UNIVERSITY OF CALIFORNIA, SAN DIEGO

Regulation of Second Messenger Pathways by Cryptochrome

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biology

by

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2013

The Dissertation of Pagkapol Pongsawakul is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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2013

DEDICATION

To Mama, Watchera Pongsawakul;

Papa, Prasit Pongsawakul;

And A Gu, Suwathana Nimelaong

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ABSTRACT OF THE DISSERTATION

Regulation of Second Messenger Pathways by Cryptochrome

by

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Doctor of Philosophy in Biology

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Professor Susan S. Golden, Chair

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The circadian clock governs daily cellular, physiological, and behavioral rhythms to synchronize and anticipate the approximately 24-hour day/night cycle. In mammals, such biological processes include the sleep-wake cycle, feeding cycle, hormonal secretion, cellular metabolism, cell cycle, and gene expression. The molecular clock is driven by interactions of

interlocking transcription-translation feedback loops of activators and repressors. This includes Cryptochromes (CRY1 and CRY2) that function as potent repressors of the CLOCK and BMAL1 activators. In addition, CRYs also regulate hepatic gluconeogenesis, a process that maintains normal blood glucose levels during fasting by responding to glucagon-mediated stimulation of cAMP signaling. CRYs gate the glucagon response by directly interacting with the $G_s\alpha$ subunit of the G protein and reducing intracellular cAMP level. This led us to investigate the role of CRYs in the cytoplasm beyond their transcriptional function in the nucleus. Using molecular and pharmacological approaches, we found that cytoplasmic CRY inhibits cAMP signaling by interacting with $G_s\alpha$. We also observed a CRY inhibitory effect on $G_q\alpha$-mediated Ca^{2+} signaling by interacting with the $G_q\alpha$ subunit of the G protein. As cAMP and Ca^{2+} are regarded as integral cytosolic components that drive the transcriptional oscillator in the circadian master pacemaker, the suprachiasmatic nucleus, we provide reciprocal evidence of a core clock repressor that regulates cytosolic signals. Our study places CRY as a molecular link connecting the core transcription-translation oscillator and cytosolic cAMP and Ca^{2+} signaling.

Chapter 1: Introduction

1.1 Circadian Rhythms and the Molecular Oscillator

The approximately 24 hour biological rhythms, named circadian rhythms, are found ubiquitously in organisms from cyanobacteria to plants and humans (1). Circadian clocks have evolved to synchronize many biological processes from gene expression to complex behaviors with the environmental time cues such as light and temperature throughout the day-night cycle. By synchronizing biological processes with the daily cycle, circadian clocks provide an adaptive advantage to organisms such as enhancing fitness in cyanobacteria and increasing photosynthesis, growth, and survival in plants (2, 3). Daily rhythms in behaviors, physiology, and cellular processes are controlled by the molecular clock present in cells and tissues throughout organisms. In mammals, such rhythms include the sleep-wake cycle, feeding cycle, hormonal secretion, cellular metabolism, cell cycle, and gene expression (4-6).

1.1.1 Molecular Clocks Across Phyla

Since the first circadian clock gene *period* was identified by a genetic screen in fruit fly *Drosophila melanogaster*, many clock genes were identified in different model organisms throughout phylogeny (7). *Frequency* (*frq*), *kaiA-C*, *TIMING OF CAB EXPRESSION 1* (*TOC1*), and *Circadian locomotor output*

cycles kaput (*Clock*) were subsequently identified in filamentous fungus *Neurospora crassa,* cyanobacterium *Synechococcus elongatus,* plant *Arabidopsis thaliana,* and mouse *Mus musculus,* respectively (8). Although many of these clock genes are not evolutionarily conserved, organisms share negative feedback loop mechanisms for circadian timing (9, 10). In eukaryotes, positive elements (activators) turn on genes that are translated into negative elements (repressors), which, after a delayed interval, suppress the activation of their own genes. This transcriptional-translational negative feedback loop is a common hallmark of the eukaryotic circadian clocks. In addition, post-translational modifications of clock proteins such as phosphorylation, ubiquitination, and acetylation are important for regulation of protein stability and degradation, and necessary for maintaining proper circadian rhythms (11). Unlike eukayotes, cyanobacterial oscillations can occur without transcription. The circadian rhythm of *S. elongatus* KaiC phosphorylation can be reconstituted *in vitro* by mixing purified KaiA, KaiB, and KaiC proteins and ATP in absence of genes and transcripts (12).

Recent studies have shown that there are circadian rhythms in oxidation-reduction reactions that persist independently of the canonical transcription-translation feedback loops in eukaryotes. Reddy and colleagues illustrated circadian rhythms in oxidation-reduction of peroxiredoxins (PRX), antioxidant proteins, in mature human red blood cells (13). Their key evidence

showed that hyperoxidized state of peroxiredoxin (PRX-$SO_{2/3}$) oscillates in a circadian manner in anucleate human red blood cell samples. Additionally, circadian rhythms of PRX-$SO_{2/3}$ exist in a pico-eukaryote *Ostreococcus tauri* in constant darkness in the absence of light-driven transcription (14). Reddy, O'Neil, and colleagues later showed that oxidation-reduction cycles of peroxiredoxin exist through all forms of life: bacteria, archaea, and eukaryota (15).

1.2 Mammalian Circadian System

1.2.1 Mammalian Molecular Clock

The mammalian core transcriptional-translational negative feedback loop comprises basic helix-loop-helix (bHLH) – PAS (Period-Arnt-Single-minded) domain containing transcription factors CLOCK (or its paralogue neuronal PAS domain protein 2, NPAS2) and brain and muscle ARNT-like 1 (BMAL1 or ARNTL) as activators; and PERIODs (PER1, 2, and 3) and CRYPTOCHROMEs (CRY1 and 2) as repressors (1, 16). CLOCK and BMAL1 heterodimerize and activate transcription of genes that contain E/E'-box enhancer elements in their promoters, including those of *Per* and *Cry* genes. Once translated in the cytoplasm, PERs and CRYs accumulate, form complexes with CKIδ/ε, undergo post-translational modifications, and translocate back to the nucleus to repress their own transcription by inhibiting

CLOCK/BMAL1 activity (17, 18) (Fig. 1-1). When the PER/CRY complexes are degraded, the repression is relieved, and a new transcription cycle can be reinitiated (19). The delayed timing of the negative feedback loop from transcription to self-repression is crucial and believed to confer the approximately 24-hour period of the circadian clock (20).

In addition to the core loop, the mammalian circadian clock contains other interlocking loops. One of these loops involves the nuclear hormone receptors REV-ERBs (α and β) and RORs (α, β, and γ). Repressors REV-ERBs and activators RORs compete to bind retinoic acid-related orphan receptor response elements (ROREs) present in the promoters of *Clock* and *Bmal1* genes (21, 22). *Rev-erb* genes are also direct targets of CLOCK/BMAL1 mediated-transcription via E-box (Fig. 1-1). Recent evidence supports the necessity of either REV-ERBα or REV-ERBβ in regulating proper circadian behavior and metabolism (23, 24).

