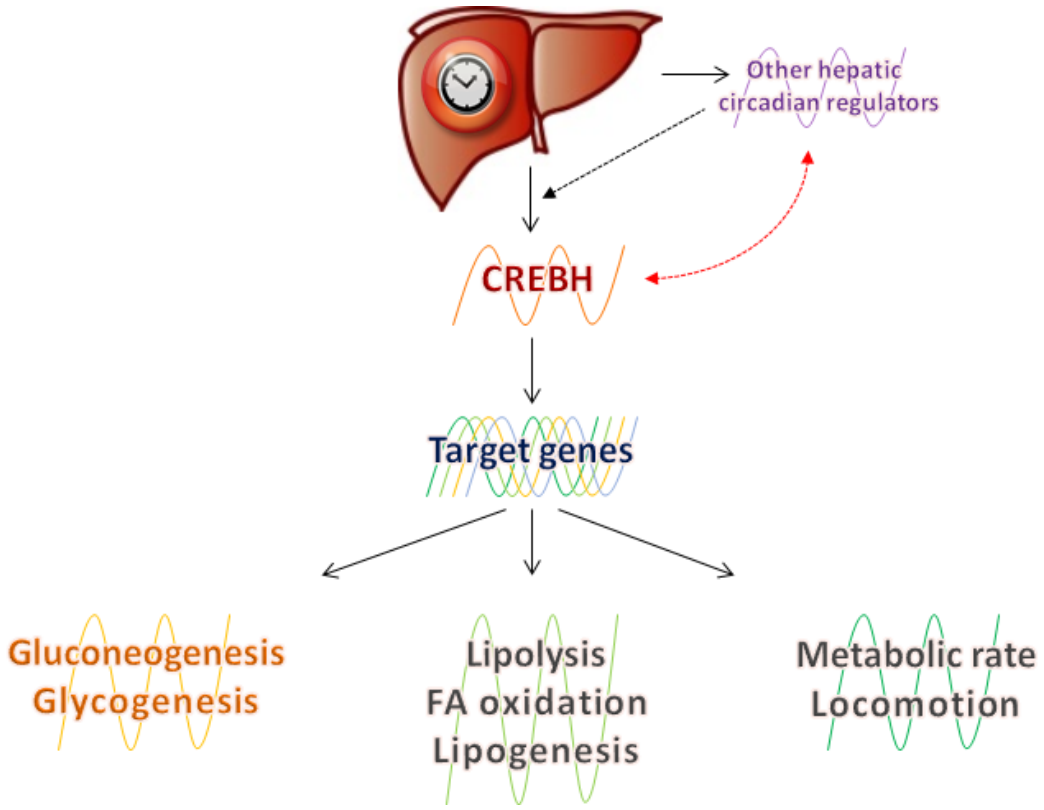

Figure 1. Major conclusions of this dissertation.

CREBH Gene Structure and Functions

The human CREBH gene is located at19p13.3 and spans 19,420 bp of genomic DNA. It has 10 constitutive exons encoding a 2586 bp mRNA transcript, with a 1385 bp open reading frame. The mouse CrebH gene is located at chromosome 10qC1 and spans 14540 bp of genomic DNA. It has 12 constitutive exons encoding a 2283 bp mRNA transcript, with a 1439 bp open reading frame (Figure 2).

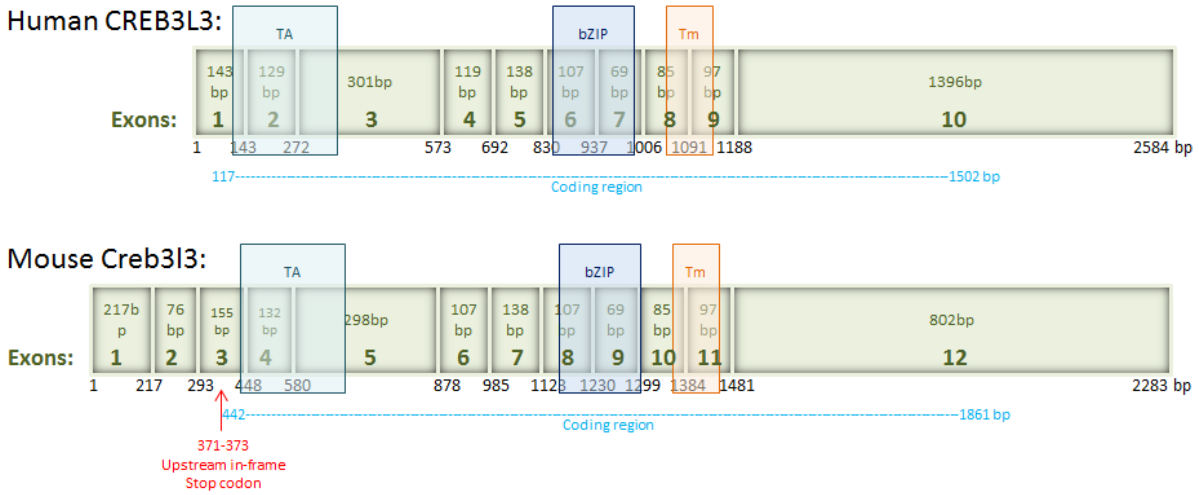

Figure 2. Domain scheme of human and mouse CREBH.
TA, transcriptional activation domain; bZIP, basic leucine zipper domain; Tm, transmembrane domain; bp, nucleotide base pair number of the mRNA.

CREBH is a b-ZIP transcription factor conserved from Caenorhabditis elegans (C. elegans) to human, and it is primarily expressed in hepatocytes, the parenchymal cell in liver, as well as in small intestine and stomach tissues at lower expression levels (Omori, Imai et al. 2001; Zhang, Shen et al. 2006; Fabbrini, Sullivan et al. 2010). In response to regulated intramembrane proteolysis (RIP) induced by ER stress, acute inflammation, and hepatic metabolic stress, CREBH can be translocated from ER membrane to Golgi, cleaved by site-1 protease (S1P) and site-2 protease (S2P), and then trafficked into the nucleus (Brown, Ye et al. 2000; Stirling and O'Hare 2006; Zhang, Shen et al. 2006; Zhang, Wang et al. 2012). Under normal conditions, CREBH protein is regulated by the ER-associated degradation (ERAD) pathway, and both full-length and cleaved CREBH have half-lives of less than 1 or 2 hours (Bailey, Barreca et al. 2007). The full-length CREBH protein molecular weight is ~75kDa. The cleaved 50 kDa CREBH fragment is gradually increased in the nuclear fraction (Zhang, Shen et al. 2006) after ER stress challenge. Several domains have been characterized, including transcriptional activation (TA) domain, basic leucine zipper (bZIP) domain, transmembrane (Tm) domain, and ER luminal domain (Chan, Kok et al. 2011). The interactions between CREBH and other bZIP domain-containing transcription factors have been reported by previous studies. CREBH forms a homodimer, or a heterodimer with activating transcription factor 6 (ATF6) through the bZIP dimerization domain to activate the expression of acute phase response (APR) genes, serum amyloid P-component (SAP), and C-reactive protein (CRP) upon ER stress (Zhang, Shen et al. 2006). CREB-Zhangfei (CREBZF), as known as SMILE, repressively heterodimerizes with CREBH through the bZIP domain and inhibits CREBH-mediated, but not ATF6-mediated, transcriptional activity. This inhibitory effect is achieved by

competing with the CREBH binding ability to peroxisome proliferator activated receptor gamma coactivator 1 alpha (PGC1α) (Misra, Chanda et al. 2011). Unlike ATF6, the ER luminal tail of CREBH is not required for Golgi translocation, and it does not bind to BiP (Llarena, Bailey et al. 2010). However, the N-linked glycosylation at the C-terminus of CREBH ER luminal domain is required for the stress-induced cleavage and nuclear transport (Chan, Mak et al. 2010).

In rodent models, the expression of CREBH can be greatly induced after 16 hours fasting in the wild-type (WT) mouse liver (Lee, Chanda et al. 2010; Lee, Giannikopoulos et al. 2011; Zhang, Wang et al. 2012), and in the liver tissue from db/db mice with insulin resistance (Lee, Chanda et al. 2010). Interestingly, enhanced CREBH activation by proteolysis without changing the *CrebH* mRNA level has been reported in the wild-type mice fed an AHF diet for 6 months, which developed significant insulin resistance and non-alcoholic steatohepatitis (NASH) (Zhang, Wang et al. 2012). This indicated a possible adaption stage for CREBH expression after chronic high-fat dietary stress.

Many hepatocyte-enriched genes are transcriptionally regulated by CREBH. Hepcidin, as well as the APR genes, SAP and CRP, are transcriptionally up-regulated by CREBH upon ER stress (Zhang, Shen et al. 2006) (Figure 3). CREBH is also required to activate the expression of the genes encoding functions involved in *de novo* lipogenesis, fatty acid (FA) elongation and oxidation, triglyceride (TG) and cholesterol biosynthesis, lipolysis, and lipid transport in liver. In hepatocytes, CREBH binds to the promoter of Fgf21, apolipoprotein A-IV (Apoa4), apolipoprotein A-II (ApoC2), phosphoenolpyruvate carboxykinase 1 (Pck1) and glucose-6-phosphatase (G6pc) and activates their transcriptions in response to fasting-induced TG lipolysis and gluconeogenesis (Lee,

Chanda et al. 2010; Lee, Giannikopoulos et al. 2011; Zhang, Wang et al. 2012). CREBH also activates transcriptions of other TG lipolysis regulators Apoa5, Apof, Lcat, Scp2, Acot4, and G0s2, lipid droplet formation regulator Cidec, FA elongation enzymes Elovl2, Elovl5, Elovl6, and Pecr, FA oxidation or cholesterol synthesis enzymes Cpt1a, Cyp4a10, Cyp4a14, Cyp2b9, Cyp2b13, Fads1, Fads2, Acox1, Pparα, Dhcr24, and Acs1, and TG synthesis enzymes Fasn, Acc1, Acc2, Scd1, and Dgat2 (Lee, Giannikopoulos et al. 2011; Zhang, Wang et al. 2012).

Recent studies indicated association between CREBH and liver-related disorders in human. The increased *CrebH* mRNA levels were found in liver biopsy samples of chronic infection of hepatitis C virus (HCV) which can either directly induce hepatic steatosis, or promote existing steatosis with fibrosis (Yoon and Hu 2006; Asselah, Bieche et al. 2010). The association between multiple non-synonymous single nucleotide polymorphism (SNP) mutations in CREBH N-terminal region with hypertriglyceridemia was recently reported (Lee, Giannikopoulos et al. 2011; Johansen and Hegele 2012).

Taken together, CREBH is an important regulator of hepatic acute-phase inflammation, fatty acid oxidation, lipogenesis, lipolysis, and gluconeogenesis under metabolic stress conditions, and the dysfunction of CREBH is associated with the pathogenesis of NASH, hypertriglyceridemia, and insulin resistance. Despite of this progress, the general mechanism by which CREBH regulates the different metabolic pathways remains unclear.

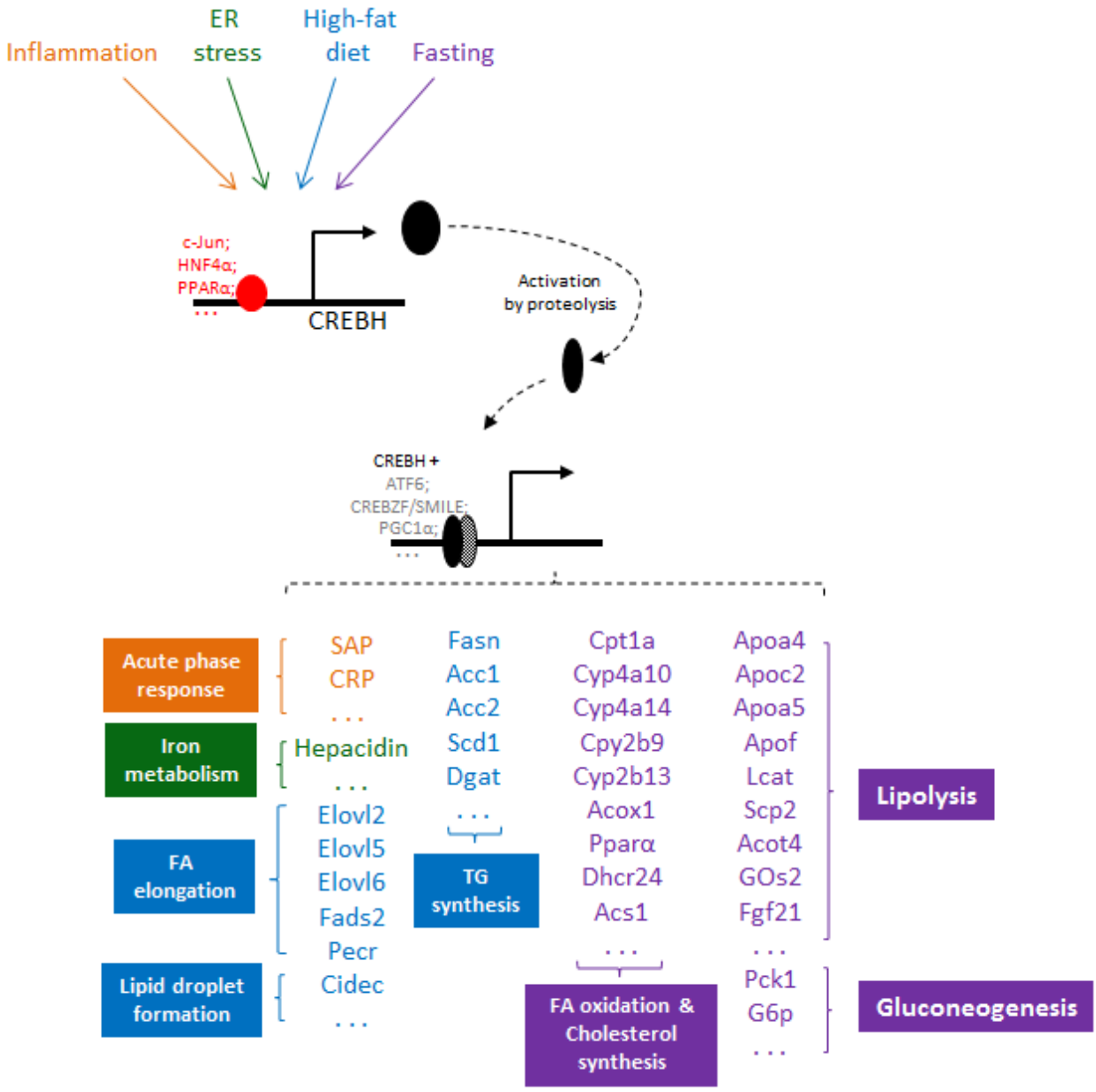

Figure 3. CREBH-mediated signaling pathways.

Transcriptional Regulation of CREBH

Transcription of CREBH is regulated by c-Jun in response to the activation of cannabinoid 1 receptor (CB1R) (Chanda, Kim et al. 2011), as well as hepatocyte nuclear factor 4α (HNF4α), which is a nuclear hormone receptor involved in the determination of hepatocyte differentiation (Fabbrini, Sullivan et al. 2010) (Figure 3). The peroxisome proliferator-activated receptor alpha (PPARα) agonist up-regulates CREBH expression in both mouse and human hepatocytes (Rakhshandehroo, Hooiveld et al. 2009). PGC1α, the transcriptional co-activator of PPARγ for the lipid metabolism, also enhances CREBH expression in primary hepatocytes (Lee, Chanda et al. 2010). Increased FA uptake activates CREBH expression in hepatocytes *in vitro* (Gentile, Wang et al. 2010; Zhang, Wang et al. 2012), and this increased transcription can be blocked by insulin signals (Gentile, Wang et al. 2010). Interestingly, the chronic AHF diet significantly promoted CREBH activation without affecting the transcription levels (Zhang, Wang et al. 2012). This suggests an adaptation of the transcription of CREBH, but an accumulation of cleaved CREBH protein level after chronic metabolic stress. After chronic AHF diet, CREBH-null mice display hypertriglyceridemia and massive accumulation of hepatic TG compared to the WT mice (Lee, Giannikopoulos et al. 2011; Zhang, Wang et al. 2012). On the other hand, fasting, which can lead to acute hepatic steatosis, FA oxidation, lipolysis and gluconeogenesis, can significantly induce both mRNA transcription and protein activation of CREBH (Lee, Chanda et al. 2010; Zhang, Wang et al. 2012). All these observations suggest that CREBH is an important hepatic metabolic regulator.

CHAPTER II: Identification of CREBH as a clock-regulated regulator in liver

Summary

Circadian rhythm is the biological oscillation on the scale of 24 hours observed in most living creatures on earth. The peripheral organs, such as liver and kidney, are known to have local rhythms synchronized by the master clock oscillators located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus to orchestrate rhythmic physiology and behavior (Reppert and Weaver 2001). Approximately 2%-10% of all genes exhibit circadian oscillations according to several microarray analyses for gene expression in Drosophila, honey bees and mouse (Akhtar, Reddy et al. 2002; Lin, Han et al. 2002; Duffield 2003; Keegan, Pradhan et al. 2007; Hughes, DiTacchio et al. 2009; Doherty and Kay 2010; Rodriguez-Zas, Southey et al. 2012). Those genes are categorized as clock-controlled genes (CCGs). The genes that are directly regulated by the core circadian transcription factors CLOCK/BMAL1 heterodimer are referring to as first-order CCGs. The second-order CCGs are defined as rhythmic genes regulated by the first-order CCGs which are also transcription factors (Hughes, DiTacchio et al. 2009; Lee and Sancar 2011). The hepatic metabolic homeostasis is regulated by rest-activity cycle and feeding behavior, and it is coordinated by neural, hormonal and behavioral signals (Stokkan, Yamazaki et al. 2001). Till now, several ER membrane-localized hepatic metabolic regulators were found to be regulated by circadian clock, such as the activation of IRE1α–XBP1 and SREBP-Insig2 pathways (Le Martelot, Claudel et al. 2009; Cretenet, Le Clech et al. 2010). The role of circadian clock in liver metabolism is critical and fundamental in controlling nutrient and energy homeostasis (Li and Lin 2009; Maury,

Ramsey et al. 2010). However, the communication between liver local clock and the central clock for the synchronization of molecular oscillation still remain unclear.

In this dissertation, I first answered the questions of whether circadian clock regulates the expression of CREBH in liver, and its proteolytic activation process. We established animal models for circadian study by using CREBH-null and WT control mice, and we found both CREBH expression and its proteolytic activation exhibit significant, but distinct, circadian rhythmic patterns. Interestingly, although BMAL1 directly regulates CREBH transcription in liver under normal physiological condition, other regulatory mechanisms may be also involved in CREBH expression and activation under stresses, such as prolonged fasting.

The transcriptional activation of CREBH mediated by circadian rhythm represents an elegant signal transduction network. Delineation of this regulatory network increased our understanding of the fundamental process and the synchronization of the liver local clock and the hepatic metabolic pathways. CREBH protein requires activation, inactivation, and degradation processes in response to the different circadian clock-controlled physiological events. Identification of the regulatory mechanism of CREBH activity and the sensing mechanism for the different circadian physiological events are important addition to the knowledge of hepatic circadian metabolic regulation. Based on these findings, the CREBH rhythmic gene regulation networks could be exploited as novel therapeutic targets that modulate hepatic lipid and glucose homeostasis under circadian rhythm disruption and metabolic syndromes.