Another interlocking loop involved in the circadian regulation includes PAR bZip-family genes that encode for transcription factors DBP, TEF, HLF and E4BP4 (or NFIL3) (25). These transcription factors bind to D-boxes in the promoter or introns of many clock genes including *Per1, Per2, Per 3, Rev-erbα*, *Rev-erbβ, Rorα, Rorβ,* and *Cry1* (26) (Fig. 1-1). The combination of E/E'-box, RORE, and D-box is believed to confer proper phases of the circadian gene transcripts and contribute to the delay in feedback repression

through *Cry1* expression that is necessary for mammalian circadian clock function (20, 27).

1.2.2 Suprachiasmatic Nuclei as the Master Circadian Pacemaker

In mammals, the suprachiasmatic nucleus (SCN), located in the anterior hypothalamus, acts as the master circadian pacemaker that coordinates and synchronizes peripheral clocks throughout the body (28). Comprising approximately 20,000 neurons, SCN receives direct photic input from the retina via retino-hypothalamic tract (RHT) and, therefore, is able to synchronize with the external day/night cycle as well as adapt to the seasonal changes in day length (29). SCN neurons are coupled by neuronal connection via synapses and by neurotransmitters such as vasoactive intestinal polypeptide (VIP), arginine vasopressin (AVP), γ-aminobutyric acid (GABA), and gastrin-releasing peptide (GRP) (30). Dispersed SCN neurons are autonomous oscillators and can generate independent neuronal firing rhythms and PER2 rhythms with ranges of periods and phases (31, 32). Coupled SCN neurons, however, exhibit increased robustness of the neuronal firing and PER2 rhythms by consolidating phases and periods among individual neurons. In addition, the intercellular coupling also confers robustness against genetic perturbations in the absence of *Per1* or *Cry1* (32).

In the SCN, approximately 10% of total genomic transcripts cycle in a circadian manner in constant conditions. These cycling genes include many of

the clock genes such as *Per1, Per2, Bmal1, Cry1, Rev-erbα, Rev-*erb*β, Dbp,* and *E4bp4* (33, 34). Others include genes responsible for locomotor activity, peptide neurotransmitters, peptide synthesis and secretion, and redox and energy flux (33). At the proteome level, approximately 13% of SCN soluble proteins are clock-regulated. These cycling proteins are enriched in pathways involving metabolism, protein trafficking, and synaptic vesicle recycling (35).

1.2.3 Central and Peripheral Clocks

Not only are circadian clocks in the SCN and other brain regions, there are robust oscillators in different peripheral organs throughout the body (36, 37). In transgenic mice that contain a PERIOD2::LUCIFERASE fusion protein as a real-time bioluminescent reporter (*Per2*^{Luc} knockin mice), peripheral tissues such as cornea, liver, pituitary, kidney, and lung exhibit robust circadian rhythms of PER2::LUC in cultures for several weeks (38). In addition, individual fibroblasts can also act as autonomous oscillators that have diverse circadian phases and periods independently of the neighboring cells (39, 40).

At the genome level, approximately 10% of total mRNA in peripheral organs such as liver or heart also oscillates, and phases of the maximal expression are distributed throughout the day (33, 41). However, only a few dozen cycling genes (including clock genes) cycle in both liver and SCN, or liver and heart, suggesting specific circadian regulations in each clock tissue

and physiological significance of the peripheral clocks outside of the SCN (33, 34, 41).

At the proteome level, up to 20% of soluble proteins exhibit circadian oscillation in the liver (42). Surprisingly, almost half of the cycling proteins lack corresponding cycling transcripts, indicating post-transcriptional and translation regulations for the oscillation of circadian protein expressions (43, 44). At the metabolome level, many liver metabolites also undergo daily oscillations. Approximately 20% of known metabolites oscillate with 24-hour rhythmicity. These rhythmic metabolites are involved in major metabolic pathways such as synthesis of amino acids, lipids, and peptides; and xenobiotics (45). In *Clock*$^{-/-}$ liver, only one third of metabolites cycle compared to that of the wild-type liver, suggesting an influence of the circadian systemic cues in hepatic metabolomics oscillations (45, 46).

1.2.4 Transcriptional Architecture of the Core Circadian Clock

Using mouse liver as a model clock, Takahashi and colleagues have elucidated the molecular transcriptional architecture of the core circadian clock at the genome level (19). They performed time-specific chromatin immunoprecipitation sequencing (ChIP-seq) to identify DNA binding sites of core clock proteins CLOCK, NPAS2, BMAL1, PER1, PER2, CRY1, and CRY2 as well as RNA polymerase II and co-activators, CBP and p300, over a circadian day. Takahashi and colleagues found temporal dynamic controls of

circadian clock proteins in a stepwise manner over a circadian period: a transcriptional poised state, an activation state followed by a global peak of transcription, and the transcription repression state. At circadian time 0 (CT0, the start of subjective day) or a poised state, CRY1 is bound to its transcription targets and stalls the CLOCK/BMAL1 activation. At CT6-8, CRY1 occupancy declines causing derepression, and binding of CLOCK(NPAS2)/BMAL1 occurs to initiate transcription by recruitment of transcription co-activator p300. A few hours later, the global transcription peaks at CT15 followed by the repression state with maximal occupancies of PER1, PER2, CRY2, and CBP toward the end of the subjective night at CT16-20 (19). This study provided the first comprehensive evidence of dynamic regulations of the core clock proteins toward global circadian gene transcription.

1.3 Cryptochromes

CRYPTOCHROMEs (CRYs) are flavin-containing proteins that are found in prokaryotes, plants, and animals (47). They were first identified in *Arabidopsis thaliana* as blue-light photoreceptors important for growth and development (48). In *Drosophila*, CRY also functions as a blue-light photoreceptor in central clock neurons as well as a circadian repressor in peripheral tissues (49, 50). In mammals, CRY1 and 2 act as potent

repressors of CLOCK/BMAL1 and are critical components of the core clock loop, yet lack the photoreceptive functions (51, 52). Interestingly, monarch butterflies possess both *Drosophila*-like CRY1, which is photoreceptive, and mammal-like CRY2, which is a light-insensitive potent circadian repressor of CLK and CYC (the insect homologs of CLOCK and BMAL1, respectively) (53).

1.3.1 Cryptochrome/Photolyase Family

Photolyases are light-responsive DNA repair enzymes present in bacteria, archaea, and eukaryotes. Photolyase protein family can be categorized by its UV-damaged DNA targets of either (6-4) photoproducts or cyclobutane pyrimidine dimer (CPD) (47). CRYs are closely related to and share structural domain with the photolyase proteins (54). However, CRYs do not have DNA repair activity nor bind directly to the DNA. Furthermore, plant and animal CRYs have carboxy terminal domains extending from the conserved photolyase structure, suggesting that CRYs may have evolved from the photolyase ancestor (55).