Materials and Methods

Animal model

All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee (IACUC) of the Wayne State University. Male wild-type and CREBH knockout C57BL6 mice of 4-month-old were housed in 12-hour light/12-hour dark (LD) cycles with free access to food and water for at least 2 weeks before switching to constant darkness (DD) for 24 hours to allow endogenous clocks to free run. Mice were euthanized with isoflurane followed by rapid cervical dislocation. Liver samples from 3-5 mice per time point per genotype group were collected in constant darkness every 4 hours for a 24-hour period. Circadian time 0 (CT0) is the onset of light phase. CT is shown in hours.

Quantitative real-time reverse-transcription PCR (qRT-PCR) analysis

Total RNAs from mouse livers were isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instruction. RNA was reverse-transcribed into cDNA using a High-capacity cDNA Reverse Transcription Kit (Invitrogen). For quantitative real-time PCR analysis, the reaction mixture containing cDNA template, primers, and SYBR Green PCR Master Mix (Applied Biosystems) was run in a 7500 Fast Real-time PCR System (Applied Biosystems, Carlsbad, CA). The sequences of real-time PCR primers used in this study are described in Table 1. Fold changes of mRNA levels were determined after normalization to internal control *Arbp* (acidic ribosomal phosphoprotein P0) mRNA levels.

Table 1. Primers for gene expression qPCR.

Target region	Organism	5'- Sequence -3'	Product length
Arbp	Mus	ccgatctgcagacacacact	90
		accctgaagtgctcgacatc	
Fgf21	Mus	gctgctggaggacggttaca	108
		cacaggtccccaggatgttg	
Per2	Mus	TGTGCGATGATGATTCGTGA	70
		GGTGAAGGTACGTTTGGTTTGC	
RevERBa	Mus	ctactggctccctcacccagga	177
		gacactcggctgctgtcttcca	
Hnf4a	Mus	ACTCCTGCAGGTTTAGCCGA	126
		GGTCCCGCTCATTTTGGACA	
Bdh1	Mus	AGATGCGGCTAGTGGCAAAG	148
		CAGTTCCTTGACCCCAGCAT	
G6pc	Mus	CTGTCACCTGTGAGACCGGA	147
		AGATGACGTTCAAACACCGGAA	
Acc1	Mus	CAGTAACCTGGTGAAGCTGGA	142
		GCCAGACATGCTGGATCTCAT	
Comt	Mus	GTGTCAGAGCCCGTGTCCG	187
		AGGACTCTCTCATCCCCTCGT	
E4bp4	Mus	GAGCAGAACCACGATAACCCA	95
		AGGACTTCAGCCTCTCATCC	
Clock	Mus	CAGGCACGTGAAAGAAAAGCA	118
		GCCGTCTTCTGTGTGACTGA	
Bmal1	Mus	GCAACTACAGTGGCCCTTTG	118
		TCCACAGGATTTGACTGGGG	
Ppara	Mus	GGGAACTTAGAGGAGAGCCAAG	145
		CCATGTTGGATGGATGTGGC	
Cebpb	Mus	GCAATCCGGATCAAACGTGG	74
		CCCGGCTGACAGTTACACG	
Srebf1	Mus	ACTTTTCCTTAACGTGGGCCT	147
		AGCATGTCTTCGATGTCGTTCA	
Lxra	Mus	ACGCGACAGTTTTGGTAGAGG	117
		AACTCCGTTGCAGAATCAGGA	

Immunoblotting analyses

Total cell lysates were prepared from mouse livers or cultured cells using IPA 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 (Bio-Rad). 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-Rad). 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, Mendez 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.

Preparation of membrane and nuclear protein fractions

Cellular membrane and nuclear protein fractions were prepared from mouse liver tissues utilizing a Subcellular Protein Fractionation Kit (Thermo Scientific) according to the manufacturer's instruction. Male wild-type C57BL6 mice of 4-month-old were housed in 12-hour light/12-hour dark (LD) cycles with free access to food and water for at least 2

weeks before switching to constant darkness (DD) for 24 hours to allow endogenous clocks to free run. Liver tissues were collected from the mice every 4 hours over a 24-hour circadian cycle. Equal amounts (weight) of liver tissues from the mice at each time point (3 mice per time point) were pooled for the extraction of subcellular protein fractions. The protein concentration of each fraction was determined by using the Bradford Protein Assay (Bio-Rad).

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 minutes at 4°C. Following a washing step, nuclear pellets were cross-linked with 1% fresh formaldehyde in PBS for 10 minutes at room temperature. Cross-linking was terminated by addition of 200mM Tris-HCl (pH 9.4) and 1mM DTT for 10 minutes and centrifuged at 1160 g for 15 minutes at 4°C. Nuclear pellets were suspended in SDS lysis buffer containing protease inhibitors, incubated for 10 minutes on ice, and sonicated in a cold-water bath using chiller circulator-equipped Bioruptor Sonication Device (Diagenode) (Kapatos, Vunnava et al. 2007; Kfoury and Kapatos 2009).

ChIP assays with mouse liver chromatin

Mouse liver chromatin was fragmented to an average size of 500 bp by sonication (see above), and then cleared of debris by centrifugation at 20000 g for 30 minutes at 8°C. The supernatant was harvested and diluted 10-fold with ChIP Dilution Buffer (0.01% SDS; 1.1% Triton X-100; 1.2 mM EDTA; 167 mM NaCl; 16.7 mM Tris-HCl, pH8.0). Approximate 10 µg of fragmented chromatin was pre-cleared by incubating with 2µg/mL

of rabbit IgG (Santa Cruz) for 1 hour at 4°C, followed by 1 hour of incubation with 50 µL protein G agarose (Invitrogen) at 4°C with rotation. BMAL1- or CREBH-binding complexes were pulled down by using 2 µg/mL of a rabbit anti-BMAL1 antibody (Novus Biologicals, NB100-2288) or the rabbit anti-CREBH antibody developed in our laboratory (Kim, Mendez et al. 2014). As controls, the pre-cleared chromatin samples were pulled down using a rabbit anti-HA antibody (2µg/mL). Immunoprecipitated chromatin fragments were reverse cross-linked, digested by proteinase K, and purified using QIAquick PCR Purification Kit (Qiagen, Germantown, MD). Presence of BMAL1 or CREBH in gene promoters under different circadian phases were quantified by qRT-PCR and expressed relative to the input genomic DNA as previously described (Kapatos, Vunnava et al. 2007; Kfoury and Kapatos 2009; Shimomura, Kumar et al. 2013). The sequences of the primers used for the ChIP-PCR assay are described in Table 2.

Table 2. Primers for ChIP-qPCR.

Target region	Organism	5'- Sequence -3'	Product length
E-boxes on Crebh promoter	Mus	CCTAATGCAGGTGCAAAGGC	160
		TGTAGGAGCAAAGCAGGAGC	
E-box on Crebh promoter	Mus	ATGAGGCCAAGGGTGAACTG	120
		AGCAGCGATAAAGGCTCTGG	
E-box unrelated region on Crebh promoter	Mus	CTGGGTGTGGTGGTCAGC	137
		CCCTGCTCCAGGTGTTACAG	
Rplp0 promoter	Mus	CTTCTCCCTCCCTCACCCC	179
		CTTCTTGGCCCTCAGCAGTG	
Fgf21 promotor	Mus	CGC CCT GGC CAC GGT GGA AT	227
		CTC CGG TGC CCA GCA GGG AT	
Pck1 promotor	Mus	CACCTAGTGAGGTAACACAC	151
		TCATATGTTGCTGGCTGCAC	
Acc1 promotor	Mus	ATTCATCAGCCCAGGGACTG	158
		CTTGTGAAGGCAGCAGCTGT	
G6P promotor	Mus	TACGTAAATCACCCTGAACATG	143
		CAAGGCACAGACTGATAGCA	
Gys2 promoter	Mus	CTC ACC ACA ATC CAG CTT GTA C	207
		CCT TTA GAC CAA ACT CCT ACA C	
Apoc2 promoter	Mus	CACACTGTTTAGGAAAGGAGGCA	154
		CTGCTGTACTCCACTCTTTCAC	
Fads2 promoter	Mus	CCAGCAGGGCTTAACTCCAT	89
		AGGATCTTTCGAAGGCCAGC	
Bdh1 promoter	Mus	TGCTTGCCAGAGGGTCAAAT	88
		CGTGTTTGTCATCGAACGGG	
Lipasin promoter	Mus	GTTGGATGTGAAGGGAGCCA	117
		GAGGTAGTGGGCAAGCTCAG	
Cpt1a promoter	Mus	CAGAGAAGTTTACGGGCGGA	172
		TAAGTCCCGAGCTTGCCAAC	
LXRa promoter	Mus	CAAAGAGCCTCCAGGGTGAG	96
		CCCTGTCCCCTACCCTCTAC	
E4BP4 promoter	Mus	AATGGGCAAAAGGGTCCTGG	144
		CAGTCCGCGTCCTTCTCTG	
SREBF1 promoter	Mus	ATGTCTGGGCTGGGTCTAGT	75
		GGGCCTTGGCTTCTTCTGTA	
Cebpb promoter	Mus	CAATGACGCGCACCGAC	92
		AGCGGGAGGTTTATAAGGCG	
PPARa promoter	Mus	GCAGTCCCTTCACCTAACCC	140
		CTGGACGGCAGTGTCTGATT	

Statistics

The results of experiments were analyzed by several statistical methods. Unpaired Mann-Whitney U test was used for non-parametric comparisons. One-way ANOVA test was used for parametric comparisons. Two-way ANOVA was used to distinguish the effects of genotypes from the effects of circadian time on gene expression, levels of mouse blood lipid and blood metabolites, and quantification of food intake. In all cases, p value less than 0.05 was used to attribute statistical significance. When multiple testing procedures were implemented (i.e. multiple t tests), the Bonferroni correction was used.

Results

CREBH is a clock-controlled gene (CCG) in mouse liver

Previously, we demonstrated that inflammatory metabolic stimuli induce expression and/or activation of CREBH in the liver (Zhang, Wang et al. 2012). To test whether circadian rhythm for CREBH is present in mouse liver, we examined the 24-hour expression profile of CREBH. Expression levels of the *CrebH* mRNA in the liver peaked at circadian time (CT) 44 and reached a trough at CT56 (Figure 4A). Expression of the *CrebH* mRNA in mouse liver exhibited a trend to increase during the late phase of daytime and decrease during the late phase of nighttime. We next examined levels of precursor and activated forms of CREBH proteins in mouse liver across the day-night cycle. Production of the activated CREBH protein involves translocation of CREBH precursor from the ER to the Golgi where it is cleaved by S1P and S2P protease (Zhang, Shen et al. 2006), and therefore, levels of the activated form of CREBH can be evaluated by examining cleaved CREBH proteins. Western blot analysis with total liver protein lysates from animals under the circadian cycle demonstrated that levels of CREBH precursor protein during the “daytime” phases were higher than those of the “nighttime” phases (Figure 4B). In contrast, levels of cleaved CREBH protein during the “daytime” phases were lower than those of the “nighttime” phases. To further delineate circadian rhythmic levels of CREBH precursor and activated proteins, we performed Western blot analyses with the membrane and nuclear protein fractions collected from pooled liver tissues of the mice under the circadian circle. Levels of the membrane-bound CREBH precursor protein peaked at CT44 and reached a trough at CT56 (Figure 4A, C-D), which is consistent with the rhythmic mRNA expression profile. In an opposite manner, levels of the activated,

nuclear CREBH protein reached a bottom at CT44 and peaked at CT56, 12 hours after the peak production of the CREBH precursor protein (Figure 4C-D). These data indicates that the expression and proteolytic activation of CREBH in the liver are both rhythmically regulated during the day-night cycle although they exhibit distinct circadian rhythmic patterns.

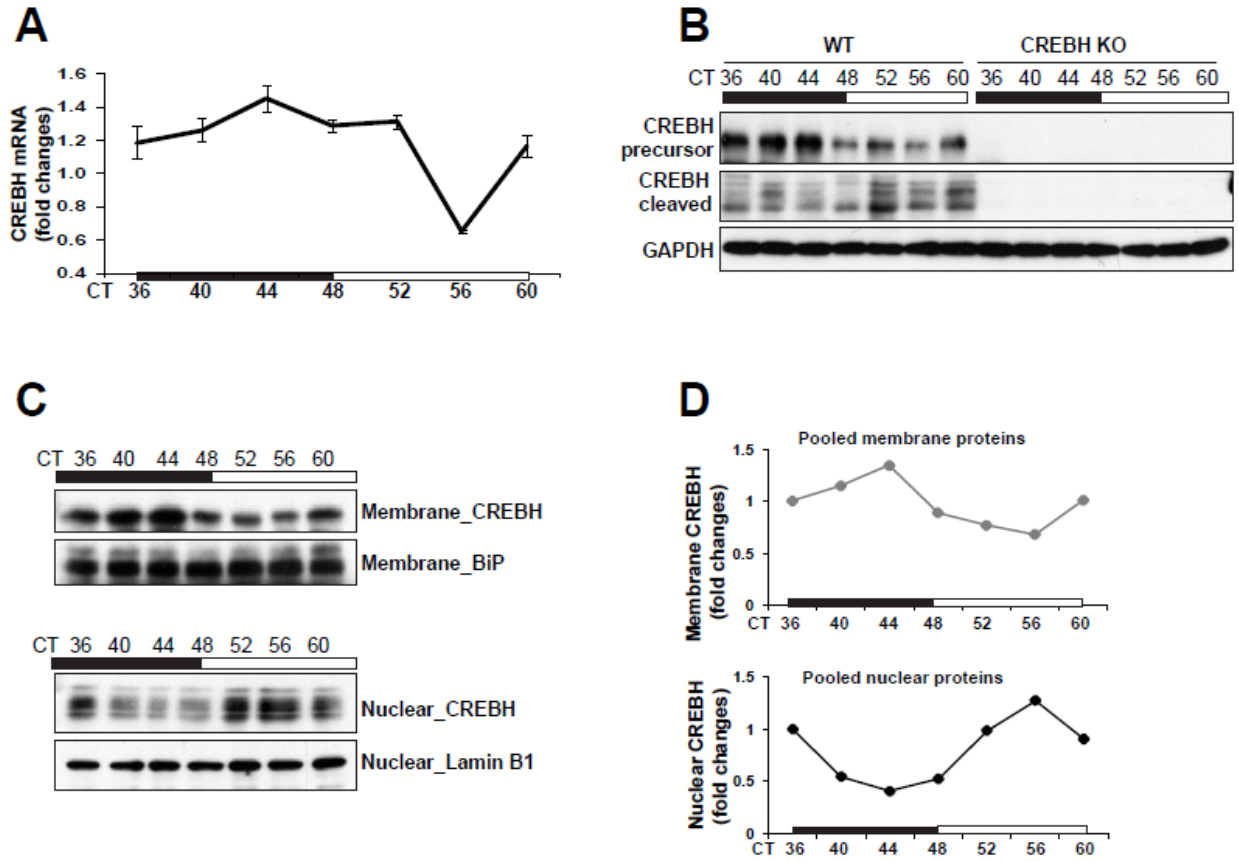

Figure 4. CREBH is a clock-controlled gene (CCG) in mouse liver

(A-B) Circadian oscillations of the *CrebH* mRNA expression (A) and protein levels (B) in male WT mouse liver tissues collected every 4 hours over a 24-hour period in constant darkness (DD), determined by qRT-PCR and Western blot analyses, respectively. Fold changes of mRNA levels are shown by comparing to the nadir mRNA levels at CT56. Each point denotes the mean ± SEM (n=3). Circadian time (CT) is shown in hours. (C) Western blot analysis of levels of membrane-bound CREBH precursor and nuclear proteins in mouse livers over the circadian circle. Cellular membrane and nuclear protein fractions were prepared from pooled liver tissues of WT mice collected every 4 hours over a 24-hour circadian cycle (n=3 mice per time point). (D) Quantification of the CREBH precursor and nuclear protein signals in the mouse livers under the circadian clock. CREBH protein signals in the pooled liver membrane and nuclear protein fractions were determined by Western blot densitometry. Fold changes of the protein levels are shown by comparing the protein signals to that at CT36 (defined as 1). Three mouse liver tissue samples per time point were pooled for the Western blot analysis, as described in panel C.

The expression of CREBH is rhythmically regulated by BMAL1 in liver

One of the key identifying features of clock-controlled genes (CCGs) is the presence within the proximal promoter of one or more canonical E-box elements that bind the core circadian CLOCK/BMAL1 heterodimer (Kumaki, Ukai-Tadenuma et al. 2008; Ukai and Ueda 2010). There are several E-box binding elements found in the mouse *CrebH* gene promoter regions (Figure 5).

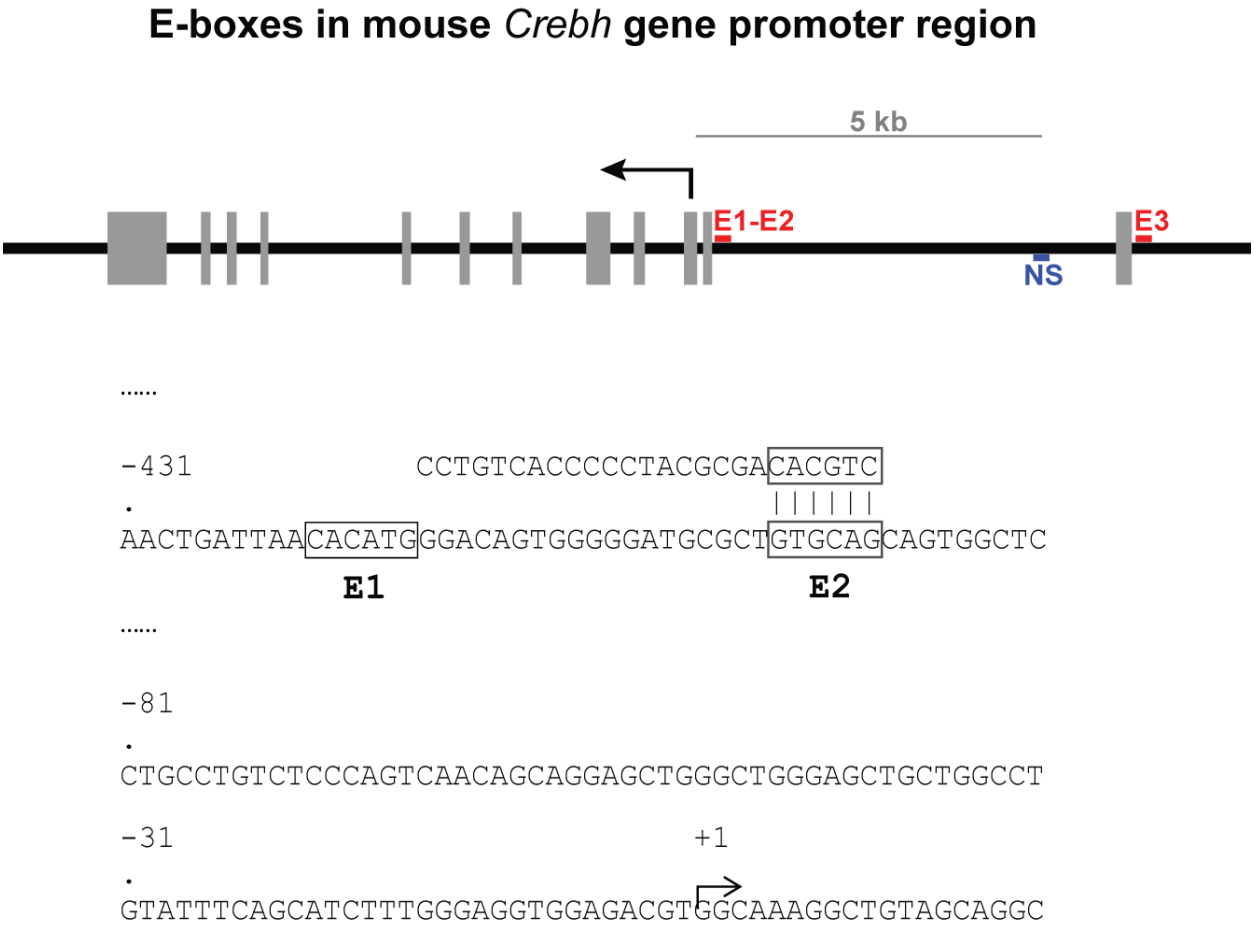

Figure 5. Illustration of E-boxes in mouse CrebH gene promoter region.
Non-canonical E-boxes E1 (CACATG) and E2 (CACTGC) locate in the promoter region of mouse CrebH gene.

To reveal whether BMAL1 regulates CREBH expression, we first examined expression levels of the *CrebH* mRNA in liver-specific Bmal1 conditional knockout (*Bmal1* LKO) and control mouse liver samples collected every 6 hours during a 24-hour circadian period (Molusky, Ma et al. 2012). Expression levels of *CrebH* mRNA across the day-night period were decreased in the livers of *Bmal1* LKO mice, compared to those in the control mouse liver (Figure 6A). To determine whether BMAL1 binds to the *CrebH* gene promoter, chromatin immunoprecipitation (ChIP)-qPCR analysis was performed on mouse livers from different circadian phases. ChIP-qPCR analysis demonstrated increased enrichment of BMAL1 in the *CrebH* gene E-box-containing promoter region at CT8, a time point when levels of the *CrebH* mRNA reached a nadir and began a sharp increase (Figure 4A, 6B-C).

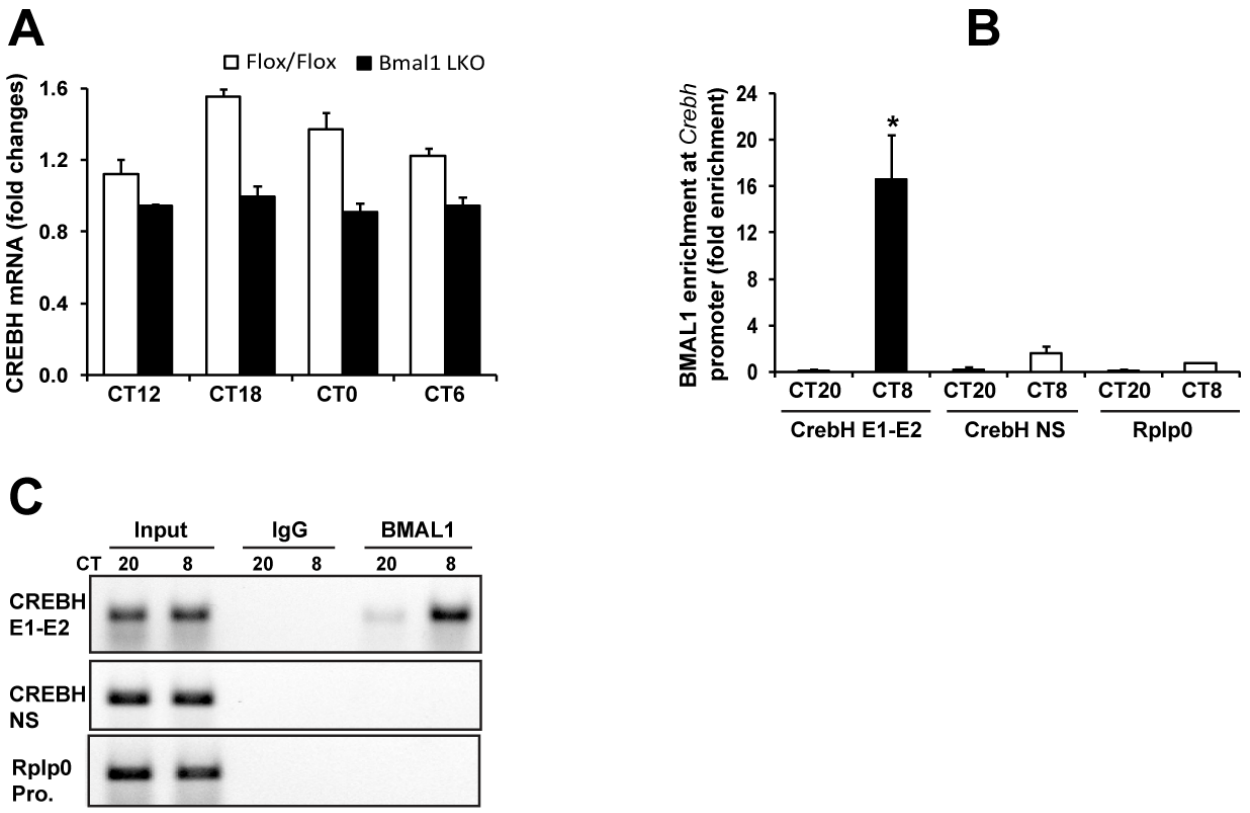

Figure 6. CrebH is directly regulated by BMAL1 in mouse liver

(A) Expression of the *CrebH* mRNA in the livers of *Bmal1* LKO and *flox/flox* control mice during different circadian phases. The liver samples from *Bmal1* LKO and *flox/flox* control mice were collected every 6 hours over a 24-hour circadian period. The mean expression values were obtained with pooled liver cDNAs from 3-5 mice per time point per genotype as previously described (n= 3 experimental repeats) (Molusky, Ma et al. 2012). (B-C) ChIP analysis of the enrichment of BMAL1 in the *CrebH* gene promoter in mouse liver under different circadian phases. Presence of the endogenous BMAL1 to the E-boxes (E1-E2)-containing promoter region of the *CrebH* gene was quantified by ChIP-qPCR (F) in the WT mouse livers collected from CT8 and CT20 (n=3). A non-specific region of the *CrebH* gene distal from the promoter and a housekeeping gene *Rplp0* promoter were amplified used as negative controls of ChIP assays. Quantification of BMAL1 enrichment in the *CrebH* gene promoter at different circadian phases was determined by comparing ChIP-qPCR signals from the samples pulled down by the anti-BMAL1 antibody to that pulled down by a rabbit anti-HA antibody. Each bar donates mean ± SEM (n=3 mice per time point). * $p < 0.05$ (CT8 vs CT20). The enrichment of BMAL1 in the *CrebH* gene promoter was confirmed by ChIP-PCR (C) utilizing the same templates and primers used for the ChIP-qPCR analysis.

Interestingly, this regulatory pattern is distinct from that of typical BMAL1-regulated circadian genes whose expression levels are usually increased in few hours after BMAL1 enrichment in the promoter regions. One possible explanation is that other metabolic trans-activators may also be involved in the transcriptional activation of the *CrebH* gene upon energy fluctuations. This is supported by the observation that the diurnal expression profile of the *Bmal1* mRNA was distinguished from that of CrebH in the livers of wild-type mice (Figure 7). Under metabolic stress, such as fasting, the diurnal expression profile of the *CrebH* mRNA, but not the *Bmal1* mRNA, was significantly altered (Figure 7A-B), indicating that CREBH is not a typical CCG. Additionally, we examined expression and activation of CREBH in livers of *Bmal1* LKO and control mice under normal feeding conditions or after a 24-hour period of fasting (Molusky, Ma et al. 2012). Levels of the *CrebH* mRNA were significantly reduced in *Bmal1* LKO livers under both fasting and feeding conditions (Figure 7C). Immunoblotting analysis shown a slight decrease in the precursor form of CREBH protein in the *Bmal1* LKO liver, compared to the control liver, under both feeding and fasting conditions (Figure 7D). The cleaved/activated CREBH protein was however diminished in the livers of *Bmal1* LKO mice under the feeding, but not the fasting condition. These results confirm the regulation of CREBH by BMAL1 under the normal physiological conditions and suggest that additional regulatory mechanisms are also contributing to CREBH expression and activation.

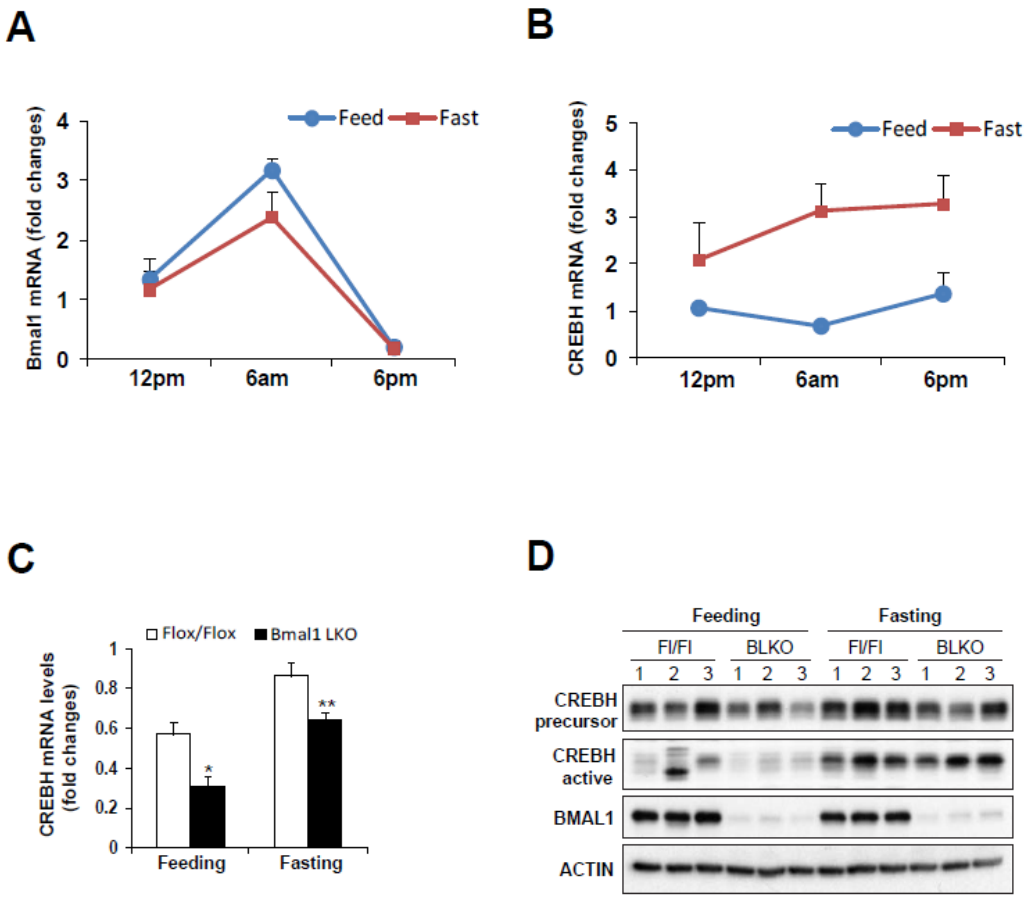

Figure 7. CREBH is not a typical CCG.

(A-B) Diurnal expression profiles of the *Bmal1* and *CrebH* mRNAs in the wild-type mice under feeding or fasting conditions. 3-month old wild-type mice were subjected to fasting or feeding for 6, 12, or 24 hours. The experiment was started at 6 pm. The groups of animals were euthanized to collect liver samples at 12 am, 6 am, and 6 pm, respectively. Expression values of mRNAs were determined by qRT-PCR and normalized to the β-actin mRNA levels. Fold changes of mRNA levels are determined by comparing the expression values to that of one of the liver samples collected at 12 pm under the feeding condition (n=3 mice per time point under the fasting condition or 2 mice per time point under the feeding condition). (C-D) Levels of the *CrebH* mRNA (C) and protein (D) in the livers of *Bmal1* LKO and *flox/flox* control mice under the feeding condition or after 16-hour overnight fasting. The levels of the *CrebH* mRNA were determined by qRT-PCR analysis, and the levels of the CREBH protein were determined by Western blot analysis. In A, expression values were normalized to the *Arbp* mRNA levels. Fold changes of mRNA levels are shown by comparing to that of one of the control mice under the feeding condition. Each bar donates mean ± SEM (n=4 mice per group).

CHAPTER III: CREBH Functions under Circadian Rhythm

Summary

The disruption of circadian rhythm is associated with the pathogenesis of hepatic and gastrointestinal metabolic syndromes, such as non-alcoholic fatty liver diseases (NAFLD) (Hoogerwerf 2009). CREBH transcriptionally regulated many hepatic metabolic enzymes involved in lipogenesis, FA oxidation, lipolysis, and gluconeogenesis (Figure 3). There are significant rhythmic patterns in the expression and activation of CREBH (Figure 4). These observations enable us to hypothesize that CREBH may function as an organ-specific clock-controlled transcription factor that coordinates different pathways under circadian rhythm to maintain hepatic metabolic homeostasis. By using circadian animal models, we found that CREBH is required to keep circadian profiles of blood triglycerides, fatty acids, and glucose as well as hepatic glycogen storage. Intriguingly, the expression levels and amplitudes of the key genes regulated by CREBH are involved in bi-directional metabolic pathways of both energy utilization and storage. CREBH deficiency leads to increased metabolic rates, hyper-locomotion, and phase-shifted feeding behavior in mice.

These findings are significant and innovative because they indicate that the liver local rhythm is critical for the maintenance of hepatic lipid and glucose homeostasis. CREBH may sense the liver local clock and mediate the transcription of different metabolic pathways, such as lipolysis, lipogenesis, FA oxidation, gluconeogenesis, and glycogenesis in the different circadian phases. The new revealed functions of CREBH result in a paradigm shift regarding our understanding of the molecular basis of liver

circadian rhythm, hepatic lipid/glucose homeostasis, and the pathological progression of metabolic syndromes.

Materials and Methods

Rhythmic lipid and glucose profile analyses and food intake measurement

To profile circulating, approximate 20 μL blood serum per mouse per time point was collected every 6 hours for 48 hours in constant darkness from the tail vein using 20μL K+ EDTA-containing microcapillary tubes. Blood serum TG and FFA were measured by colorimetric assays (BioAssay Systems, Hayward, CA). To quantify hepatic TG or glycogen, liver tissues from similar lobe regions of CREBH-null and WT control mice under the circadian clock were collected and subjected to measurements of TG and glycogen using commercial enzymatic kits following the manufacturer's instructions (BioAssay Systems, Hayward, CA). Levels of hepatic TG or glycogen were presented after normalization to liver mass. The amount of animal chow left in the individual mouse cages was carefully measured at each time point, and serial subtraction was calculated for the measurement of food intake. Levels of blood glucose of the mice under constant darkness were measured every 6 hours for 36 hours with an OneTouch Ultra Blood Glucose Meter (LifeScan, Milpitas, CA).

Histological staining and quantitative analysis of hepatic glycogen

Periodic-acid staining of hepatic glycogen was performed according to the standard protocol (Zhang, Wang et al. 2012; Zheng, Xu 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 5 minutes. The oxidized tissue sections were incubated in Schiff's reagent (Sigma) for 15 minutes. 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, Wang et al. 2012; Zheng, Xu 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.

Locomotor activity

After 2 weeks on LD cycles, mice were released into DD for an additional 30 days as previously described (Liu, Li et al. 2007; Siepka, Yoo et al. 2007). To collect locomotion variable during 44 days in total, individual mice were housed in chambers surrounded by an infrared photocell array interfaced with a computer running VersaMax/VersaDat programs (AccuScan Instruments, Columbus, OH). Measures included distance travelled (in centimeters) and stereotypy count (number of beam breaks at the same photocell array) every 6 minutes over the 44 days (Bishop and Walker 2004). Activity level in certain periods was calculated by averaging the total distance travelled per 6 minutes in centimeters or the stereotypic movement counting of each mouse in the specific circadian time period, as indicated in Figure 6, over 14 consecutive days during the LD cycles. The period of rhythmic activities was calculated by the onset of major activities of each mouse from two successive days over 30 days in DD.

Indirect calorimetry

Each mouse was monitored individually in the computer-controlled OxyScan open circuit indirect calorimetry system (AccuScan Instruments, Columbus, OH) (Bishop and

Walker 2004) with free access to food and water. Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were measured for 48 hours. Gas analyzers were calibrated to room air drawn through each chamber at a rate of 0.5 L/minute.

Results

CREBH regulates circadian rhythmic levels of TG and FA by activating the genes encoding functions in lipolysis, FA oxidation, and lipogenesis.

We recently demonstrated that CREBH is a key regulator of energy homeostasis under metabolic stress (Zhang, Wang et al. 2012). To elucidate whether CREBH regulates energy homeostasis under the day-night cycle, we characterized rhythmic profiles of circulating lipids in CREBH-null and WT control mice. Compared to the WT control mice, CREBH-null mice exhibited significantly higher levels of serum TG and FA over a 48-hour period of constant darkness (Figure 8A-B). Hepatic TG levels in CREBH-null mice were insignificantly lowered, compared to that in the control mice, at the night time (CT40) when mice usually take most of their meals of the day (Figure 9). These observations were consistent with the established roles of CREBH in TG lipolysis, FA oxidation, and lipogenesis upon fasting or atherogenic high-fat feeding, as we previously described (Zhang, Wang et al. 2012; Kim, Mendez et al. 2014).

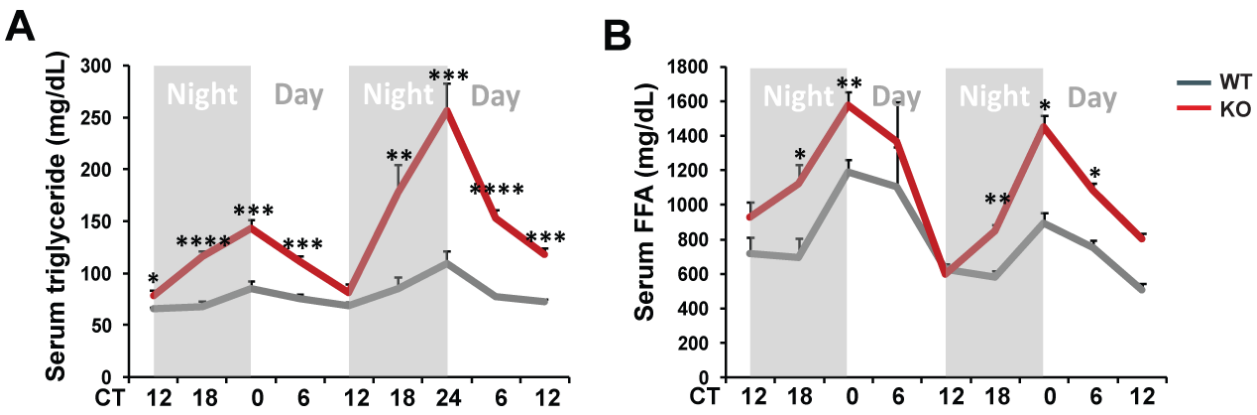

Figure 8. CREBH regulates rhythmic levels of circulating lipids in mice.
(A-B) Levels of circulating TG (A) and FFA (B) in CREBH-null and WT control mice under the circadian clock. Blood samples were collected every 6 hours for 48 hours in constant darkness for measuring TG and FFA. Data was presented as mean ± SEM (n=8 mice per time point) at each time point. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

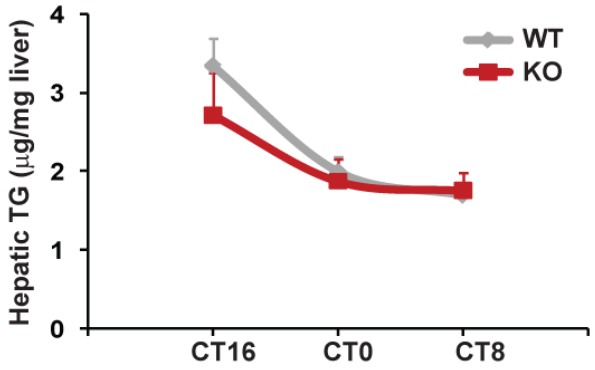

Figure 9. Levels of hepatic TG in CREBH-null and WT control mice livers.
Quantitative enzymatic analysis of hepatic TG in the livers of CREBH-null and WT control mice at the circadian time of CT40, 48, and 56, respectively. Each bar donates mean ± SEM (n=3 mice per group per time point).