The first CRY family member was identified in the *hy4* mutant of *Arabidopsis*. The seedlings of this mutant exhibited prolonged elongation growth under blue light (48). The distinct carboxy terminal domains (CTD) of *Arabidopsis* CRY1 and CRY2 are involved in light signaling, and their interaction with COP1, an E3 ubiquitin ligase, facilitates light dependent

proteasome-mediated degradation. *Arabidopsis* CRYs function not only in the entrainment of the circadian clock and photoperiodic responses, they also have diverse roles in plant development, hormone signaling, defense response, stress response, photosynthesis, and metabolism (56).

1.3.2 Non-mammalian Cryptochrome

Drosophila CRY (dCRY) is one of the most studied non-mammalian animal CRYs. It was first identified in cry^{baby} (cry^{b}) mutant, which is a point mutation for FAD-binding site. cry^{b} mutant flies cannot entrain to the light-dark cycle (49). In pacemaker neurons, dCRY functions as a blue-light photoreceptor (57). dCRY targets TIMELESS (TIM), a *Drosophila* core clock repressor, for proteasome-mediated degradation in a light-dependent manner to reset the clock (58). The light degradation processes of TIM and dCRY are mediated by an F-box protein JETLAG, a component of the E3 ubiquitin ligase complex (59). The CTD of dCRY is responsible for light-dependent TIM interaction, dCRY stability, and photosensitivity. dCRY also functions as a transcriptional repressor in fly peripheral tissues (50). Recent finding uncovered that dCRY mediates magnetosensitivity similar to that of butterflies (60, 61).

1.3.3 Mammalian Cryptochrome

Mammalian Cryptochrome genes were first identified by homology search of the photolyase-like genes. There are two Cryptochrome genes in

mice (*Cry1* and *Cry2*) and human (*CRY1* and *CRY2*). Mice lacking both *Cry1* and *Cry2* genes (*Cry1*$^{-/-}$;*Cry2*$^{-/-}$) exhibit arrhythmic locomotor activity under constant darkness (62, 63). Interestingly, mice lacking *Cry1* gene have short locomotor activity period while mice lacking *Cry2* gene have long locomotor activity period in constant darkness (62, 63). Recent evidence from chemical biology and mouse mutation analysis concurs that CRY1 is more effective period regulator than CRY2 and supports the notion that CRY2 is a less effective decelerator of the intrinsic clock, instead of a period accelerator of the clock as previously speculated (64, 65). Biochemically, CRY1 and CRY2 interact with PER proteins as well as CLOCK and BMAL1 (52, 66). CRYs function as potent transcription repressors of the E-box mediated CLOCK/BMAL1 activity. Mammalian Cryptochrome genes and proteins oscillate in SCN and many other peripheral tissues throughout the body (17, 63). This evidence established CRYs as core circadian clock components necessary for rhythmicity in mammals. However, unlike plant and fly CRY, mammalian CRYs are light-independent in their repressive activity and interactions with other clock proteins.

1.3.4 Cryptochrome and the Circadian Repressor Complex

Cell-based genetic screening of human *CLOCK* and *BMAL1* mutants that were insensitive to CRY repression revealed the significance of CRY repression for mammalian circadian clock function. The CRY-insensitive

CLOCK and *BMAL1* mutants lose PER/CRY interaction and do not sustain normal circadian rhythmicity when co-expressed with *PER2-Luciferase* reporter in NIH3T3 cells (67). The crystal structure of CLOCK/BMAL1 heterodimer supported this notion such that the CRY-insensitive *CLOCK* mutants map to the residues near the solvent-exposed β-sheet face of CLOCK PAS-B domain that is accessible for CRY interaction (68).

In the liver, CRYs and PERs form complexes in a time-specific manner and exhibit peak of nuclear abundance at CT15-CT22. Interestingly, CRY1 and CRY2 are more abundant in the cytoplasm throughout the circadian cycle, and the timing of CRYs nuclear translocation coincides with those of PERs (17). The physical interaction of PER/CRY complex with CLOCK/BMAL1 is believed to be necessary for the repression. However, detailed mechanisms of which CRYs (and PERs) inhibit E-box-mediated CLOCK/BMAL1 activity have been elusive (69, 70).

Employing mass spectrometry technique, Schibler and colleages have identified proteins in a large PER1-associated complex (approximately > 1 mega Dalton) including RNA-binding protein NONO and a histone methyltransferase complex subunit WDR5 (71). Using affinity purification and mass spectrometry in the mouse liver, Weitz and colleagues further identified common PER1- and PER2-associated proteins: PTB-associated splicing factor (PSF) and RNA-helicases, DEAD-box polypeptide 5 (DDX5) and

DEAH-box protein 9 (DHX9). Two possible repression mechanisms arose from the studies of these PER-associated proteins. First, PER/CRY complex may recruit the SIN3-HDAC complex, a negative regulator of transcription, by direct interaction with the corepressor recruiter PSF at the promoter regions (72). Supporting this notion, previous studied have also identified the interaction of CRY1 with SIN3B, HDAC1, and HDAC2 *in vivo* and *in vitro* (73). The second possible mechanism involves RNA helicases DDX5 and DHX9 that promote transcription termination of RNA polymerase II at the 3' end of the pre-mRNA targets (74).

1.3.5 Cryptochrome modifications, stability, and degradation

Post-translational modifications such as phosphorylation, acetylation, and sumoylation regulate nuclear/cytoplasmic localization, stability, and degradation of the core clock proteins. Mutations that affect the stability of the clock proteins alter expressions of other clock genes and proteins that result in shortening or lengthening of the endogenous circadian period. CRYs undergo post-translational modifications by different enzymatic protein partners leading to changes in subcellular localization or proteasome-mediated degradation (11). CKIε interacts with and phosphorylates CRY1 and CRY2 when forming the complex with PERs (75). Mitogen-activated protein kinase (MAPK) interacts with and phosphorylates CRY1 and CRY2 at residue Ser247 and Ser265, respectively. Phospho-mimic form of CRY1 (S247D) or

CRY2 (S265D) reduces MAPK-phosphorylation and exhibits attenuated repression of CLOCK/BMAL1 activity (76). Dual-specificity tyrosine-phosphorylated and regulated kinase 1A (DYRK1A) phosphorylates CRY2 at residue Ser557 and primes glycogen synthase kinase 3β (GSK3β) phosphorylation at residue Ser553. Phosphorylation of CRY2 at Ser553 and Ser557 leads to degradation in the cytoplasm and delayed nuclear accumulation (77).

Genetic screening in mice has identified regulators that target CRY for degradation. By screening *N*-ethyl-*N*-nitrosourea (ENU)-mutagenized mice that have altered periods of wheel-running activity, two mouse mutations *after hours* (*Afh*) and *overtime* (*Ovtm*) were identified and mapped to *Fbxl3* gene (78, 79). This gene encodes F-box protein FBXL3, a member of the E3 ubiquitin ligase complex. FBXL3 interacts with and targets CRY1 and CRY2 for ubiquitin-mediated proteasome degradation (78, 80). Adenosine monophosphate-activated protein kinase (AMPK) signals CRY1 for FBXL3-mediated degradation in the nucleus by phosphorylating CRY1 at Ser71 and Ser280 (81). Furthermore, another F-box protein FBXL21 was recently identified as a CRY regulator via forward genetics (an ENU-mutagenesis screening in mice) and reverse genetics (FBXL21-targeted knockout mice) (82, 83). Similar to FBXL3, FBXL21 also interacts with and ubiquitinates CRY1 and CRY2. Surprisingly, FBXL21 stabilizes CRYs in the cytoplasm

whereas FBXL3 degrades CRYs in the nucleus, suggesting antagonizing mechanisms that fine-tune CRY stability in different subcellular compartments (82, 83).