To check whether CREBH rhythmically regulates expression of the genes involved in lipid metabolism in the liver, we determined rhythmic expression profiles of the genes encoding key enzymes or regulators in lipolysis, FA oxidation, and lipogenesis in CREBH-null and WT control mice under the endogenous circadian clock. Quantitative real-time PCR (qRT-PCR) analysis indicated that rhythmic expression levels and amplitudes of the following genes were altered in CREBH-null mice (Figure 10): 1) the gene encoding the key enzyme in lipolysis, apolipoprotein C-II (ApoC2); 2) the genes encoding the key enzymes or regulators in FA oxidation, including carnitine palmitoyltransferase 1A (CPT1α), 3-hydroxybutyrate dehydrogenase 1 (BDH1), and FGF21; and 3) the genes encoding the key enzymes in lipogenesis, including fatty acid desaturase 2 (FADS2) and Acetyl-CoA Carboxylase 1 (ACC1). Consistent with the mRNA expression profiles, protein levels of CPT1α, BDH1, FADS2, and ACC1 were decreased in the livers of CREBH-null mice (Figure 11). Additionally, rhythmic expression levels of other key metabolic genes involved in lipolysis, FA oxidation, and lipogenesis, including *Dhcr24, Lcat, Acot4, Hmgcl, Dgat2*, and *Elvol6*, only insignificantly altered in the CREBH-null livers (Figure 12).

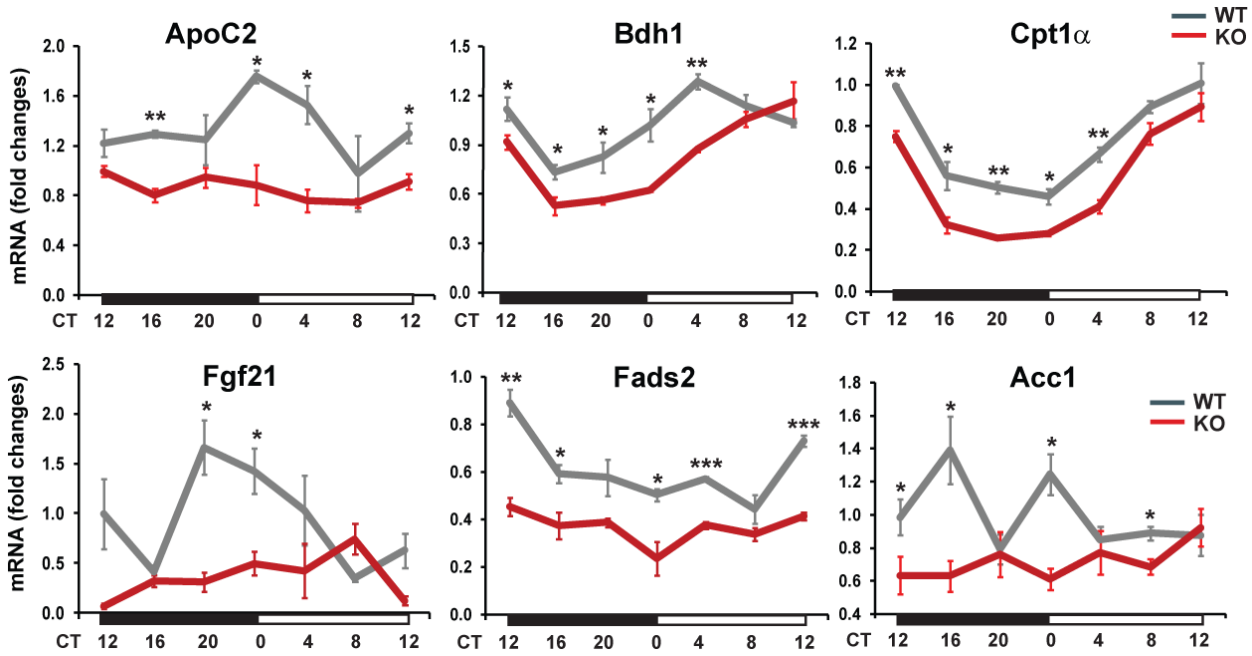

Figure 10. CREBH regulates rhythmic expression of the genes involved in lipolysis, FA oxidation, and lipogenesis in mice under the circadian clock.

Rhythmic expression levels of the CREBH-target genes involved in TG lipolysis, FA oxidation, and lipogenesis, including *ApoC2, Bdh1, Cpt1a, Fgf21, Fads2,* and *Acc1* in CREBH-null and WT control mouse livers. 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 time point).

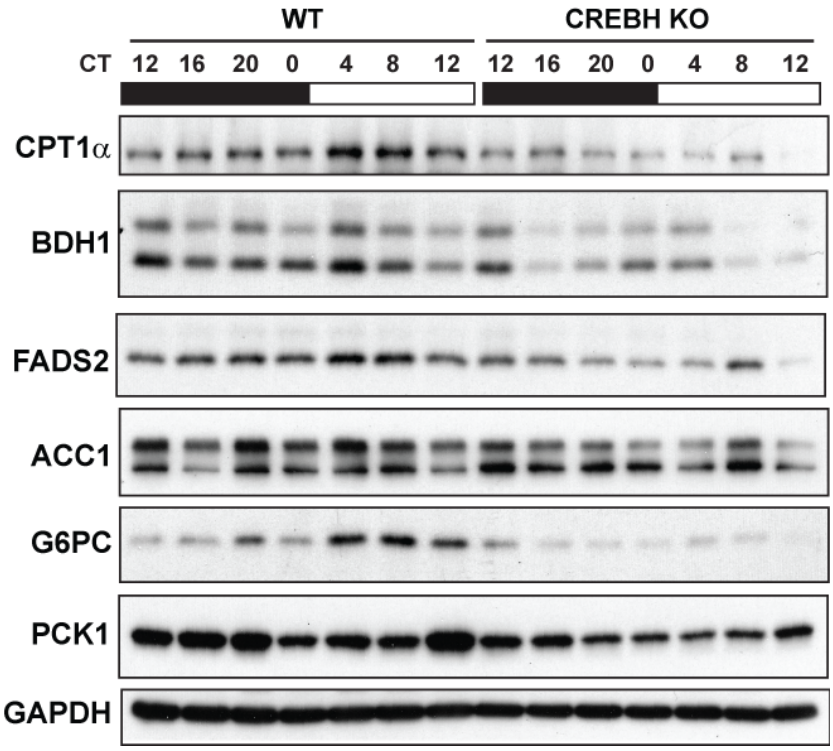

Figure 11. Rhythmic protein levels of the key metabolic enzymes or regulators in the livers of CREBH-null and WT control mice.

Rhythmic protein levels of the CREBH-target genes encoding PCK1, G6PC, FADS2, CPT1α, BDH1, and ACC1 in the livers of CREBH-null and WT control mice. The liver tissue samples from CREBH-null and WT control mice were collected every 4 hours in a 24-hour circadian period. Pooled liver protein lysates from 3-5 mice per genotype group per time point were used for the Western blot analyses. Levels of GAPDH were determined as loading controls.

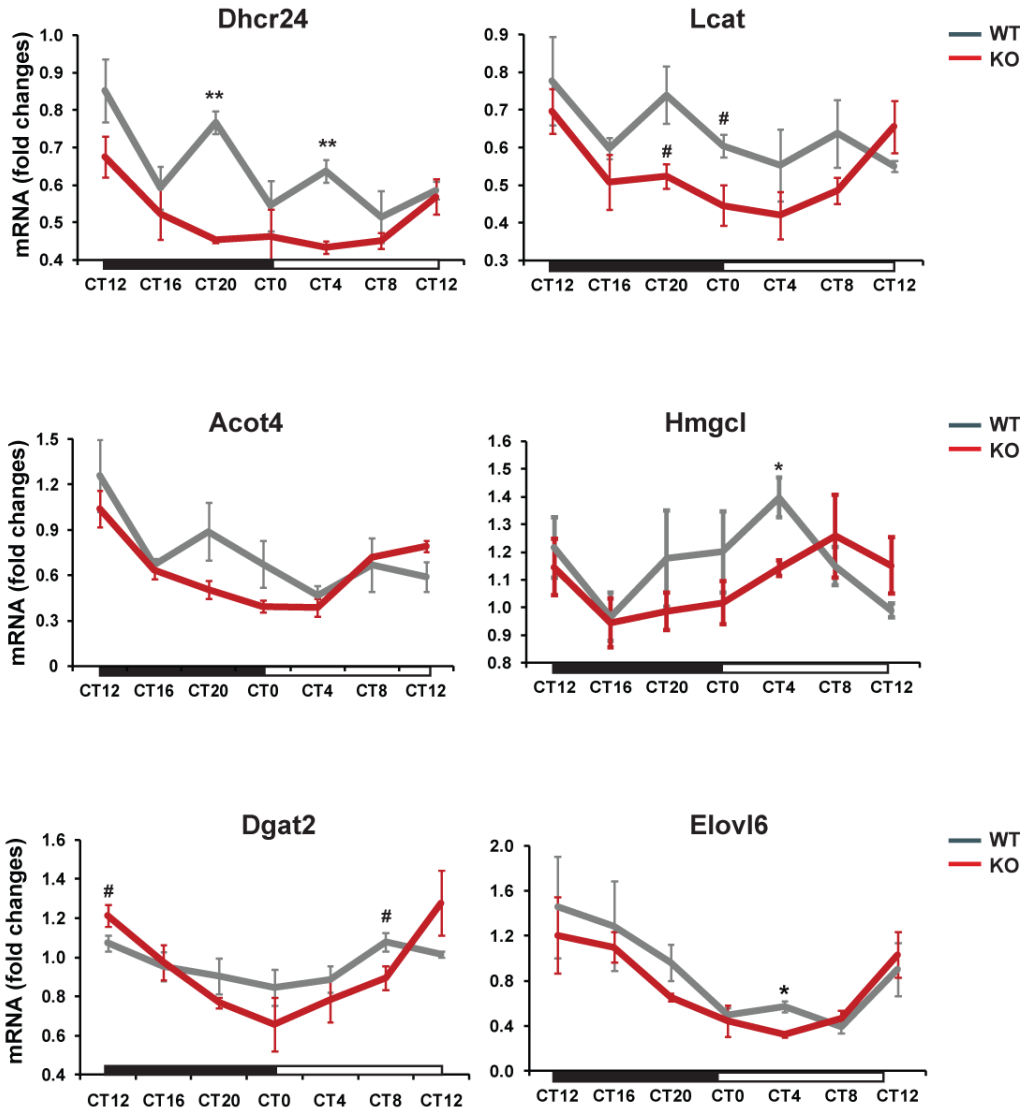

Figure 12. Insignificant alterations of metabolic gene rhythmic expressions in the livers of CREBH-null and WT control mice.

(B) Expression profiles of the genes encoding key enzymes involved in lipolysis, FA oxidation, and lipogenesis, including 24-Dehydrocholesterol Reductase (Dhcr24), Lecithin-Cholesterol Acyltransferase (Lcat), Acyl-CoA Thioesterase 4 (Acot4), 3-hydroxymethyl-3-methylglutaryl-CoA lyase (Hmgcl), diacylglycerol O-acyltransferase 2 (Dgat2), and ELOVL fatty acid elongase 6 (Elovl6), in the livers of CREBH-null and WT control mice under circadian clock. The liver samples from CREBH-null and WT control mice were collected every 4 hours over a 24-hour period. These RNAs were subjected to quantitative real-time RT-PCR analysis. Expression values were normalized to the *Arbp* mRNA levels. Fold changes of mRNA levels are shown by comparing to that of one of the WT control mice at the starting circadian time point. Asterisks indicate significant differences (* $p < 0.05$, ** $p < 0.01$) between WT and CREBH-null mice by post-hoc analyses followed by two-way ANOVA. Data represent mean ± SEM (n=3-5 mice per group per time point).

To determine whether CREBH directly regulates its target genes under the circadian cycle, we performed ChIP-qPCR analysis to determine CREBH enrichment in the promoter regions of metabolic genes whose rhythmic expression profiles were altered in CREBH-null mouse livers. ChIP-qPCR analyses with WT mouse livers collected at different circadian phases indicated that CREBH binds in a circadian phase-dependent manner to *the* promoters of *ApoC2, Bdh1, Cpt1a, Fgf21, Fads2*, or *Acc1* genes that possess one or multiple CRE-binding elements (Figure 13, Table 3). Increased enrichment of CREBH in the *ApoC2* gene promoter was detectable at CT40 and peaked at CT52, which is consistent with the rhythmic expression profile of the *ApoC2* mRNA in the liver. Similarly, consistent with the mRNA expression profiles, the enrichments of CREBH in the *Fads2* and *Acc1* gene promoter reached peak levels at CT52, CT56, and CT40, respectively (Figure 13). Taken together, these results indicate that CREBH activates expression of genes involved in bi-directional metabolic pathways of both energy utilization (lipolysis and FA oxidation) and storage (lipogenesis) depending upon the circadian cycle.

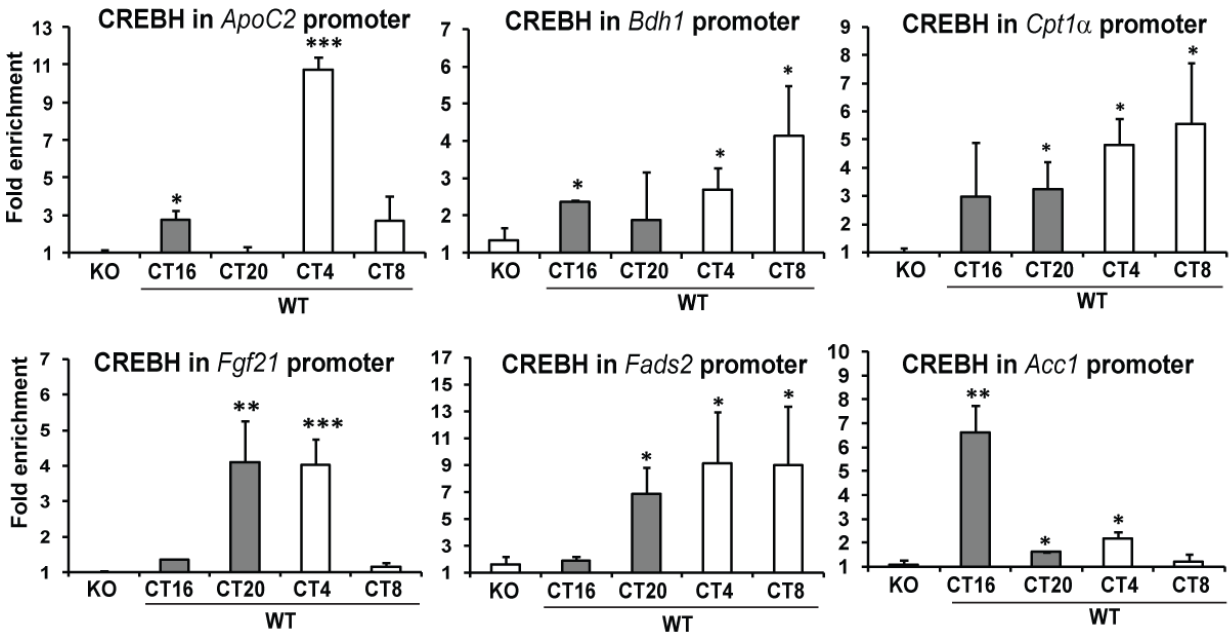

Figure 13. CREBH regulates rhythmic levels of lipids and expression of the genes involved in lipolysis, FA oxidation, and lipogenesis in mice under the circadian clock.
CREBH enrichment in the CREBH-target gene promoters in the WT mouse livers under different circadian phases determined by ChIP-qPCR. CREBH-null liver nuclei were used as negative control for the endogenous CREBH ChIP assays. Quantification of CREBH enrichment in the gene promoters at different circadian phases was determined by comparing ChIP-qPCR signals from the samples pulled down by the anti-CREBH antibody to that pulled down by a rabbit anti-IgG antibody. Each bar donates mean ± SEM (n=3 mice per time point).

Table 3. CRE binding motifs in the promoter regions of mouse genes.

	Promoter sequences	Nucleotide (nt) regions
***Apoc2* promoter**	TGGCCTCTGACTGTCACTGT	nt -117 to nt -113
***Acc1* promoter**	CTAACGCTGACCTTCTTTAC CTTTCTCATGAACTTTATTT	nt -315 to nt -310 nt -271 to nt -265
***Fgf21*promoter**	CCACTCCTGACGCGTGATAT	nt -63 to nt -67
***Bdh1* promoter**	GTGAGGTGACCAATCCCCCT	nt -452 to nt-434
***Cpt1a* promoter**	TCATTCTCTGATGTTAGACAAGC TTCCTTACTGACCTCCTCCCCGCA	nt -568 to nt -562 nt -245 to nt -240
***Fads2* promoter**	AGGTCAGACACGTCGCCGACCG	nt -599 to nt -594
***Gys2* promoter**	GTTGTACACTGACAAATACAGA CATAATACTTGACATTTAAAAT GATAGGGATTGACAATCAACCA	nt -591 to nt -587 nt -437 to nt -433 nt -375 to nt -371
***Ppara* promoter**	ACAGGGGTGACGGGGGC	nt -323 to nt -319
***Cebpβ* promoter**	GGGCGGGCTGGCGTCACCCGC ACCGCAGT CGGGCAATGACGCGCACCGA CCCAGCGTGACGCAGCCCGT	nt -344 to nt -337 nt -206 to nt -202 nt -160 to nt -156
***Lxra* promoter**	GGAACGCTGACTCTGGAGGCT GTGGGGGTGACTGAGAAGCAG	nt -184 to nt -180 nt -151 to nt -147
***E4bp4* promoter**	CCGCCGCCCGTCACGGCGGGG GCAGT	nt -160 to nt -156
***G6pc* promoter**	CTGGATTGACCTACAGACTG	nt -68 to nt -63
***Pck1* promoter**	CTTCTCATGACCTTTGGCCG TGGGAGTGACACCTCACAGC GGTGTTTTGACAACCAGCAG	nt -450 to nt -445 nt -431 to nt -427 nt -407 to nt -403

The binding motifs are highlighted (red underline). The complementary sequences (blue underline) are presented if the binding motifs locate in the negative strand.

CREBH regulates rhythmic hepatic glycogen storage and blood glucose levels by activating the key genes involved in glycogenesis and gluconeogenesis.

We next examined whether CREBH regulates glucose homeostasis across the day-night cycle. Periodic acid-Schiff (PAS) staining of hepatic glycogen of CREBH-null and WT control mice under the endogenous circadian clock indicated that production of hepatic glycogen in WT mice exhibited a circadian rhythmic pattern, which was increased from CT40 to CT52 and depleted at CT56 (Figure 14A). In contrast, hepatic glycogen storage in CREBH-null mice lost its rhythmic pattern, as the distribution and levels of glycogen in the livers of CREBH-null mice exhibited marginal changes over the circadian period. This observation was confirmed by quantitative enzymatic assay of hepatic glycogen levels (Figure 14B). Further, we measured blood glucose levels of CREBH-null and WT control mice across the day-night cycle. A phase-shifted rhythmic pattern of serum glucose levels is revealed in CREBH-null mice (Figure 14C), and the blood glucose levels in CREBH-null mice were lower than those in the control mice during the daytime period. These phenotypes suggest that CREBH functions as a key regulator of glucose homeostasis under the circadian control.

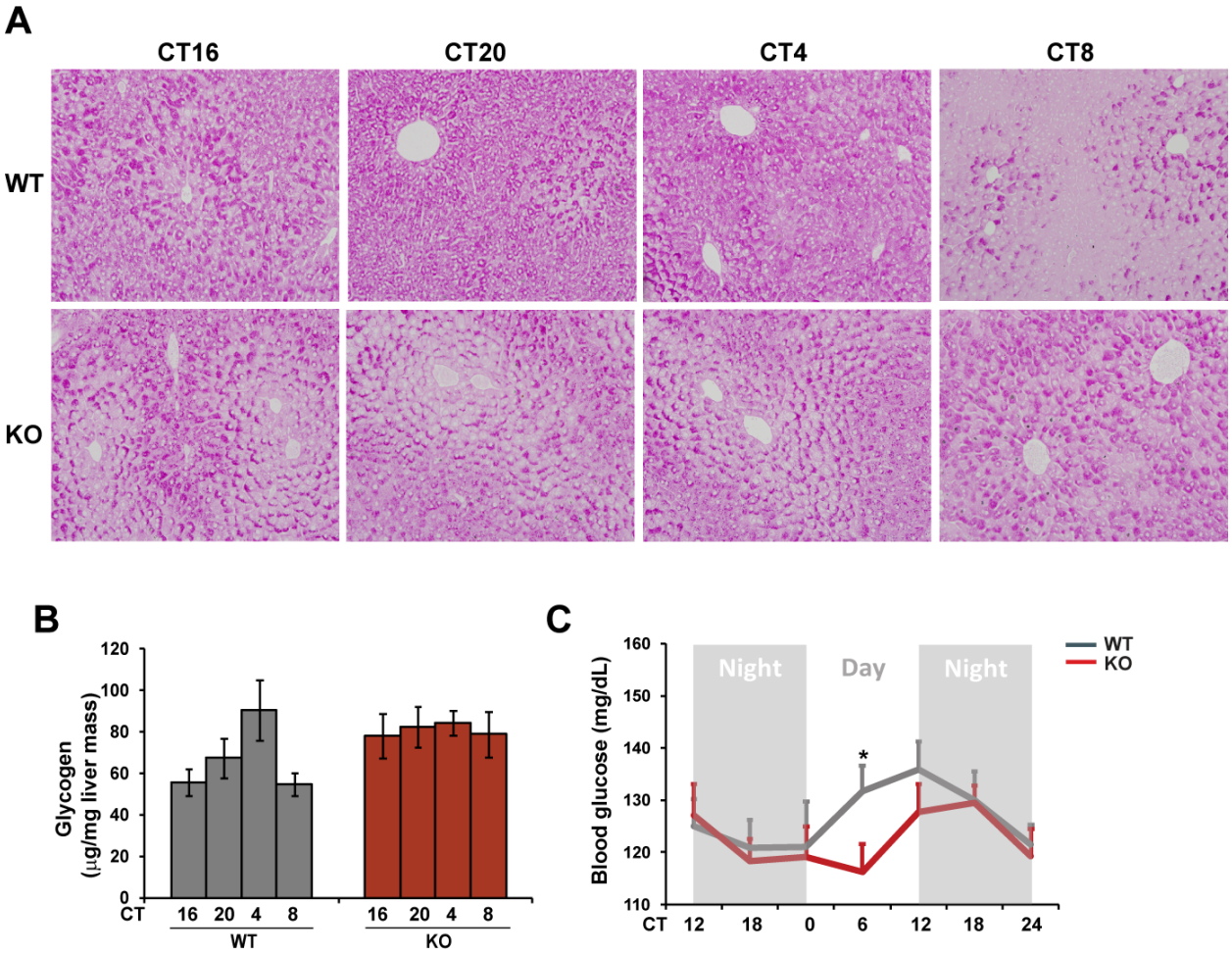

Figure 14. CREBH regulates rhythmic levels of blood glucose and hepatic glycogen storage in mice under the circadian clock.

(A) Periodic-acid Schiff (PAS) staining of hepatic glycogen in the livers of CREBH-null and WT control mice at the circadian time of CT40, 44, 52, and 56, respectively (magnification: 200×). (B) Quantitative enzymatic analysis of hepatic glycogen in the livers of CREBH-null and WT control mice at the circadian time of CT40, 44, 52, and 56, respectively. Each bar donates mean ± SEM (n=3 mice per group per time point). (C) Levels of blood glucose in CREBH-null and WT control mice under the circadian clock. Blood glucose were measured every 6 hours for 36 hours in constant darkness. Data was presented as mean ± SEM (n=8 mice per time point) at each time point.

To understand the molecular basis for the altered rhythmic profile of hepatic glycogen storage and blood glucose in CREBH-null mice, we examined expression of phosphoenolpyruvate carboxykinase 1 (Pck1), glucose-6-phosphatase (G6pc), and glycogen synthase 2 (Gys2), the rate limiting enzymes of hepatic gluconeogenesis and glycogenesis, respectively (Roach, Depaoli-Roach et al. 2012), in CREBH-null and WT control mouse livers. Compared to WT mice, CREBH-null mice exhibited decreased rhythmic expression of the *Pck1* and *G6pc* genes in the livers (Figure 15A). Interestingly, CREBH-null mice displayed an inverse rhythmic expression pattern of the *Gys2* gene in the liver (Figure 15A). Expression levels of the *Gys2* mRNA in the WT mice peaked at CT40 and reached a trough at CT56, whereas *Gys2* mRNA expression in the CREBH-null mice reached its nadir at CT40 and peaked at CT56. These results suggest that CREBH is required to maintain the normal rhythmic expression of the *Gys2* gene in the liver. In the absence of CREBH, however, an alternative transcriptional mechanism likely exists to enable expression of the *Gys2* gene in a reverse rhythmic pattern. Our results indicated that CREBH-null mice do not have sufficient Gys2 for hepatic glycogenesis upon feeding in the night, and therefore, they display lower levels of glycogen during the night time (Figure 14A-C). During the resting phases, however, CREBH-null mice produce higher levels of Gys2 (due to inverse rhythmic expression) but lower levels of blood glucose (due to the defect in gluconeogenesis). The combined effects of the altered Gys2 expression and the repressed gluconeogenesis may explain the loss of rhythm in hepatic glycogen storage in CREBH-null mice (Figure 14A-C).

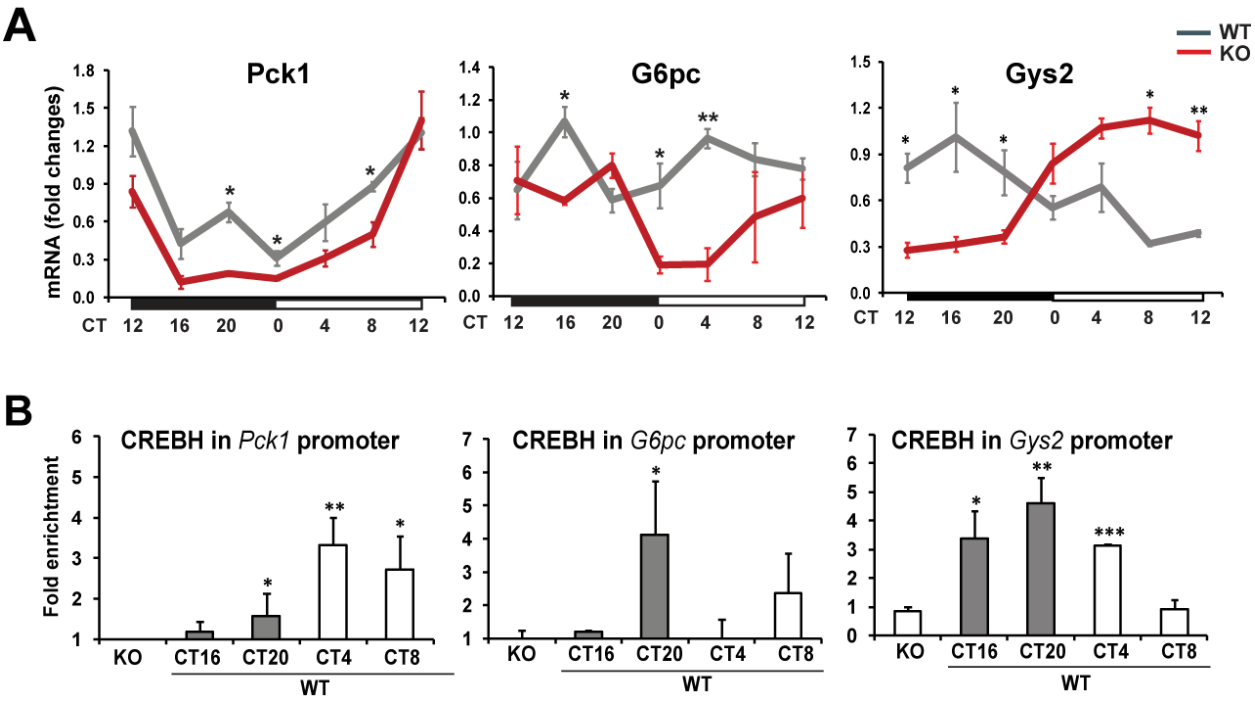

Figure 15. CREBH regulates rhythmic expression of the genes involved in gluconeogenesis and glycogenesis in mice under the circadian clock.

(A) Rhythmic expression levels of the CREBH-target genes involved in gluconeogenesis and glycogenesis, including Pck1, G6pc, and Gys2 in CREBH-null and WT control mouse livers. 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 time point). (B) CREBH enrichment in the *Pck1, G6pc*, and *Gys2* gene promoters in the WT mouse livers under different circadian phases determined by ChIP-qPCR. CREBH-null liver nuclei were used as negative control for the endogenous CREBH ChIP assays. Quantification of CREBH enrichment in the gene promoters at different circadian phases was determined by comparing ChIP-qPCR signals from the samples pulled down by the anti-CREBH antibody to that pulled down by a rabbit anti-IgG antibody. Each bar donates mean ± SEM (n=3 mice per time point). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Many typical CRE-binding elements present in the promoter regions of the *Pck1, G6pc*, and *Gys2* genes (Table 3). To evaluate whether CREBH, as a transcriptional activator, can directly target on the *Pck1, G6pc*, and *Gys2* gene promoters under the circadian cycles, ChIP-qPCR analysis were performed to quantify enrichment of CREBH in the gene promoter regions in mouse liver tissues collected at different circadian phases. ChIP-qPCR analyses indicated that enrichment of CREBH at the *Pck1* and *G6pc* gene promoters peaked at CT52 and CT44, respectively, consistent with the mRNA expression profiles (Figure 15B). In the *Gys2* gene promoter, enrichment of CREBH was increased during the circadian night period from CT40 to CT44, a time of the day when mice usually take their meals (Figure 15B). During the daytime period, enrichment of CREBH in the *Gys2* gene promoter was decreased at CT52 and not detectable at CT56. These results suggest that CREBH maintains Gys2 rhythmic expression levels by directly regulating transcription of the *Gys2* gene. Additionally, in the absence of CREBH, an alternative transcriptional mechanism likely exists to enable expression of the Gys2 gene in a reverse rhythmic pattern. What the other transcription regulators of Gys2 expression and whether CREBH interacts with these factors are interesting questions to be elucidated in the future. Therefore, similar to the regulatory roles of CREBH in lipid metabolism, CREBH regulates rhythmic expression of the key genes in bi-directional glucose metabolic pathways of both energy utilization (gluconeogenesis) and storage (glycogenesis).

CREBH-null mice exhibit hyper-locomotion, increased metabolic rate, and phase-shifted feeding behavior.

To further evaluate the physiological role of CREBH in the metabolic of whole animal body, we examined locomotor activity, metabolic rate, and feeding behavior of CREBH-null and WT control mice across the day-night cycle. We monitored locomotor activity for 14 days during the normal light-dark cycle followed by 30 days in constant darkness (Bishop and Walker 2004). Analysis of total distance travelled collected during the 30 days in constant darkness showed that CREBH-null mice exhibited a 5.17-minute shorting of the daily locomotor activity (23.86 hours/period), compared to WT control mice (23.94 hours/period) (Figure 16A-B). Interestingly, there was no difference between groups in respect to average distance travelled during the 12-hour light period (CT48-60) (Figure 16A, C). However, CREBH-null mice exhibited significant hyper-locomotion during the 12-hour dark period (CT36-48). We also monitored stereotypic movements, which are characterized by small movements without travelling distance, such as grooming, body shaking, and feeding. CREBH-null mice consistently exhibited increased stereotypic movements during the second 6 hours of the dark period (CT42-48) (Figure 16A, D).

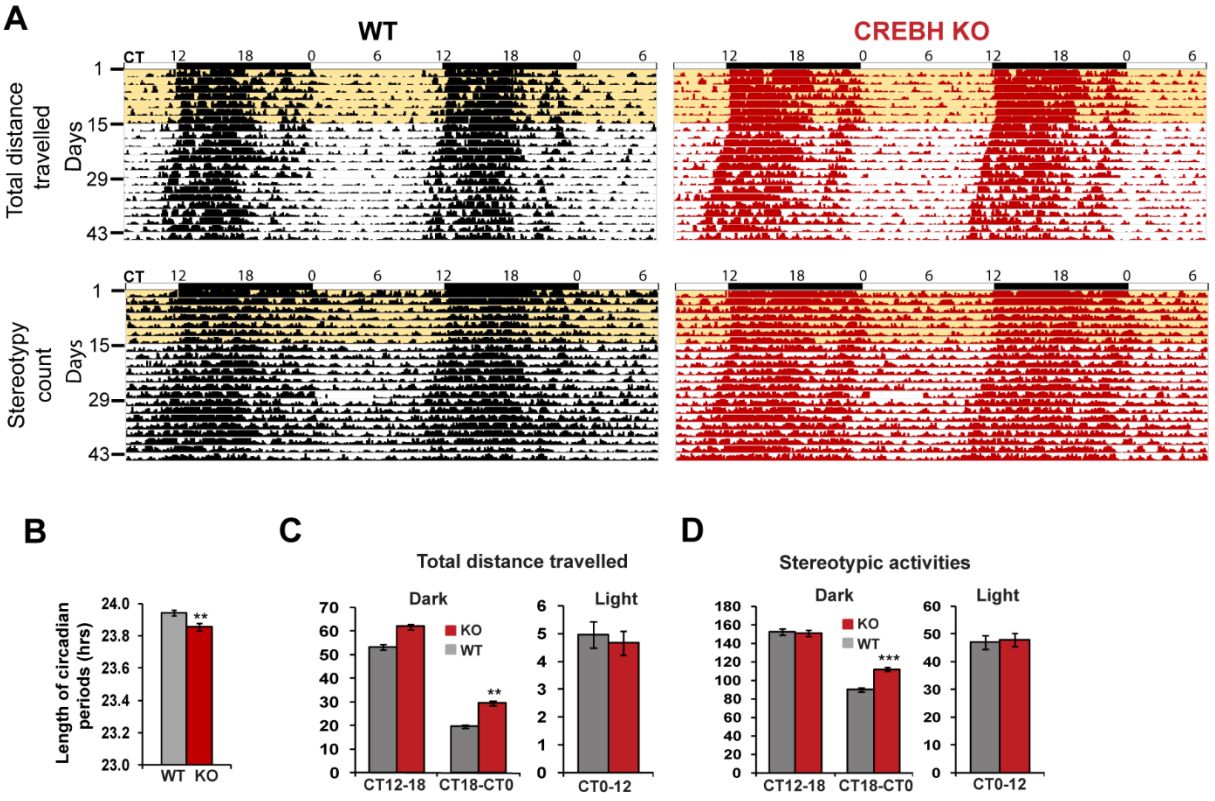

Figure 16. CREBH-null mice display hyper-locomotion and increased stereotypic activities during the night time.

(A) Circadian rhythmic profiles of locomotor activities of CREBH-null and WT control mice under the circadian clock. Total distance travelled in centimeter (upper panel) and stereotypic movement (lower panel) of CREBH-null and WT control mice were monitored every 6 minutes under 12-hour/12-hour LD cycles for 14 days and under DD for 30 days (n=6 mice per group). (B) Lengths of circadian periods of CREBH-null and WT control mice calculated based on the circadian locomotor activities during 30 days in DD (n=6 mice per group). (C-D) Average activities of distance travelled (C) and stereotypic movement (D) of the CREBH-null and WT control mice over the circadian periods of CT36-48, CT48-56, or CT54-60 during the first 14 days in LD. Each bar donates mean ± SEM (n=6 mice per time point). ** $p < 0.01$, *** $p < 0.001$.

To further explore the pathophysiological effects related to the hyper-locomotion of CREBH-null mice during the late dark phase, we characterized rhythmic feeding behavior and metabolic rates in CREBH-null and WT control mice. A significant time-shift in food intake was observed with CREBH-null mice across the 36-hour circadian period (Figure 17A). Compared to the control mice, CREBH-null mice exhibited approximately a 6-hour delay in taking their biggest meal of the day (peak at CT42-48). The metabolic rates, as reflected by the rates of oxygen consumption, of CREBH-null mice were significantly higher than those of WT control mice across the 12-hour dark period, which is consistent with the hyper-locomotion of CREBH-null mice during the 12-hour dark period (CT36-48) (Figure 17B, 16C-D). Moreover, a dramatic increase in metabolic rates was observed in CREBH-null mice during the second 6 hours of the dark period, concord with the increased stereotypic movements and delayed feeding behavior of CREBH-null mice during the same phase (Figure 17A-B, 16A, 16D). Additionally, we found that compared to that of WT control mice, total food intake of CREBH-null mice over 48-hour circadian period was modestly increased (Figure 17C). As CREBH is required for hepatic glycogenesis and lipogenesis during the night (Figures 8-15), the impaired energy catabolism may entrain CREBH-null mice to consume more dietary energy metabolites. Additionally, high levels of blood TG and FFA may stimulate satiety-related signals that lead to delayed food intake behavior and hyper-metabolic rates in the CREBH-null mice during the late night time, an interesting question to be further elucidated in the future.

It is also interesting to discuss why Gys2 expression pattern is inversed, but glycogen storage is not, in CREBH-null mice liver (Figure 14-15). It's explainable if combining glucose and food intake data (Figure14-15, 17). In WT mice, upon feeding,

Gys2 increased after feeding to store free glucose from blood stream into glycogen in liver. Meanwhile, CREBH null-mice don’t have enough Gys2 to process glycogenesis, and therefore, they have lower glycogen during the nighttime. In WT mice, during resting phase, glycogenesis slows down (low liver glycogen storage), but glycolysis and gluconeogenesis increases to provide glucose as energy for body philological use. Meanwhile CREBH null mice exhibited a delay in increasing blood glucose levels (due to the defect in gluconeogenesis), and high levels of Gys2 which boost hepatic glycogen levels when hepatic glycogen storage was depleted in the WT control mice.