1.4 Circadian Clock Feedback Loops and the Metabolic Cycles

1.4.1 Circadian Clock and Cellular Metabolic Signals

Gene profiling experiments have shown that many critical rate-limiting enzymes in key metabolic pathways are under the control of clock or feeding (84). Not only are many metabolic processes under regulation of circadian clocks and feeding cycles, but circadian clocks can also be modulated directly and indirectly by metabolic signals and their regulators (85, 86). Such metabolic signals include nicotinamide adenine dinucleotide (NAD^+), adenosine triphosphate (ATP), and glucose.

NAD^+ production is under circadian clock control. Conversely, NAD^+ and NAD^+-dependent regulators can also regulate and reset the clock. A gene encoding the rate-limiting enzyme of the NAD^+ biosynthesis pathway, nicotinamide phophoribosyltransferase (NAMPT), is under E-box mediated-CLOCK/BMAL1 activation, thus controlling oscillation in *Nampt* expression and NAMPT activity (87, 88). On the other hand, the reduced form of NAD^+, NAD(P)/NAD(P)H can regulate the DNA binding of CLOCK/BMAL1 and NPAS2/BMAL1 (89). NAD^+-dependent enzymes SIRTUIN1 (SIRT1) and

poly(ADP-ribose) polymerase 1 (PARP-1) also regulate many of the core clock proteins. SIRT1 interacts with CLOCK/BMAL1, and rhythmically deacetylates BMAL1 and PER2 (90, 91). The deacetylation of PER2 reduces PER2 protein stability (90). PARP-1, which is driven by rhythmic feeding, binds and poly(ADP-ribosyl)ates CLOCK in a circadian manner. Poly(ADP-ribosyl)ation of CLOCK leads to reduction of CLOCK/BMAL1 DNA binding. Mice deficient in *Parp-1* have altered clock gene expression and circadian locomotor activity in restricted feeding, suggesting the role of PARP-1 in connecting feeding and the circadian clock (92).

Adenosine monophosphate-activated protein kinase (AMPK) is an ATP-depletion sensor in cells. It was shown in the liver and *in vitro* that AMPK phosphorylates CRY1, and that phosphorylation reduces PER2 interaction and increases FBXL3 interaction, leading to ubiquitin-mediated CRY degradation (78, 80, 81). AMPK also phosphorylates CK1ε. As a result, CK1ε enhances PER2 phosphorylation activity, leading to PER2 degradation (93). This evidence suggests that AMPK can regulate and reset clocks by sensing cellular energy.

Glucose down-regulates expression of *Per1*, *Per2,* and *Bmal1* as well as up-regulating many other transcription factors including transforming growth factor (TGF)-β-inducible early gene 1 (*Tieg1*) or krüppel-like factor 10 (*Klf10*). Upon glucose induction, TIEG1/KLF10 represses *Bmal1* expression

via GC boxes (94). *Tieg1/Klf10* expression is rhythmic and under clock control. Mice lacking *Tieg1/Klf10* are hyperglycemic during fasting and exhibit disruption in lipogenic gene expression (95). These data indicate that TIEG1/KLF10 is a circadian regulator that links glucose, the circadian clock, and liver metabolism.

1.4.2 Circadian Clock and Cytosolic Signals

Second messengers such as adenosine 3',5'-monophosphate (cAMP) and Ca^{2+} have been established as important entrainment signals for the SCN. Neuropeptide VIP primarily mediates SCN interneuronal communication by binding to $VPAC_2$ (VIP receptor 2, a G protein-coupled receptor (GPCR)), leading to activation of adenylyl cyclase (AC) for cAMP production via $G_s\alpha$, a G protein subunit. Mice deficient in VIP or $VPAC_2$ exhibit disrupted behavioral rhythms. Many of the SCN neurons of these mice still have rhythmic firing but lack synchrony among the neurons (96, 97). Other interneuronal signaling peptides in the SCN include GRP and AVP, and both signal through their respective GPCRs via $G_q\alpha$, phospholipase C and intracellular Ca^{2+} signaling (98).

Both cAMP and Ca^{2+} display rhythmic concentrations in the SCN, and the rhythms of cAMP and Ca^{2+} can drive rhythmic transcription of genes containing cAMP response element (CRE) in their promoters, including that of *Per1* and *Per2* (99). The photic input resets the clock by activating CRE-

binding protein (CREB) via cAMP, inducing transcription of *Per1* and *Per2* (100). The rhythms of cAMP and Ca^{2+} level precede the rhythm of CRE-reporter gene in the SCN, supporting the notion that cAMP/Ca^{2+} drives CRE rhythm of the SCN (101). Pharmacological perturbations of cAMP pathway in the SCN disrupt the circadian clock oscillator. Treatment with an AC inhibitor severely diminishes the rhythm of PER2::LUC rhythms in the SCN. An antagonist that slows down the rate of cAMP synthesis lengthens periods of PER2:LUC rhythms in the SCN slices of WT and *Clock* mutant mice, implying an integral role of cAMP for SCN rhythmicity (102). Furthermore, selective ligand that targets $G_q\alpha$-mediated Ca^{2+} signaling lengthens period as well as dampens amplitude of the PER2::LUC rhythms in the SCN (101). Conversely, SCN slices derived from the circadian mutant mice have altered cAMP and Ca^{2+} rhythms with periods altered in correlation with the circadian locomotor periods of the corresponding mutant mice. This study and others suggest that cAMP and Ca^{2+} are under circadian control (102, 103). cAMP and Ca^{2+} are not only regulated by the clock, but also important for entrainment and regulation of the clock itself, underlying a synergistic reinforcement of cytosolic signals and core transcription-translation feedback loops for robust circadian rhythmicity.

1.5 Circadian Regulation of CREB Target Genes?

Upon phosphorylation, CREB activates CRE-containing genes upon changes in intracellular cAMP in response to hormonal signals or neurotransmitters. In the liver, glucagon mediates cAMP-signaling dependent gluconeogenesis, a process that generates *de novo* glucose. Under fasting, a rhythmic behavior under circadian clock control, glucagon stimulation increases expression of two key gluconeogenic enzymes encoded by glucose-6-phosphatase (*G6pc*) and phophoenolpyruvate carboxykinase-1 (*Pck1*). Both *G6pc* and *Pck1* express in a rhythmic manner in mouse liver. Moreover, phophoenolpyruvate carboxykinase-1 also exhibits circadian rhythm in enzymatic activity (104). These data suggest that circadian clock mediates hepatic gluconeogenesis by rhythmically regulating gluconeogenic genes, and many of these genes are CREB targets. Comparing several genome-wide databases of clock-controlled and CREB targets, we observed a circadian variation in numbers of CREB targets that are clock-controlled. By comparing circadian phases of peaks of expression of the clock-controlled CREB targets in the liver, the number of peaking genes is highest at CT 14, and lowest at CT 22 (33, 34, 105). The lowest abundance of peaking CREB targets expression at CT 22 suggests that there might be a circadian inhibitory mechanism that regulates CRE-containing genes during the transition of fasting to feeding.

Previous reports have identified cAMP signaling as the pathway to reset the clock via phosphorylation of CREB in the SCN (100). In a photosensitive cell-based experiment, *Per2*^{Luc} fibroblasts were genetically engineered to express photopigment melanopsin. As a result, these transgenic melanopsin-expressing fibroblasts were photosensitive, similar to the intrinsically photosensitive retinal ganglion cells. By performing a light pulse at different circadian phases, Panda and colleagues observed a rhythmic binding of phosphorylated CREB at *Per2* promoter CRE site (106). This result suggested a mechanism by which the circadian clock gates cAMP signaling. With rhythmic expression of CREB target genes, including gluconeogenic genes *Pck1* and *G6pc* and the circadian gating of phosphorylated CREB activity, we aim to investigate the regulation of circadian clock in the cAMP-signaling pathway. Specifically, we would like to identify the component of the transcription-translation feedback loops that mediates rhythmic cAMP signaling. Because CREB regulates many metabolic pathways in diverse tissues, elucidating the interaction of circadian clock and cAMP signaling may provide another level of regulation for circadian control in metabolism.

1.6 Figure

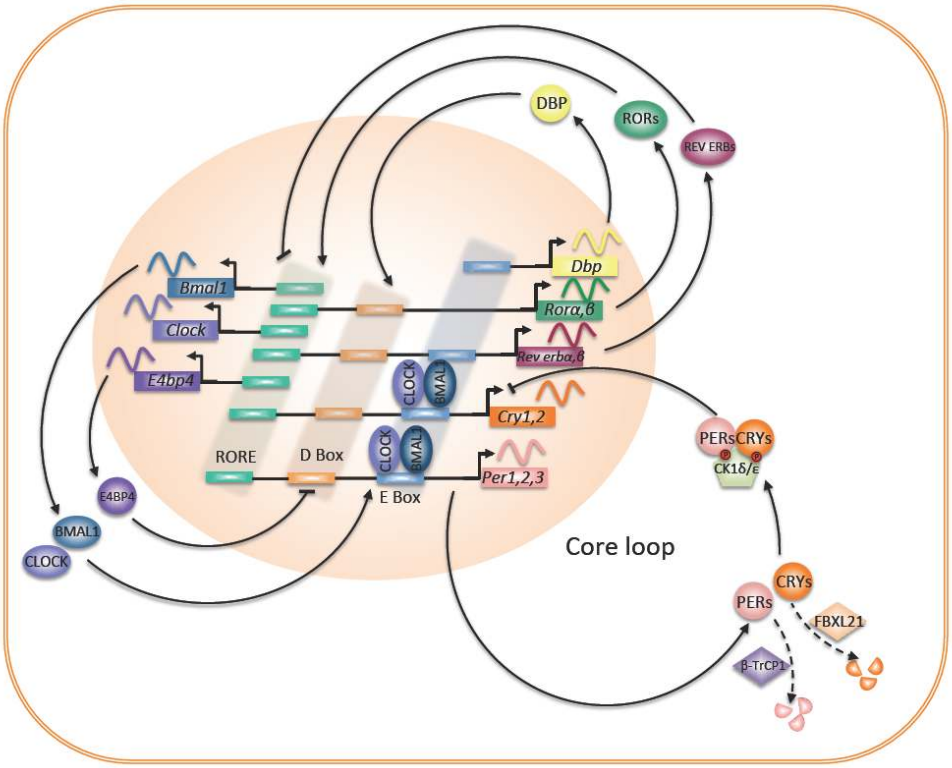

Figure 1-1. The interlocking transcription/translation feedback loop of the mammalian circadian clock

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Chapter 2:

Cryptochrome Mediates Circadian Regulation of cAMP Signaling and Hepatic Gluconeogenesis

2.1 Abstract

During fasting, mammals maintain normal glucose homeostasis by stimulating hepatic gluconeogenesis (1). Elevations in circulating glucagon and epinephrine, two hormones that activate hepatic gluconeogenesis, trigger the cAMP-mediated phosphorylation of cAMP response element-binding protein (Creb) and dephosphorylation of the Creb-regulated transcription coactivator-2 (Crtc2) - two key transcriptional regulators of this process (2). Although the underlying mechanism is unclear, hepatic gluconeogenesis is also regulated by the circadian clock, which coordinates glucose metabolism with changes in the external environment (3-6). Circadian control of gene expression is achieved by two transcriptional activators, Clock and Bmal1, which stimulate cryptochrome (Cry1 and Cry2) and Period (Per1, Per2 and Per3) repressors that feed back on Clock-Bmal1 activity. Here we show that Creb activity during fasting is modulated by Cry1 and Cry2, which are rhythmically expressed in the liver. *Cry1* expression was elevated during the night-day transition, when it reduced fasting gluconeogenic gene expression

by blocking glucagon-mediated increases in intracellular cAMP concentrations and in the protein kinase A-mediated phosphorylation of Creb. In biochemical reconstitution studies, we found that Cry1 inhibited accumulation of cAMP in response to G protein-coupled receptor (GPCR) activation but not to forskolin, a direct activator of adenyl cyclase. Cry proteins seemed to modulate GPCR activity directly through interaction with $G_s\alpha$. As hepatic overexpression of *Cry1* lowered blood glucose concentrations and improved insulin sensitivity in insulin-resistant *db/db* mice, our results suggest that compounds that enhance cryptochrome activity may provide therapeutic benefit to individuals with type 2 diabetes.

2.2 Results

We used an adenovirally encoded reporter, in which a cAMP response element (CRE) drives the expression of luciferase (CRE-luc), to evaluate the rhythmicity of hepatic Creb activity under fasting conditions (7). To control for potential variations in pancreatic glucagon secretion among the different mice in our experimental population, we examined effects of intraperitoneal (i.p.) glucagon administration at different times of the day. I.p. glucagon administration increased the adenoviral CRE-luc reporter activity 40-fold better in mice fasted at Zeitgeber time 10–13 (ZT10-13, the day-to-night transition) than at ZT22-1, corresponding to the night-to-day transition (Fig. 2-

1a and Supplementary Fig. 2-1). Consistent with a rhythmic oscillation in Creb activity, glucagon re-injection to the same mice fasted at ZT6-9, near the day-to-night transition, caused a robust induction in hepatic CRE-luc reporter activity (Supplementary Fig. 2-1). These results suggest that the amplitude of hepatic Creb activation by fasting is gated in a circadian fashion.