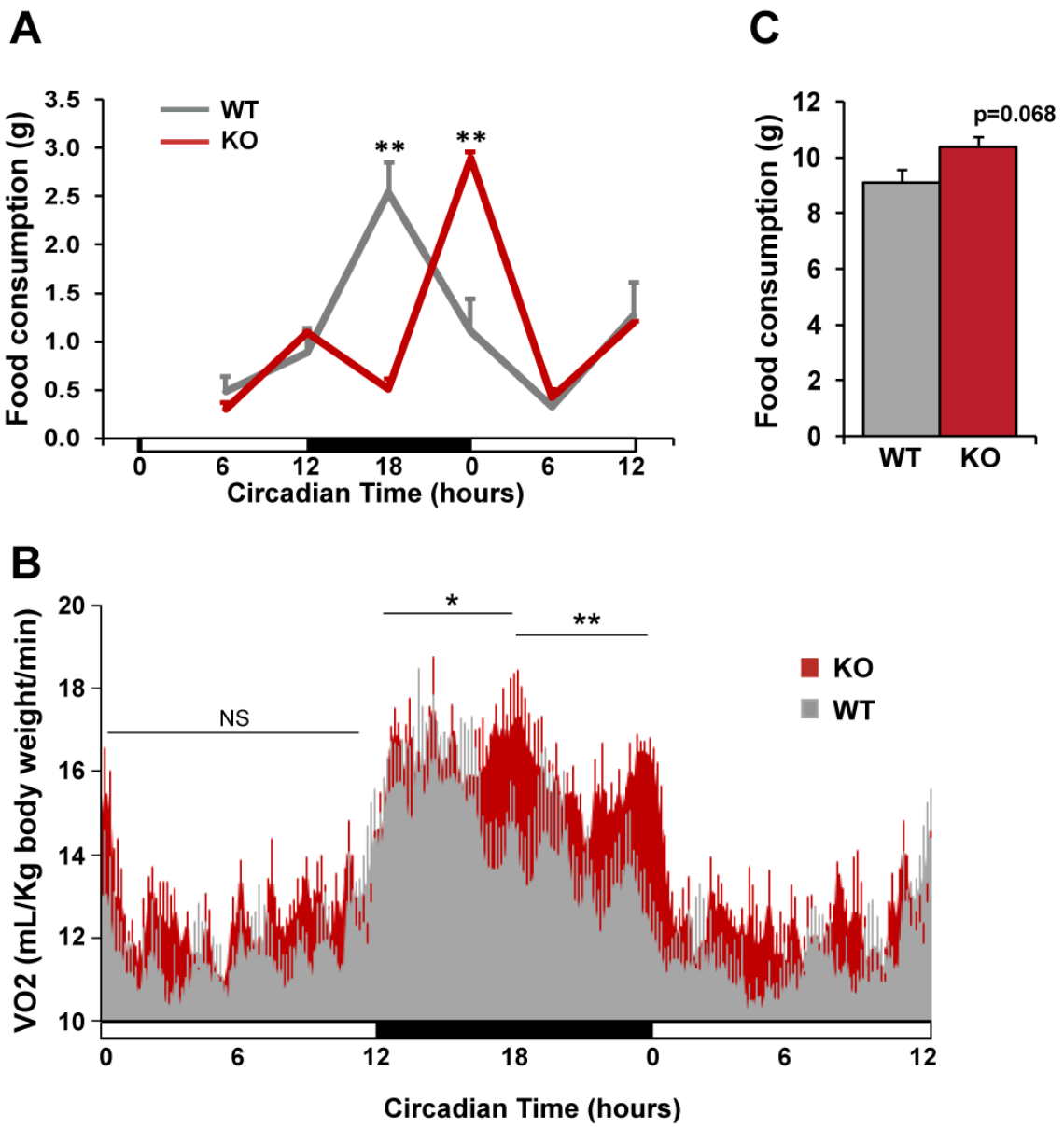

Figure 17. CREBH deficiency leads to phase-shifted feeding behavior and increased metabolic rates.

(A) Food intakes of CREBH-null and WT control mice under the circadian clock. Food intakes of individual animals were measured every 6 hours over 36-hour period in DD. Each point donates mean ± SEM (n=5 mice per group per time point). ** $p < 0.01$. (B) Metabolic rates, represented by oxygen consumption, of CREBH-null and WT control mice under the circadian clock. Oxygen consumption (VO2) normalized by body weight of individual mice was recorded every 10 minutes over a 36-hour period by a computer-controlled OxyScan open circuit indirect calorimetry systems. Average VO2 levels of each mouse over the phases of CT36-48, CT48-54, or CT54-60 were calculated for the statistical analysis (n=4 mice per group). * $p < 0.05$; ** $p < 0.01$; ns, non-significant. (C) Accumulative food intakes of CREBH-null and WT control mice over the 48-hour period. (D) Illustration of CREBH working model as a circadian metabolic oscillator.

CHAPTER IV: Crosstalk of CREBH and Other Circadian Regulators

Summary

The hypothesis in this dissertation is that CREBH may function as, or interact with, the local first-order CCG to regulate the synchronization of rhythmic transcription of hepatic metabolic enzymes. The transcriptional function of D-element binding protein (DBP), a known local first-order CCG, can be repressed by the interleukin 3-regulated nuclear factor (NFIL3, also known as E4BP4), a bZIP domain containing rhythmic transcription factor regulated by the repressor REV-ERBa (Clayton, Kyriacou et al. 2001; Reppert and Weaver 2002; Ueda, Chen et al. 2002). A recent study showed E4BP4 negatively regulates the rhythmic transcription of FGF21, a metabolic hormone that regulates lipid and glucose metabolism, and this repressive regulation can be inhibited by an unknown fasting-inducible factor (Tong, Muchnik et al. 2010). Since CREBH is a fasting-inducible factor that promotes FGF21 transcription (Lee, Giannikopoulos et al. 2011; Zhang, Wang et al. 2012), we hypothesize that CREBH may bind to E4BP4 and repress the negative regulation of E4BP4 to up-regulate the gluconeogenesis and TG lipolysis pathways during the day time under circadian rhythm. To answer the questions, we used both *in vivo* and *in vitro* methods to discover that CREBH regulates, and interacts with, the circadian transcriptional activators PPARα and C/EBPβ or the repressor E4BP4 to modulate CREBH transcriptional activities.

Materials and Methods

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 immunoprecipitated 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).

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 hours 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.

Results

Altered rhythmic expression of core clock genes in CREBH-null mice liver.

It is known that both the activation and function of circadian regulatory proteins are regulated through intimate and reciprocal interactions with other circadian transcriptional activators and repressors (Rutter, Reick et al. 2002; Bass and Takahashi 2010). To understand whether CREBH, as a liver local circadian oscillator, regulates, and/or interacts with, other circadian regulators, we examined expression of core clock genes in the livers of CREBH-null and WT control mice. Deletion of CrebH resulted in insignificantly altered rhythmic expression amplitudes of the genes encoding the core circadian oscillators including *Clock, Dbp*, and *Rev-erbα* in mouse livers (Figure 18). Rhythmic expression of Hnf4α, a previously identified regulator of CREBH (Luebke-Wheeler, Zhang et al. 2008), was reduced in CREBH-null mice. Western blot analysis confirmed that rhythmic expression of the circadian regulators BMAL1 and HNF4α were repressed in CREBH-null livers (Figure 19).

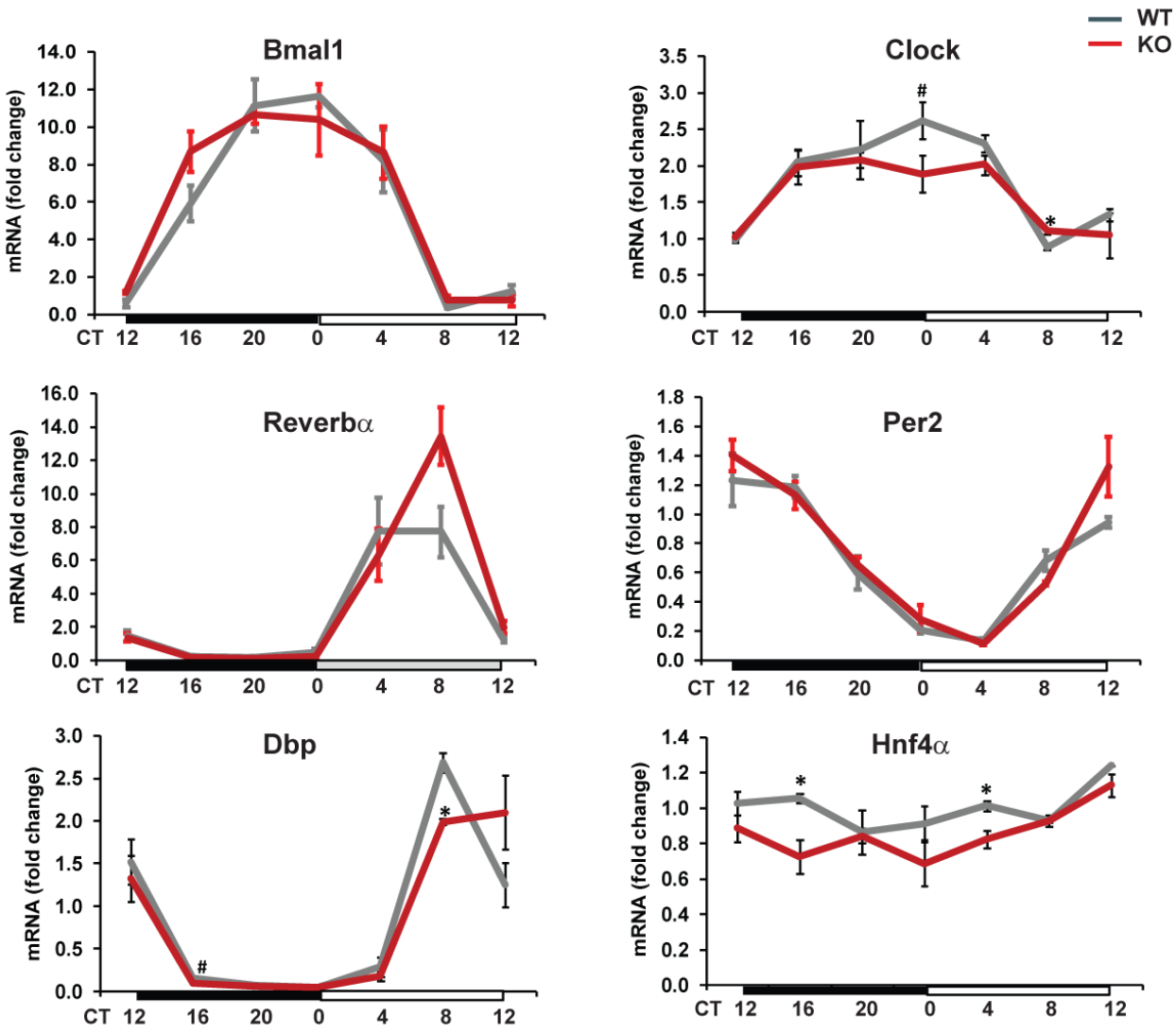

Figure 18. Rhythmic expression levels and amplitudes of core clock genes in the livers of CREBH-null and WT control mice.

Expression profiles of the clock genes, including *Bmal1, Clock, Reverbα, Per2, Dbp*, and *Hnf4α*, in the livers of CREBH-null and WT control mice under the circadian clock. The liver samples from the CREBH-null and WT control mice were collected every 4 hours over a 24-hour period. Expression values of mRNAs were determined by qRT-PCR and normalized to the *Arbp* mRNA levels. Fold changes of mRNA levels are shown by comparing to that of one of the wild-type control mice at the starting circadian time (CT) point. Asterisks indicate significant differences (* $p < 0.05$) between WT and CREBH-null mice by post-hoc analyses followed by two-way ANOVA. Data represent mean ± SEM (n=3 mice per group per time point).

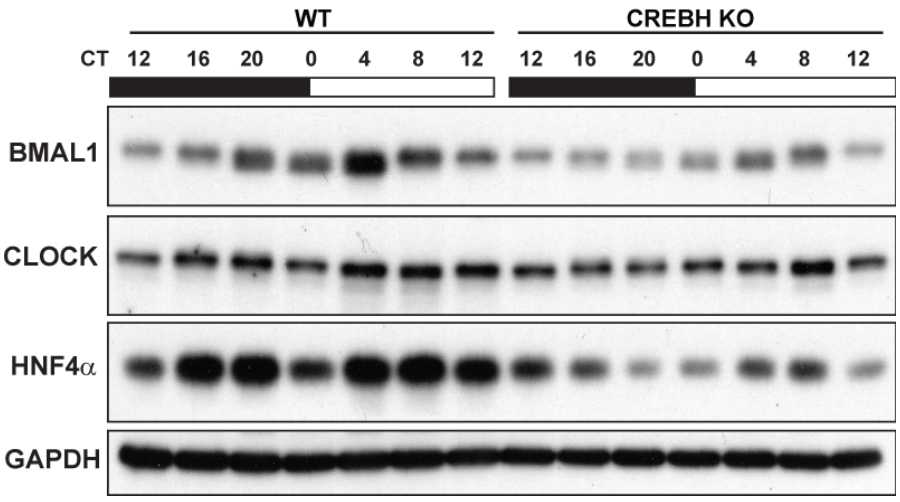

Figure 19. Rhythmic regulation of core clock proteins by CREBH in the liver.
Rhythmic levels of BMAL1, CLOCK, and HNFα proteins in CREBH-null and WT control mouse livers collected every 4 hours in a 24-hour period. Levels of proteins were determined by Western blot analysis. Pooled liver protein lysates from 3-5 mice per time point per genotype group were used. Levels of β-actin or GAPDH were included as loading controls.

CREBH rhythmically regulates circadian transcriptional activators C/EBPβ and PPARα, and the repressor E4BP4.

To elucidate whether CREBH regulates, and/or interacts with, other circadian regulators, we examined expression of core clock genes in the livers of CREBH-null and WT control mice. PPARα is a liver-enriched, clock-regulated nuclear receptor that plays key roles in regulating lipid metabolism during the starvation phase (Oishi, Shirai et al. 2005). C/EBPβ is a bZIP-containing transcriptional regulator known to rhythmically regulate autophagy in the liver, facilitating degradation of glycogen and lipid droplets for body energy (Ma, Panda et al. 2011). Liver X receptor α (LXRα) is a nuclear receptor that regulates lipogenesis by forming heterodimers with members of the retinoid X receptor (RXR) family (Willy, Umesono et al. 1995). Gene expression analyses showed that rhythmic expression levels of the *Pparα* and *Lxrα* mRNAs were significantly repressed in the CREBH-null livers, while rhythmic levels of the *C/ebpβ* mRNA in the CREBH-null mice were only marginally changed, compared to those in the control mice (Figure 20A-C). The mouse *Pparα, C/ebpβ,* and *Lxrα* gene promoters possess one or multiple CRE-binding motifs (Table 3). ChIP-qPCR analysis indicated that in mouse livers CREBH binds in a day-night dependent manner to the *Pparα, C/ebpβ*, or *Lxrα* gene promoters (Figure 20A-C), suggesting that Pparα, C/ebpβ, and Lxrα are circadian-dependent targets of CREBH. Moreover, Western blot analysis indicated that rhythmic levels of PPARα, C/EBPβ, and LXRα proteins were decreased in the CREBH-null livers (Figure 20E). Note that the discrepancy in C/EBPβ protein and mRNA levels in CREBH-null mice implies alternative regulation of C/ebpβ gene expression independent of CREBH or potential involvement of CREBH in C/EBPβ protein stability.

Further, we found that CREBH regulates rhythmic expression of E4BP4, a bZIP domain-containing circadian transcriptional repressor (Clayton, Kyriacou et al. 2001; Reppert and Weaver 2002; Ueda, Chen et al. 2002). Loss of CREBH resulted in a phase-inversed expression pattern of hepatic E4bp4 mRNA (Figure 20D). Western blot analysis showed that expression levels and the oscillation amplitude of E4BP4 protein were repressed in CREBH-null livers (Figure 20E). ChIP-qPCR analysis of mouse liver chromatins indicated that CREBH robustly binds to the E4bp4 gene promoter at CT4 (Figure 20D), suggesting that rhythmic expression of E4BP4 is regulated by CREBH in mouse livers.

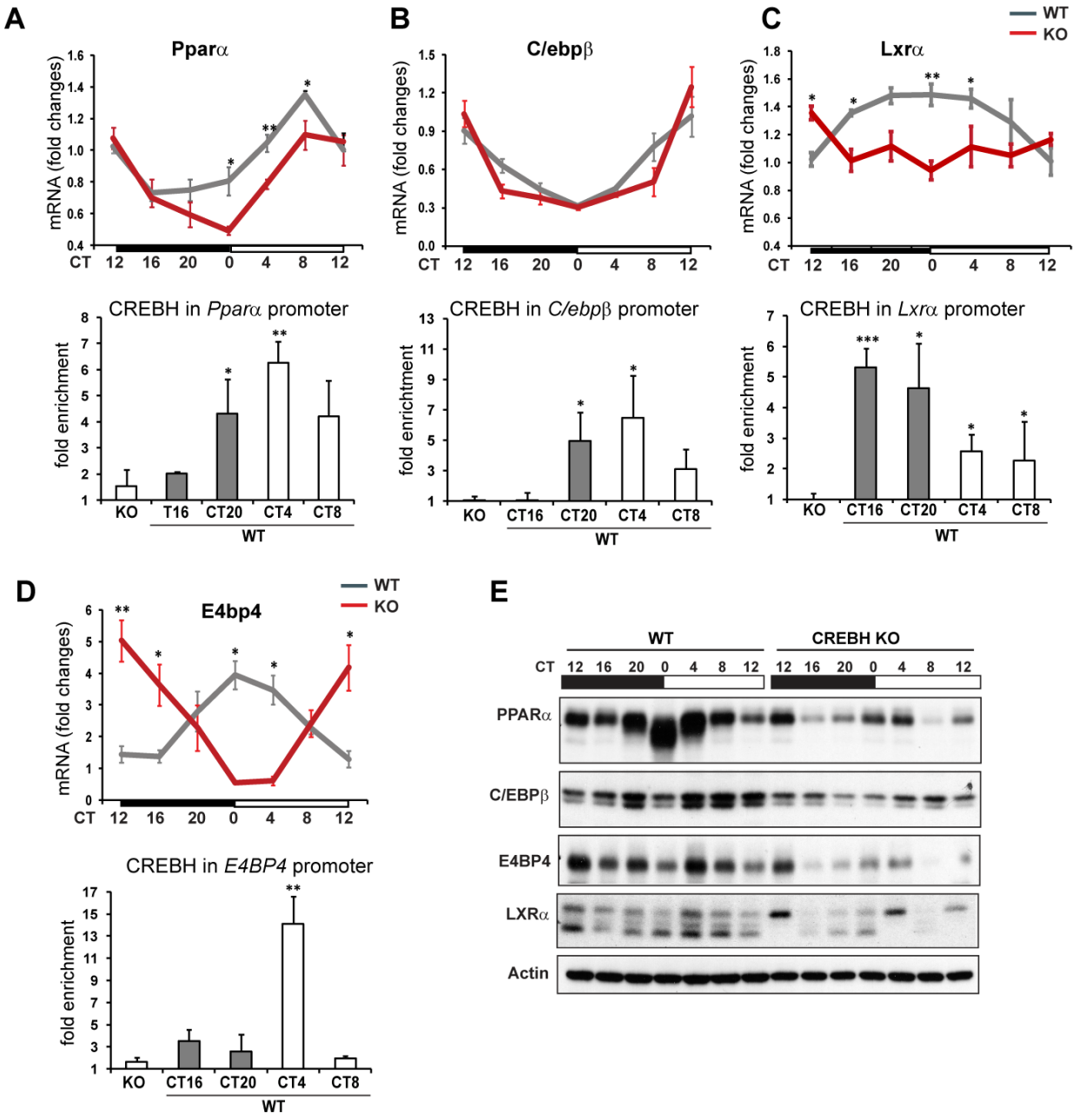

Figure 20. Rhythmic regulation of circadian transcriptional activators and repressor by CREBH in the liver.