On the basis of the changes in fasting CRE-luc reporter activity, we hypothesized that the circadian clock modulates the Creb- and Crtc2-mediated effects on the gluconeogenic gene expression program. Supporting this idea, glucagon administration increased the gene expression of two key gluconeogenic factors (glucose-6-phosphatase (*G6pc*) and phosphoenolpyruvate carboxykinase-1 (*Pck1*)) to a greater extent in mice fasted at ZT10-13 (Fig. 2-1b). Consistently, i.p. glucagon administration increased hepatic Creb phosphorylation and Crtc2 dephosphorylation robustly in ZT10-13 fasted mice, but the increase was smaller in in ZT22-1 fasted mice (Fig. 2-1c). Together, these results indicate that the clock modulates fasting gluconeogenesis in part by regulating Creb and Crtc2 activities, as indicated by changes in their phosphorylation statuses. These observations are consistent with recent reports showing that the phosphorylation of Creb is rhythmic *in vivo* (8).

Owing to the rhythmic Creb phosphorylation, we considered that the liver clock may exert global effects on the expression of cAMP-inducible

genes. In line with this idea, we identified 194 Creb targets among a set of 652 clock-controlled genes (CCGs) in published microarray databases of mouse liver (9-11). To examine the relationship between Creb signaling and clock regulation, we plotted the distribution of the Creb-targeted CCGs according to the circadian timepoints of their peak expression. We found that not only the absolute number of Creb-targeted CCGs but also the ratio (versus the numbers of CCGs at the given circadian timepoints) showed a clear correlation at circadian time 22 (CT22) (Supplementary Fig. 2-2) with the reduced levels of phosphorylated Creb that we observed at ZT22-1 (Fig. 2-1c). These rhythmic patterns generally coincided with the trough phase of *Per2* (Supplementary Fig. 2-2), a clock gene whose temporal expression reflects the endogenous clock activities, suggesting a common inhibitory mechanism between the Creb signaling and clock regulation at CT22.

We then evaluated the extent to which the rhythmic regulation of Creb activity is cell autonomous. Exposure to vasoactive intestinal peptide (VIP), a GPCR ligand, increased CRE-luc activity in mouse adult fibroblasts harboring the receptor Vipr2 (12) (Fig. 2-2). After synchronizing the cells by serum shock, we exposed them to VIP at specific times and found CRE-luc reporter activation that showed a rhythmic pattern, with peak (24 and 48 h) and trough (36–39 h) activities coinciding with those for a *Per2* promoter-driven luciferase (Per2-luc) reporter (Fig. 2-2a). These results show the regulation of Creb

pathway induction by a cell-autonomous circadian clock and are reminiscent of the circadian gating seen in the light signaling induction of a Per2::luc knockin reporter in mouse fibroblasts containing melanopsin (a light sensor GPCR) (13).

Because CRE-luc activity in liver and in synchronized fibroblasts is lowest when Per-Cry activity is highest (ZT22), we tested the potential role of Crys in regulating Creb signaling. Overexpression of *Cry1* or *Cry2* in mouse fibroblasts ectopically expressingVipr2 and CRE-luc (designated Vipr2-CRE-luc cells) or HEK293T cells attenuated CRE-luc activity in cells exposed to VIP (Fig. 2-2b-d), whereas overexpression of *Per1* did not show a significant effect (Fig. 2-2c). The repression was dose dependent (Fig. 2-2d). Consistent with the absence of E-boxes, known to be inhibited by Crys through Clock-Bmal1, within the CRE-luc reporter, Clock-Bmal1 did not activate CRE-luc expression, suggesting that the inhibitory effect of Crys on CRE-luc reporter is irrelevant to Clock-Bmal1-mediated transcriptional regulation (Supplementary Fig. 2-3) (10). Furthermore, *Cry1* or *Cry2* overexpression reduced the effects of urocortin-3, another GPCR ligand, on CRE-luc activity in cells expressing its receptor corticotropin-releasing factor receptor-2β (14) (Supplementary Fig. 2-4). Thus, these data indicate that Crys may exert more general effects on GPCR-mediated increases in Creb- and Crtc2-dependent transcription.

Given the inhibitory effects of Cry proteins on multiple GPCR signaling

in fibroblasts and HEK293T cells, it may also modulate the effects of glucagon on liver gene expression through the glucagon receptor, another GPCR. Supporting this notion, hepatic expression of adenovirally encoded Cry1 (Ad-Cry1) reduced glucagon-induced CRE-luc activity in liver of fasted mice at ZT10–13, when endogenous *Cry1* expression is low (Fig. 2-3a). Moreover, Ad-Cry1 also downregulated gluconeogenic gene expression (*G6pc* and *Pck1*) in fasted mice given glucagon i.p. (Fig. 2-3b). Consequently, Ad-Cry1-expressing mice showed lower circulating blood glucose concentrations compared to Ad-GFP-expressing controls (Fig. 2-3c).

We tested whether depletion of Cry, either by targeted disruption of the *Cry1* and *Cry2* genes (15) or by RNAi-mediated knockdown in the liver *in vivo*, was sufficient to increase Creb activity. By contrast with the circadian oscillation of hepatic CRE-luc induction in wild-type mice, glucagon-stimulated CRE-luc activity was constitutively elevated in $Cry1^{-/-};Cry2^{-/-}$ mice (Supplementary Fig. 2-5). Similarly, RNAi-mediated depletion of hepatic *Cry1* and *Cry2* increased glucagon-stimulated CRE-luc activity at ZT1 relative to control mice expressing nonspecific shRNA (USi) (Fig. 2-3d). As a result, hepatic mRNA levels for gluconeogenic genes (*G6pc* and *Pck1*) were higher in mice with knocked down *Cry1* and *Cry2* expression compared to controls (Fig. 2-3e). Circulating glucose concentrations were elevated after *Cry1* and *Cry2* depletion (Supplementary Fig. 2-6), suggesting that circadian changes in

hepatic *Cry* expression are sufficient to modulate the gluconeogenic genetic program, in part through their inhibitory effects on the Creb-Crtc2 pathway. Indeed, knockdown of *Cry1* and *Cry2* also increased glucose production in primary hepatocytes after exposure to glucagon, indicating that Cry effects on hepatic glucose metabolism are probably cell autonomous (Fig. 2-3f).

We also wondered whether Cry would similarly modulate circulating glucose concentrations in the setting of insulin resistance. Supporting this idea, adenoviral expression of *Cry1* reduced fasting blood glucose concentrations and improved whole-body insulin sensitivity in insulin-resistant *db/db* mice, as measured by glucose tolerance testing (Fig. 2-3g). Pointing to a functional effect on hepatic gluconeogenesis, *Cry1* overexpression also lowered glucose excursions in pyruvate tolerance–testing studies (Fig. 2-3h).