(A-C) Rhythmic expression levels and CREBH enrichment in the promoters of the *Pparα*, *C/ebpβ*, *Lxrα and E4bp4* genes in the livers of CREBH-null and WT control mice under the circadian clock. Fold changes of the mRNA levels were determined by qRT-PCR (upper panel). Each bar denotes mean ± SEM (n = 3 mice per time point). Rhythmic enrichment of endogenous CREBH in the target gene promoters in the WT mouse livers under different circadian phases were determined by ChIP-qPCR (lower panel). Each bar donates mean ± SEM (n=4 mice per time point). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (D) Rhythmic levels of PPARα, C/EBPβ, E4BP4, and LXRα proteins in CREBH-null and WT control mouse livers collected every 4 hours in a 24-hour period. Levels of proteins were

determined by Western blot analysis. Pooled liver protein lysates from 3-5 mice per time point per genotype group were used. Levels of β-actin or GAPDH were included as loading controls.

CREBH rhythmically interacts with circadian transcriptional activators and repressor.

Next, we were interested about whether CREBH interacts with the circadian transcriptional regulators to modulate its transcriptional activity. CREBH interaction with PPARα and C/EBPβ were proved by IP-Western blot analyses using mouse livers collected across the day-night cycle (Figure 21). The interaction between CREBH and C/EBPβ peaked at CT56, a time period when lipolysis, FA oxidation, and gluconeogenesis are highly activated upon energy demands. The CREBH-PPARα interaction was detected from CT44 to CT52 (Figure 21). We were able to detect a robust interaction between CREBH and the transcriptional repressor E4BP4, which began at CT36, gradually increased over the night period, and peaked at CT48 (Figure 21). Interestingly, the phase and intensity of the CREBH-C/EBPβ interaction roughly opposes that of the CREBH-E4BP4 interaction, implying that C/EBPβ and E4BP4 may compete to interact with CREBH in a circadian phase-dependent manner.

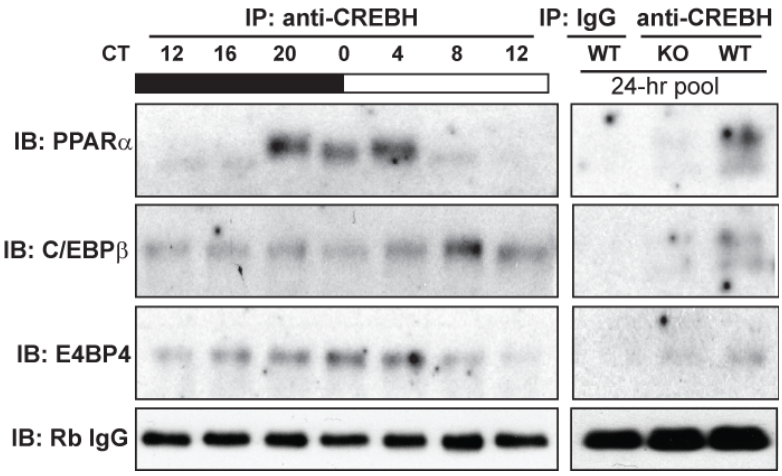

Figure 21. Rhythmic interactions between CREBH, the circadian transcriptional activators PPARα and C/EBPβ, and the repressor E4BP4 in the liver.

Interactions between endogenous CREBH and PPARα, C/EBPβ, or E4BP4 in mouse livers under different circadian phases were determined by IP-Western blot analysis. Liver protein lysates pooled from 3 WT mice were pulled down by a rabbit anti-CREBH antibody and then probed with an antibody against PPARα, C/EBPβ, or E4BP4. As a loading control, the liver protein lysates pulled down by the anti-CREBH antibody was probed with the rabbit anti-IgG. Liver protein lysates pooled from WT or CREBH-null mice under the 24-hour circadian phases were included as positive and negative controls, respectively.

To explore the functional significance of the interactions between CREBH and C/EBPβ, PPARα, or E4BP4, we performed reporter analysis with the *Pck1* gene promoter, a common target of CREBH, C/EBPβ, and PPARα (Nizielski, Arizmendi et al. 1996; Juge-Aubry, Pernin et al. 1997; Lee, Chanda et al. 2010; Peeters and Baes 2010). Indeed, the promoter region of the mouse *Pck1* gene possesses multiple binding motifs for CREBH, PPARα, C/EBPβ, and E4BP4, respectively (Figure 22). While over-expression of the active form of CREBH, C/EBPβ, or PPARα alone can significantly increase *Pck1* gene promoter activity, co-expression of CREBH with C/EBPβ or PPARα further augmented the reporter activity (Figure 23A). In contrast, co-expression of CREBH with E4BP4 significantly decreased *Pck1* promoter activity either when compared to expression of CREBH alone or co-expression of CREBH with GFP. These results suggest that C/EBPβ and PPARα function as co-activators of CREBH in driving *Pck1* gene transcription, while E4BP4 acts as a repressor of CREBH-dependent *Pck1* gene expression. Moreover, we observed that co-expression of E4BP4 with the combination of CREBH and C/EBPβ or PPARα repressed expression of the *Pck1* gene reporter, compared to co-expression of CREBH with C/EBPβ or PPARα (Figure 23B), thus supporting the suppressive effect of E4BP4 on CREBH transcriptional activity through competition with the co-activator C/EBPβ or PPARα. Given that the rhythmic expression of E4BP4 is decreased in CREBH-null livers (Figure 21), the repressive effect of E4BP4 on CREBH activity may serve as a negative feedback regulation of CREBH under the circadian constrain.

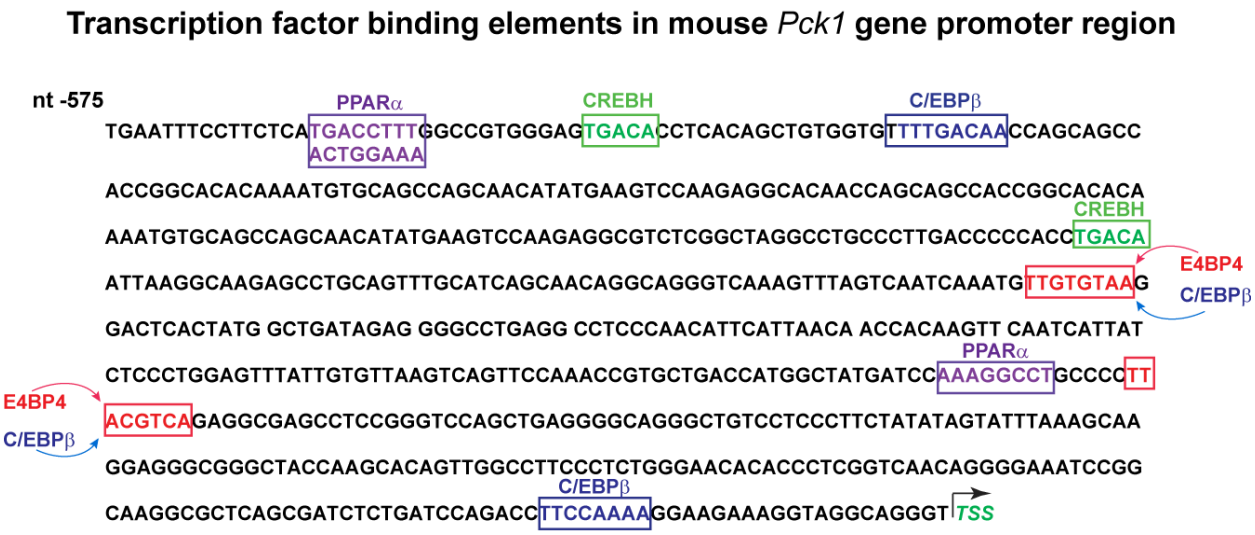

Figure 22. Potential CREBH-, PPARα-, C/EBPβ-, and E4BP4- binding sequences in the promoter region of mouse Pck1 gene.

The binding motifs are highlighted. The complementary sequence is presented if the binding motif locates in the negative strand.

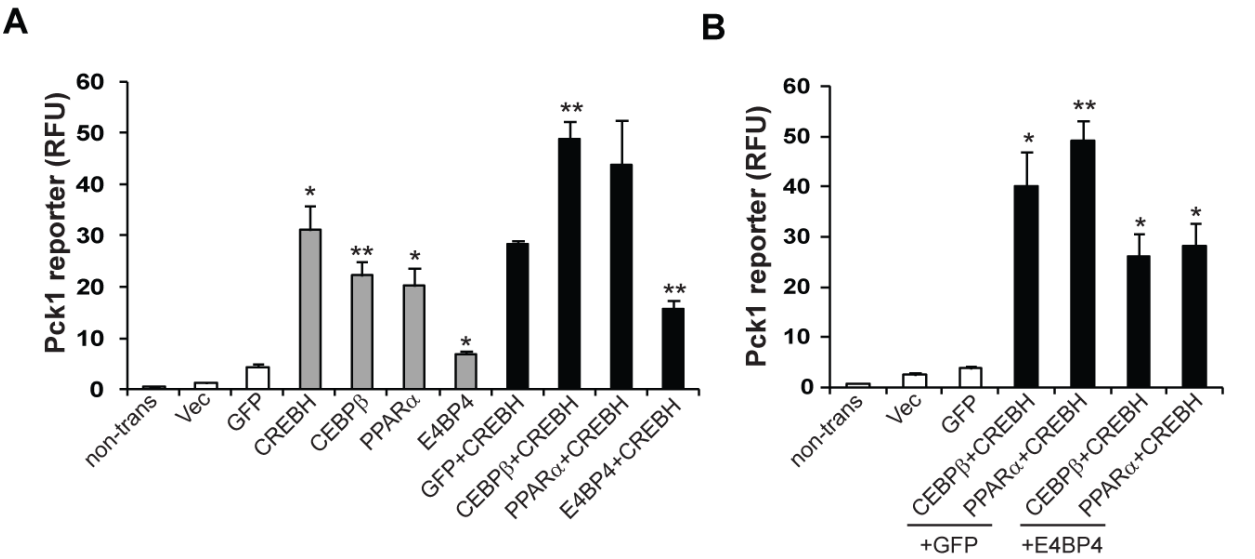

Figure 23. Rhythmic interactions between CREBH, the circadian transcriptional activators PPARα and C/EBPβ, and the repressor E4BP4 in the liver.

(B-C) Luciferase reporter analyses of transcriptional activation of the mouse *Pck1* gene promoter by CREBH alone or in combination with PPARα, C/EBPβ, and/or E4BP4. Mouse hepatoma cell line Hepa1-6 was transiently transduced with the Pck1 reporter vector or vehicle. After 24 hours, the transfected cells were infected with adenovirus expressing GFP (control), PPARα, C/EBPβ, and/or E4BP4, as indicated in the Figure. Renilla reporter plasmid was included in the co-transfection for normalization of luciferase reporter activities. The same amounts of adenovirus titers were used for individual infections. Each bar denotes the mean ± SEM (n=2 experimental repeats).

CHAPTER V: Conclusions and Significance

In this study, we demonstrated that CREBH functions as a circadian regulator of lipid and glucose metabolism in the liver (Figure 24). Our major findings include: 1) CREBH is a BMAL1-regulated diurnal regulator in the liver, and expression and proteolytic activation of CREBH are both rhythmically regulated during the circadian cycle; 2) CREBH rhythmically regulates expression of the genes encoding key enzymes or regulators of energy utilization processes, including lipolysis, FA oxidation, and gluconeogenesis, and of energy storage processes, including lipogenesis and glycogenesis; 3) CREBH controls rhythmic levels of circulating TG, FFA, and glucose as well as hepatic glycogens; 4) CREBH rhythmically interacts with the circadian transcriptional activators C/EBPβ and PPARα and the repressor E4BP4 to modulate CREBH transcriptional activities; and 5) disruption of CREBH leads to phase-shifted feeding behavior, increased metabolic rates, and hyper-locomotion during the dark period. These findings demonstrate that CREBH is not only a key metabolic regulator but also a liver circadian oscillator, and therefore, plays key roles in integrating energy metabolism with circadian rhythm (Figure 24). The functional activity of CREBH as a peripheral circadian metabolic regulator has profound impact on the measures of whole body physiology, such as feeding, metabolic rate, and locomotor activity. Modulation of CREBH activity may therefore have important implications in the prevention and treatment of metabolic disorders.

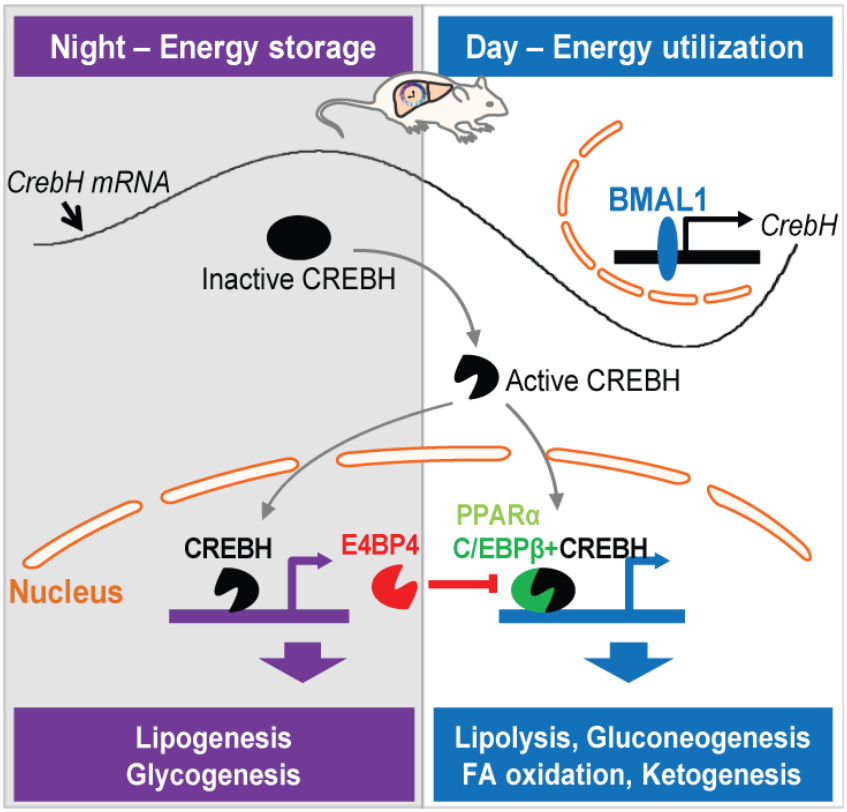

Figure 24. Illustration of CREBH working model as a circadian metabolic oscillator.

Mutation of Clock or Bmal1 damages lipid and glucose metabolism in animals, as evidenced by hyperlipidemia, hepatic steatosis, and defective gluconeogenesis (Rudic, McNamara et al. 2004; Turek, Joshu et al. 2005; Lamia, Storch et al. 2008; Shimba, Ogawa et al. 2011). However, the mechanism underlying these metabolic phenotypes remains unknown. Our data suggested that CREBH is a BMAL1-regulated liver metabolic regulator of lipolysis, FA oxidation, lipogenesis, and gluconeogenesis. Circadian profiles of circulating TG, FFA, and glucose as well as hepatic TG and glycogens were altered in CREBH-null mice. Therefore, CREBH may function as a major metabolic regulator through which the core circadian oscillators regulate hepatic energy metabolism. Previously, we identified a variety of stress signals, such as ER stress, energy fluctuations, and inflammatory challenges, which can activate CREBH (Zhang, Shen et al. 2006; Zhang, Wang et al. 2012). CREBH regulates distinct, even functionally opposite metabolic pathways, to maintain energy homeostasis under stress conditions. The current finding that CREBH is a circadian metabolic regulator brings new insights on the regulation and function of CREBH under physiological conditions. Indeed, the rhythmic regulation of CREBH activity and its roles in circadian energy homeostasis are consistent with our previous observations that CREBH is activated by distinct stress signals (Zhang, Wang et al. 2012). The regulation of metabolic pathways by CREBH during the day seems to be similar to that observed under short-term fasting conditions (Figure 24). We have shown that insulin, saturated fatty acids, and an atherogenic high-fat diet can also activate CREBH (Zhang, Wang et al. 2012). These “over-nutrient” signals may mimic feeding stimulation that is similar to mouse feeding during the night when they consume most of their daily intake. Insulin or high-fat feeding stimulates CREBH activation but have

marginal effects on *CrebH* gene expression (Zhang, Wang et al. 2012). This is consistent with the CREBH expression and activation profiles and the regulatory roles of CREBH in hepatic lipogenesis and glycogenesis during the night.

In this study, we provide expression levels and amplitudes for the CREBH-target genes across the circadian cycle. Depending on the circadian phase and energy demands, CREBH regulates expression of different target genes in lipid or glucose metabolism. During the day time, CREBH regulates expression of the genes involved in lipolysis, FA oxidation, and gluconeogenesis, including *ApoC2, Badh1, Cpt1α, Fgf21, Pck1, C/ebpβ*, and *Pparα* (Figures 8-15). During the night, CREBH regulates expression of the genes involved in lipogenesis and glycogenesis, including *Acc1, Fads2, Lxrα*, and *Gys2*. Indeed, the functions of CREBH in mouse circadian metabolism are consistent with the rhythmic activation of the CREBH protein, in which the levels of the activated, nuclear CREBH protein peaked at the daytime (CT56) but reached a trough at the nighttime period (CT44) (Figure 4C-D). Interestingly, the circadian expression pattern of the *CrebH* mRNA was opposite to the rhythmic activation of the CREBH protein in the liver (Figure 4A-D). This observation was in line with recent rhythmic proteome study showing that approximately one-half of rhythmic proteins are under significant translational or posttranslational diurnal controls and have no corresponding rhythmic mRNAs (Mauvoisin, Wang et al. 2014).

CREBH has reciprocal interactions with the circadian transcriptional regulators PPARα and C/EBPβ as well as the circadian repressor E4BP4 (Figures 18-23). CREBH regulates and interacts with PPARα or C/EBPβ to enhance CREBH transcriptional activity, which oscillates in-phase with expression of the CREBH-target genes involved in lipolysis,

FA oxidation, and gluconeogenesis. On the other hand, CREBH interacts with E4BP4 to repress CREBH transcriptional activity during the night-today transition period. Interestingly, the phase of CREBH-C/EBPβ interaction is complimentary to that of CREBH-E4BP4 interaction, suggesting that C/EBPβ and E4BP4 may compete to interact with CREBH and thereby modulate CREBH activities during different circadian phases. As a co-activator of CREBH, PPARα interacts with CREBH in the circadian phase that partially overlaps with the C/EBPβ-CREBH interaction (Figure 21). It is possible that the interaction between PPARα-CREBH may represent an enhancing mechanism that facilitates CREBH peak activity during the phases of high-energy demands.

Peripheral clocks, such as in liver, are synchronized with the central clock located in the SCN through a complicated regulatory network of neuronal, hormonal, behavioral, and environmental signals (Ueda, Chen et al. 2002). The core circadian oscillators regulate CREBH expression and activation through two layers: 1) BMAL1/CLOCK regulates transcription of CrebH; and 2) E4BP4 or C/EBPβ interacts with activated form of CREBH protein to exert suppressive or synergizing effect on CREBH activity. On the other hand, CREBH regulates mRNA and/or protein levels of the core circadian regulators, including E4BP4, C/EBPβ, PPARα, BMAL1, CLOCK, and HNF4α (Figure 18-20). The reciprocal regulation between CREBH and the key circadian regulators may provide an avenue by which local and central circadian regulators are integrated to influence whole body physiology. Because expression of CrebH in mouse is highly restricted to liver and small intestine (Luebke-Wheeler, Zhang et al. 2008) (Figure 25), the CREBH-null mouse is an excellent model to study peripheral clock-originated feedback to the master clock. Recent studies have found disturbance of circadian rhythm can lead to depression and

anxiety disorders (Salgado-Delgado, Tapia Osorio et al. 2011; Keers, Pedroso et al. 2012). CREBH-null mice exhibited increased stereotypic movements during the late night period (Figure 16), which may partially reflect depressive activities associated with metabolic and psychiatric disorders. Whether CREBH, as a liver circadian metabolic oscillator, is crucial to maintaining metabolic and thereby psychiatric wellbeing is an interesting question to be investigated in the future.