Given the rhythmic changes in hepatic Creb phosphorylation and that glucagon mediates its effects in the liver by increasing intracellular cAMP abundance and thus activating Creb, we suspected that during fasting Cry1 may disrupt the effects of glucagon on adenyl cyclase activation. Supporting this idea, i.p. administration of glucagon increased hepatic cAMP concentrations at ZT10-13, when *Cry1* expression is low, and to a much lesser extent, at ZT22-1 when *Cry1* levels are elevated (Fig. 2-4a). Furthermore, we consistently found that Ad-Cry1 expression markedly reduced hepatic cAMP concentrations in ZT10-13 fasted mice exposed to

glucagon (Fig. 2-4b). Indeed, the amount of phosphorylated Creb was lower in livers of fasted Ad-Cry1 mice compared to Ad-GFP control mice (Fig. 2-4c).

We evaluated the effects of Cry proteins on Creb-Crtc2 signaling in cultures of primary hepatocytes. Similar to its effects in liver *in vivo*, Ad-Cry1 also downregulated CRE-luc reporter activity, as well as gluconeogenic gene expression in cultured cells exposed to glucagon (Supplementary Fig. 2-7a,b). By contrast with its effect in cells exposed to glucagon, Ad-Cry1 did not reduce gluconeogenic gene expression or CRE-luc reporter activity in cells treated with forskolin, a direct activator of adenyl cyclase. Consistent with these results, Ad-Cry1 expression reduced cAMP accumulation in cells exposed to glucagon but not to forskolin (Fig. 2-4d). In addition, RNAi-mediated knockdown of *Cry1* and *Cry2* increased cAMP content in primary hepatocytes exposed to glucagon but not to forskolin (Supplementary Fig. 2-7c). Pointing to a more general effect of this repressor on GPCR signaling, *Cry1* or *Cry2* overexpression also reduced cAMP accumulation in response to VIP treatment in fibroblasts expressing *Vipr2* (Supplementary Fig. 2-8). Together, these results indicate that Cry1 acts upstream of adenyl cyclase to regulate hepatic Creb activity during fasting.

To evaluate further the mechanism by which Cry proteins inhibit cAMP signaling, we performed *in vitro* reconstitution assays. Addition of isoproterenol to plasma membranes from HEK293T cells, which express β-

adrenergic receptors, increased cAMP accumulation *in vitro* (Fig. 2-4e,f). Co-incubation of plasma membrane fractions with cytoplasmic extracts from transfected HEK293T cells expressing *Cry1* potently inhibited cAMP increases in response to isoproterenol relative to control (GFP) extracts (Fig. 2-4e). We considered that Cry1 could disrupt cAMP accumulation either directly by binding to relevant signaling components or indirectly by stimulating the expression of an intracellular inhibitor. Supporting the former hypothesis, immunodepletion of hemagglutinin (HA)-tagged Cry1 with HA-specific antiserum restored cAMP accumulation in response to isoproterenol, whereas control IgG had no effect (Fig. 2-4f). Taken together, these results demonstrate that Cry1 inhibits activation of the Creb-Crtc2 pathway in liver by disrupting cAMP production in response to ligand-dependent activation of G_s-coupled receptors.

Given the ability of Cry to disrupt GPCR-initiated, but not forskolin-mediated, increases in cAMP production, Cry would be predicted to associate with either GPCRs or with the Gsα subunit of heterotrimeric G protein. Although it did not interact detectably with the GPCR family member Vipr2, Cry associated with G_sα in pull-down assays of membrane fractions from transfected HEK293T cells incubated with recombinant GST-Cry protein (Fig. 2-4g). We also observed the Cry-G_sα interaction in reciprocal coimmunoprecipitation assays of HEK293T cells and primary hepatocytes

(Fig. 2-4h). Together, these data show a role for extranuclear Cry in regulating cAMP production.

2.3 Discussion

The circadian clock has been known to modulate hepatic gluconeogenesis for over 20 years, although the underlying mechanism has remained unclear. We found that the liver clock regulates the gluconeogenic genetic program through Cry-mediated inhibition of Creb activity during fasting. Historically regarded as a transcriptional repressor, Cry seemed to inhibit gluconeogenic gene expression primarily by blocking GPCR-mediated increases in cAMP and the subsequent phosphorylation of Creb (Fig. 2-4i). These results are consistent with recent findings that hepatic Creb phosphorylation oscillates in a circadian fashion (8). Similar to our observations in liver, cAMP concentrations have also been found to oscillate in the clock master organ, the suprachiasmatic nucleus, reaching a nadir when Cry expression is highest (16). Future studies should provide further insight in to the detailed mechanism by which Cry modulates G_s effects on cAMP in liver and other tissues.

2.4 Methods

2.4.1 Adenoviruses and Mice

We intravenously delivered adenoviruses (1 x 10^8 plaque-forming units (PFU)) Cry1, control GFP, shRNA Cry1i, shRNA Cry2i or control shRNA USi, together with CRE-luc (1 x 10^8 PFU) and RSVβ-gal (5 x 10^7 PFU) into 8- to 10-week-old male C57BL/6 J-Tyrc-2J/J mice (Jackson Laboratories), which are virtually wild-type C57BL/6J except that their pigment is completely absent from skin, hair and eyes. The targeted sequences for knockdown were 5′-GGAAATTGCTCTCAAGGAAGT-3′ (Cry1) and 5′-GCTGAATTCGCGTCTGTTTGT-3′ (Cry2). All mice were housed in colony cages with 12-h light-dark cycle for 4 weeks before study. All mouse works were conducted under the regulations of the Institutional Animal Care and Use Committee at the Salk Institute, the Genomics Institute of the Novartis Research Foundation and Chinese Academy of Sciences.

2.4.2 *In vivo* Imaging

We fasted mice beginning at ZT10 or ZT22 and injected them i.p. with glucagon (100 mg per kg body weight; Sigma) at ZT13 or ZT1, respectively, and continued fasting for 1 h. At the end of fasting (ZT14 or ZT2), we injected them i.p. with Nembutal (Abbott Laboratories) at 50 mg per kg body weight and sterile firefly d-luciferin (Biosynth AG) at 100 mg per kg body weight, and imaged them on the IVIS-100 Imaging System within 15-45 min of injection of luciferin. We analyzed the images with Living Image software (Xenogen) (7, 17).

2.4.3 *In vivo* Analysis

We sonicated mouse tissues at 4 °C, centrifuged them and reserved the supernatants for β-galactosidase activity, protein determinations and immunoblotting analysis. Blood glucose values were determined with a LifeScan automatic glucometer.

2.4.4 Cell Culture

We cultured cells in DMEM with 10% FBS and transfected them with plasmid DNA by using Lipofectamine 2000 (Invitrogen). Primary hepatocytes were isolated and cultured as previously described (7).

2.4.5 Luminometery and Luciferase Assay

We grew cells harboring Vipr2 and CRE-luc reporter to confluence in 35-mm dishes, followed by a 2-h serum shock and release into serum-free medium. We applied synthetic VIP (2 nM in saline, American Peptide) to dishes 15-48 h after serum shock at 3-h intervals. We collected data in a luminometer (Actimetrics). The luciferase reporter assay of HEK293T cells has been previously described (18).

2.4.6 mRNA Analysis

We extracted total RNA from whole liver or primary hepatocytes with the RNeasy kit (Qiagen) and measured mRNA levels as previously reported (7).