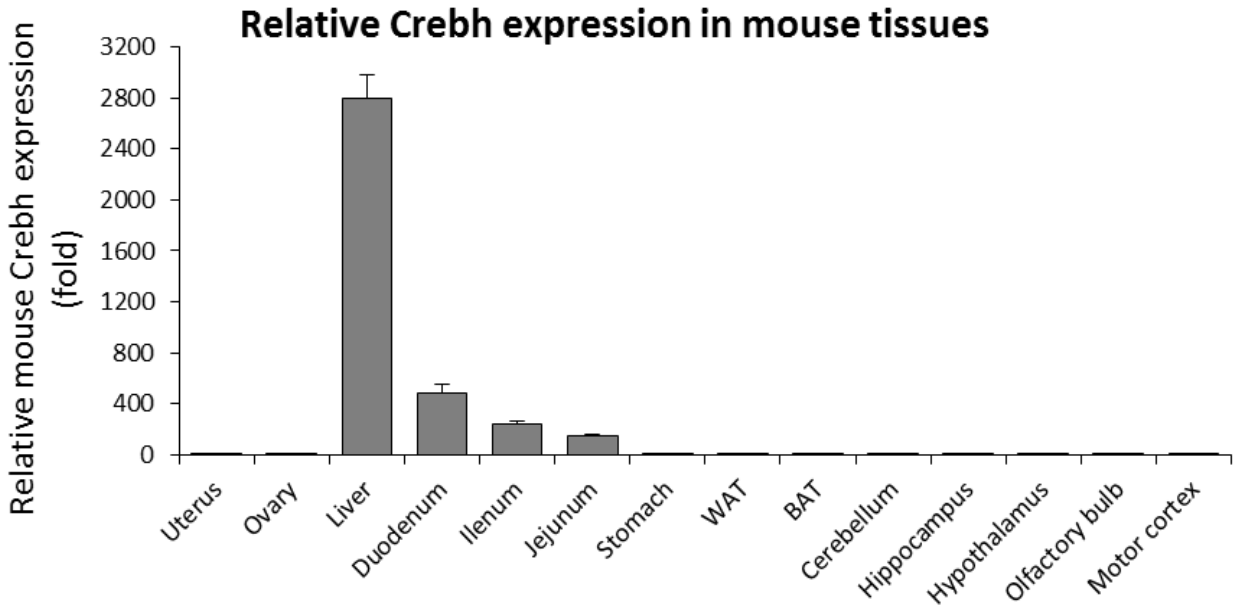

Figure 25. Tissue-specific expression of the mouse CrebH mRNA.
CrebH mRNA expression profiles in mouse various organs were determined by qPCR. Each bar donate mean ± SEM (n=3 mice). Selection of brain regions was based on the brain regions described in Allen Brain Altas (http://www.brain-map.org/) (cortex, hippocampus, cerebellum, and olfactory bulb) and anterior hypothalamus where suprachiasmatic nuclei (SCN) locates. Female WT mice were deeply anesthetized with isoflurane followed by rapid decapitation. Brain was rapidly removed and placed on ice, and the specific brain regions were dissected under microscopic control. For dissecting anterior hypothalamus and motor cortex, section was made on ice-cold mouse brain matrix between posterior cut at Bregama-2.92mm and anterior cut at Bregama-0.22mm by an experienced neurologist.

REFERENCES

Akhtar, R. A., A. B. Reddy, et al. (2002). "Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus." Curr Biol **12**(7): 540-550.

Asselah, T., I. Bieche, et al. (2010). "In vivo hepatic endoplasmic reticulum stress in patients with chronic hepatitis C." J Pathol **221**(3): 264-274.

Bailey, D., C. Barreca, et al. (2007). "Trafficking of the bZIP transmembrane transcription factor CREB-H into alternate pathways of ERAD and stress-regulated intramembrane proteolysis." Traffic **8**(12): 1796-1814.

Bass, J. and J. S. Takahashi (2010). "Circadian integration of metabolism and energetics." Science **330**(6009): 1349-1354.

Bishop, C. and P. D. Walker (2004). "Intranigral antagonism of neurokinin 1 and 3 receptors reduces intrastriatal dopamine D1 receptor-stimulated locomotion in the rat." Brain Res **1023**(1): 126-133.

Brown, M. S., J. Ye, et al. (2000). "Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans." Cell **100**(4): 391-398.

Bugge, A., D. Feng, et al. (2012). "Rev-erbalpha and Rev-erbbeta coordinately protect the circadian clock and normal metabolic function." Genes Dev **26**(7): 657-667.

Canaple, L., J. Rambaud, et al. (2006). "Reciprocal regulation of brain and muscle Arnt-like protein 1 and peroxisome proliferator-activated receptor alpha defines a novel positive feedback loop in the rodent liver circadian clock." Mol Endocrinol **20**(8): 1715-1727.

Chan, C. P., K. H. Kok, et al. (2011). "CREB3 subfamily transcription factors are not created equal: Recent insights from global analyses and animal models." Cell Biosci **1**(1): 6.

Chan, C. P., T. Y. Mak, et al. (2010). "N-linked glycosylation is required for optimal proteolytic activation of membrane-bound transcription factor CREB-H." J Cell Sci **123**(Pt 9): 1438-1448.

Chanda, D., D. K. Kim, et al. (2011). "Cannabinoid receptor type 1 (CB1R) signaling regulates hepatic gluconeogenesis via induction of endoplasmic reticulum-bound transcription factor cAMP-responsive element-binding protein H (CREBH) in primary hepatocytes." J Biol Chem **286**(32): 27971-27979.

Clayton, J. D., C. P. Kyriacou, et al. (2001). "Keeping time with the human genome." Nature **409**(6822): 829-831.

Cretenet, G., M. Le Clech, et al. (2010). "Circadian clock-coordinated 12 Hr period rhythmic activation of the IRE1alpha pathway controls lipid metabolism in mouse liver." Cell Metab **11**(1): 47-57.

Doherty, C. J. and S. A. Kay (2010). "Circadian control of global gene expression patterns." Annu Rev Genet **44**: 419-444.

Duffield, G. E. (2003). "DNA microarray analyses of circadian timing: the genomic basis of biological time." J Neuroendocrinol **15**(10): 991-1002.

Fabbrini, E., S. Sullivan, et al. (2010). "Obesity and nonalcoholic fatty liver disease: biochemical, metabolic, and clinical implications." Hepatology **51**(2): 679-689.

Feng, D. and M. A. Lazar (2012). "Clocks, metabolism, and the epigenome." Mol Cell **47**(2): 158-167.

Feng, D., T. Liu, et al. (2011). "A circadian rhythm orchestrated by histone deacetylase 3 controls hepatic lipid metabolism." Science **331**(6022): 1315-1319.

Gentile, C. L., D. Wang, et al. (2010). "Fatty acids regulate CREBh via transcriptional mechanisms that are dependent on proteasome activity and insulin." Mol Cell Biochem **344**(1-2): 99-107.

Green, C. B., J. S. Takahashi, et al. (2008). "The meter of metabolism." Cell **134**(5): 728-742.

Hatori, M., C. Vollmers, et al. (2012). "Time-restricted feeding without reducing caloric intake prevents metabolic diseases in mice fed a high-fat diet." Cell Metab **15**(6): 848-860.

Hoogerwerf, W. A. (2009). "Role of biological rhythms in gastrointestinal health and disease." Rev Endocr Metab Disord **10**(4): 293-300.

Hughes, M. E., L. DiTacchio, et al. (2009). "Harmonics of circadian gene transcription in mammals." PLoS Genet **5**(4): e1000442.

Inoue, I., Y. Shinoda, et al. (2005). "CLOCK/BMAL1 is involved in lipid metabolism via transactivation of the peroxisome proliferator-activated receptor (PPAR) response element." J Atheroscler Thromb **12**(3): 169-174.

Johansen, C. T. and R. A. Hegele (2012). "The complex genetic basis of plasma triglycerides." Curr Atheroscler Rep **14**(3): 227-234.

Juge-Aubry, C., A. Pernin, et al. (1997). "DNA binding properties of peroxisome proliferator-activated receptor subtypes on various natural peroxisome proliferator response elements. Importance of the 5'-flanking region." J Biol Chem **272**(40): 25252-25259.

Kapatos, G., P. Vunnava, et al. (2007). "Protein kinase A-dependent recruitment of RNA polymerase II, C/EBP beta and NF-Y to the rat GTP cyclohydrolase I proximal promoter occurs without alterations in histone acetylation." J Neurochem **101**(4): 1119-1133.

Keegan, K. P., S. Pradhan, et al. (2007). "Meta-analysis of Drosophila circadian microarray studies identifies a novel set of rhythmically expressed genes." PLoS Comput Biol **3**(11): e208.

Keers, R., I. Pedroso, et al. (2012). "Reduced anxiety and depression-like behaviours in the circadian period mutant mouse afterhours." PLoS One **7**(6): e38263.

Kfoury, N. and G. Kapatos (2009). "Identification of neuronal target genes for CCAAT/enhancer binding proteins." Mol Cell Neurosci **40**(3): 313-327.

Kim, H., R. Mendez, et al. (2014). "Liver-enriched transcription factor CREBH interacts with peroxisome proliferator-activated receptor alpha to regulate metabolic hormone FGF21." Endocrinology **155**(3): 769-782.

Kumaki, Y., M. Ukai-Tadenuma, et al. (2008). "Analysis and synthesis of high-amplitude Cis-elements in the mammalian circadian clock." Proc Natl Acad Sci U S A **105**(39): 14946-14951.

Lamia, K. A., K. F. Storch, et al. (2008). "Physiological significance of a peripheral tissue circadian clock." Proc Natl Acad Sci U S A **105**(39): 15172-15177.

Le Martelot, G., T. Claudel, et al. (2009). "REV-ERBalpha participates in circadian SREBP signaling and bile acid homeostasis." PLoS Biol **7**(9): e1000181.

Lee, J. H., P. Giannikopoulos, et al. (2011). "The transcription factor cyclic AMP-responsive element-binding protein H regulates triglyceride metabolism." Nat Med **17**(7): 812-815.

Lee, J. H. and A. Sancar (2011). "Circadian clock disruption improves the efficacy of chemotherapy through p73-mediated apoptosis." Proc Natl Acad Sci U S A **108**(26): 10668-10672.

Lee, M. W., D. Chanda, et al. (2010). "Regulation of hepatic gluconeogenesis by an ER-bound transcription factor, CREBH." Cell Metab **11**(4): 331-339.

Li, S. and J. D. Lin (2009). "Molecular control of circadian metabolic rhythms." J Appl Physiol **107**(6): 1959-1964.

Lin, Y., M. Han, et al. (2002). "Influence of the period-dependent circadian clock on diurnal, circadian, and aperiodic gene expression in Drosophila melanogaster." Proc Natl Acad Sci U S A **99**(14): 9562-9567.

Liu, C., S. Li, et al. (2007). "Transcriptional coactivator PGC-1alpha integrates the mammalian clock and energy metabolism." Nature **447**(7143): 477-481.

Llarena, M., D. Bailey, et al. (2010). "Different mechanisms of recognition and ER retention by transmembrane transcription factors CREB-H and ATF6." Traffic **11**(1): 48-69.

Luebke-Wheeler, J., K. Zhang, et al. (2008). "Hepatocyte nuclear factor 4alpha is implicated in endoplasmic reticulum stress-induced acute phase response by regulating expression of cyclic adenosine monophosphate responsive element binding protein H." Hepatology **48**(4): 1242-1250.

Ma, D., S. Panda, et al. (2011). "Temporal orchestration of circadian autophagy rhythm by C/EBPbeta." EMBO J **30**(22): 4642-4651.

Maury, E., K. M. Ramsey, et al. (2010). "Circadian rhythms and metabolic syndrome: from experimental genetics to human disease." Circ Res **106**(3): 447-462.

Mauvoisin, D., J. Wang, et al. (2014). "Circadian clock-dependent and -independent rhythmic proteomes implement distinct diurnal functions in mouse liver." Proc Natl Acad Sci U S A **111**(1): 167-172.

Misra, J., D. Chanda, et al. (2011). "Curcumin differentially regulates endoplasmic reticulum stress through transcriptional corepressor SMILE (small heterodimer partner-interacting leucine zipper protein)-mediated inhibition of CREBH (cAMP responsive element-binding protein H)." J Biol Chem **286**(49): 41972-41984.

Molusky, M. M., D. Ma, et al. (2012). "Peroxisomal localization and circadian regulation of ubiquitin-specific protease 2." PLoS One **7**(11): e47970.

Nizielski, S. E., C. Arizmendi, et al. (1996). "Involvement of transcription factor C/EBP-beta in stimulation of PEPCK gene expression during exercise." Am J Physiol **270**(5 Pt 2): R1005-1012.

Oishi, K., H. Shirai, et al. (2005). "CLOCK is involved in the circadian transactivation of peroxisome-proliferator-activated receptor alpha (PPARalpha) in mice." Biochem J **386**(Pt 3): 575-581.

Omori, Y., J. Imai, et al. (2001). "CREB-H: a novel mammalian transcription factor belonging to the CREB/ATF family and functioning via the box-B element with a liver-specific expression." Nucleic Acids Res **29**(10): 2154-2162.

Peeters, A. and M. Baes (2010). "Role of PPARalpha in Hepatic Carbohydrate Metabolism." PPAR Res **2010**.

Rakhshandehroo, M., G. Hooiveld, et al. (2009). "Comparative analysis of gene regulation by the transcription factor PPARalpha between mouse and human." PLoS One **4**(8): e6796.

Reppert, S. M. and D. R. Weaver (2001). "Molecular analysis of mammalian circadian rhythms." Annu Rev Physiol **63**: 647-676.

Reppert, S. M. and D. R. Weaver (2002). "Coordination of circadian timing in mammals." Nature **418**(6901): 935-941.

Rey, G., F. Cesbron, et al. (2011). "Genome-wide and phase-specific DNA-binding rhythms of BMAL1 control circadian output functions in mouse liver." PLoS Biol **9**(2): e1000595.

Roach, P. J., A. A. Depaoli-Roach, et al. (2012). "Glycogen and its metabolism: some new developments and old themes." Biochem J **441**(3): 763-787.

Rodriguez-Zas, S. L., B. R. Southey, et al. (2012). "Microarray analysis of natural socially regulated plasticity in circadian rhythms of honey bees." J Biol Rhythms **27**(1): 12-24.

Rudic, R. D., P. McNamara, et al. (2004). "BMAL1 and CLOCK, two essential components of the circadian clock, are involved in glucose homeostasis." PLoS Biol **2**(11): e377.

Rutter, J., M. Reick, et al. (2002). "Metabolism and the control of circadian rhythms." Annu Rev Biochem **71**: 307-331.

Salgado-Delgado, R., A. Tapia Osorio, et al. (2011). "Disruption of circadian rhythms: a crucial factor in the etiology of depression." Depress Res Treat **2011**: 839743.

Shimba, S., T. Ogawa, et al. (2011). "Deficient of a clock gene, brain and muscle Arnt-like protein-1 (BMAL1), induces dyslipidemia and ectopic fat formation." PLoS One **6**(9): e25231.

Shimomura, K., V. Kumar, et al. (2013). "Usf1, a suppressor of the circadian Clock mutant, reveals the nature of the DNA-binding of the CLOCK:BMAL1 complex in mice." Elife **2**: e00426.

Siepka, S. M., S. H. Yoo, et al. (2007). "Circadian mutant Overtime reveals F-box protein FBXL3 regulation of cryptochrome and period gene expression." Cell **129**(5): 1011-1023.

Stirling, J. and P. O'Hare (2006). "CREB4, a transmembrane bZip transcription factor and potential new substrate for regulation and cleavage by S1P." Mol Biol Cell **17**(1): 413-426.

Stokkan, K. A., S. Yamazaki, et al. (2001). "Entrainment of the circadian clock in the liver by feeding." Science **291**(5503): 490-493.

Tong, X., M. Muchnik, et al. (2010). "Transcriptional repressor E4-binding protein 4 (E4BP4) regulates metabolic hormone fibroblast growth factor 21 (FGF21) during circadian cycles and feeding." J Biol Chem **285**(47): 36401-36409.

Turek, F. W., C. Joshu, et al. (2005). "Obesity and metabolic syndrome in circadian Clock mutant mice." Science **308**(5724): 1043-1045.

Ueda, H. R., W. Chen, et al. (2002). "A transcription factor response element for gene expression during circadian night." Nature **418**(6897): 534-539.

Ukai, H. and H. R. Ueda (2010). "Systems biology of mammalian circadian clocks." Annu Rev Physiol **72**: 579-603.

Willy, P. J., K. Umesono, et al. (1995). "LXR, a nuclear receptor that defines a distinct retinoid response pathway." Genes Dev **9**(9): 1033-1045.

Yang, X., M. Downes, et al. (2006). "Nuclear receptor expression links the circadian clock to metabolism." Cell **126**(4): 801-810.

Yoon, E. J. and K. Q. Hu (2006). "Hepatitis C virus (HCV) infection and hepatic steatosis." Int J Med Sci **3**(2): 53-56.

Zhang, C., G. Wang, et al. (2012). "Endoplasmic reticulum-tethered transcription factor cAMP responsive element-binding protein, hepatocyte specific, regulates hepatic lipogenesis, fatty acid oxidation, and lipolysis upon metabolic stress in mice." Hepatology **55**(4): 1070-1082.

Zhang, K., X. Shen, et al. (2006). "Endoplasmic reticulum stress activates cleavage of CREBH to induce a systemic inflammatory response." Cell **124**(3): 587-599.

Zheng, Z., X. Xu, et al. (2013). "Exposure to ambient particulate matter induces a NASH-like phenotype and impairs hepatic glucose metabolism in an animal model." J Hepatol **58**(1): 148-154.

ABSTRACT

CREBH, A NOVEL LIVER CLOCK KEEPER FOR ENERGY METABOLISM

by

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Circadian rhythms play crucial roles in orchestrating diverse physiological processes that are critical for health and disease. Cyclic AMP responsive element binding protein 3-like 3 (CREB3L3, also known as CREBH) is a liver-enriched, endoplasmic reticulum (ER)-tethered transcription factor known to regulate hepatic acute-phase response and energy homeostasis under stress conditions. Here, we demonstrate that CREBH is regulated by the circadian clock and functions as a diurnal regulator of hepatic lipid and glucose metabolism. CREBH is required to maintain circadian profiles of blood triglycerides, fatty acids, and glucose as well as hepatic glycogen storage. CREBH rhythmically regulates expression levels and amplitudes of the key genes involved in bi-directional metabolic pathways of both energy utilization and storage. CREBH regulates, and interacts with, the circadian transcriptional activators PPARα and C/EBPβ or the repressor E4BP4 to modulate CREBH transcriptional activities. CREBH deficiency leads to hyper-locomotion, increased

metabolic rates, and phase-shifted feeding behavior in mice. In summary, our studies reveal that CREBH functions as a liver metabolic regulator that integrates energy metabolism with circadian rhythm.

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9. **Zheng Z**, Zhang C, Zhang K. Role of unfolded protein response in lipogenesis. *World J Hepatol* 2010.
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