2.4.7 Glucose Production Assay

We prepared primary hepatocytes as previously described (17). Briefly, we seeded 5 x 10^5 cells per well in a six-well dish with M199 medium (Invitrogen). Adenoviruses infected (multiplicity of infection of 5 for each Cry-specific shRNA and 10 for control USi) for 120 min before additional incubation for 42–45 h. We treated cells with glucagon (10 μg ml^{-1}, Sigma) for 5 h, washed them and incubated them with glucose production buffer (saline supplemented with 20 mM sodium lactate and 2 mM sodium pyruvate) for an additional 2 h. We collected the supernatant for measuring glucose concentration (BioVision) and normalized the readings with the whole-cell protein amount.

2.4.8 Glucose Tolerance Tests

We fasted mice overnight and injected them i.p. with glucose (1 g per kg body weight, Sigma). We measured blood glucose concentrations before the injection and at 15, 30, 45, 60 and 90 min after injection.

2.4.9 Pyruvate Tolerance Tests

We fasted mice for 24 h and injected them i.p. with sodium pyruvate (2 g per kg body weight, Sigma). We measured blood glucose before the injection and at 20, 40, 60 and 100 min after injection.

2.4.10 Cell Fractionation

We collected and washed HEK293T cells and resuspended them in lysis buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA and proteinase inhibitors

(Roche)) and centrifuged at 500*g* for 5 min. Supernatants were ultracentrifuged at 40,000*g* for 30 min. The resulting supernatant was allocated as the cytosol fraction, and the pellet was resuspended in extraction buffer (75 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10% glycerol, 12.5 mM $MgCl_2$ and proteinase inhibitors) as the membrane fraction. We measured protein concentrations, adjusted to 1 μg μl^{-1} and stored them in –80 °C freezer.

2.4.11 Adenyl Cyclase Activity Assay.

We mixed equal volumes of membrane and cytosol fractions, incubated them at 37 °C for 5 min, and then mixed with one volume reaction buffer (5 mM Tris-HCl, pH 7.4, 17.5 mM MgCl2, 120 μM ATP, 3 μM GTP, 1.5 μM FAD, 60 mM phosphocreatine and 0.375 U creatine phosphokinase (Sigma)) in the presence or absence of isoproterenol (100 μM) or forskolin (1 μM) (Sigma) for reaction at 37 °C for 30 min, stopped by mixing with HCl and neutralized by NaOH. We measured cAMP levels by ELISA (R&D). For the preclearance of HA-Cry1, cytosol fractions from Ad-HA-Cry1 infected cells were immunoprecipitated by HA-specific antibody or mouse IgG beads at 4 °C for 1 h.

2.4.12 GST Pull-down.

We prepared GST-Cry1 and control GST-GFP proteins with GST beads (GE Healthcare) as previously described (19). We dissolved membrane pellets in pull-down buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA,

150 mM NaCl, 2mM dithiothreitol and proteinase inhibitors) and mixed with 10 μg GST-tagged proteins. After 2 h incubation at 4 °C and washes, we suspended pellets in SDS loading buffer and applied 1% input samples for quantification.

2.4.13 Immunoprecipitation

We conducted immunoprecipitation assays with whole-cell lysates.

2.4.14 Statistical Analyses.

We performed all studies on at least three independent occasions. We reported results as means ± s.e.m. and considered differences statistically significant when $P < 0.05$.

2.5 Supplementary Detailed Methods

2.5.1 Cell Culture

HEK293T cells were cultured in DMEM with 10% FBS and transfected with plasmids by using lipofectamine2000. Mouse primary hepatocytes were isolated and cultured as previously described. For reporter studies, Ad CRE:Luc and RSVβ-gal infected hepatocytes (1 pfu virus^{-1} cell^{-1}) were exposed to GLU (100 nM) for 4–6 h. For cAMP studies, Lv or Ad-CRY1, CRY2 or GFP infected hepatocytes (1 pfu virus^{-1} cell^{-1}) were exposed to GLU (100 nM) or FSK (10 μM, Sigma) for 5 min without 3-isobutyl-1-methylxanthine (IBMX) pretreatment or 30 min with half hour IBMX (500 μM)

pre-treatment, intracellular cAMP levels were determined by cAMP Elisa kit (Roche) and normalized to protein concentration.

2.5.2 Glucose Production Assay

Primary hepatocytes were prepared and cultured as described previously (20). Briefly, liver from fed mice (8–12 week-old C57Bl/6, male) was perfused with Hank's balanced salt solution (HBSS), followed by treatment with 0.02% collagenase. Cell viability was higher than 70% as assessed by the trypan blue exclusion test. Cells were seeded as 5 x 10^5 per well in a 6-well dish in M199 Medium (Invitrogen) supplemented with 10% fetal bovine serum. After a 6 h attachment, adenoviruses were applied in 1ml fresh M199 medium at multiplicity of infection (M.O.I) of 5 for each CRYi and 10 for control USi for 120 min; replaced with 2ml fresh M199 medium supplemented with penicillin (100 units ml–1), streptomycin (100 μg ml–1), and glutamine (0.29 mM). After incubation for 42–45 h, cells were treated with glucagon (10 μg ml^{-1}, Sigma) for 5 h, gently washed 3 times with PBS, followed by incubation with glucose production buffer (PBS supplemented with 20 mM sodium lactate and 2 mM sodium pyruvate) for additional 2 h. Supernatant was collected for measuring glucose concentration using a fluorometric assay (BioVision). The readings were normalized with total protein in whole-cell lysates.

2.5.3 Cell Fractionation

HEK293T cells were collected, washed twice with PBS, resuspended in 1 ml lysis buffer (10 mM Tris-HCl, pH 7.4, 1mM EDTA, 100 μM PMSF and proteinase inhibitors), passed through 27-G needles 6 times, and then centrifuged at 500 x *g* for 5 min. The pellets were re-extracted with 1 ml lysis buffer three more times and the combined supernatants were ultracentrifuged at 40,000 x *g* for 30 min. The resulting supernatant was allocated as cytosol fraction and the pellet was resuspended in extraction buffer (75 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10% glycerol and 12.5 mM MgCl2, 100 μM PMSF and proteinase inhibitors) as membrane fraction. All the above procedures were carried either on ice or under 4 °C. Protein concentrations of both fractions were measured by Bio-Rad protein assay kit, adjusted to 1 μg μl^{-1} and kept in -80 °C freezer.

2.5.4 *In vitro* Adenyl Cyclase Activity Assay

Cytosol fraction with GFP or HA-CRY1 protein was prepared from HEK293T cells infected with Ad-GFP or Ad-CRY1 adenovirus, respectively by following the above protocol. For AC activity assay, equal volume of membrane and cytosol fractions (25 μl + 25 μl) were mixed, incubated at 37 °C for 5 min, and then mixed with 25 μl AC reaction buffer (5 mM Tris-HCl, pH 7.4, 17.5 mM MgCl2, 120 μM ATP, 3 μM GTP, 1.5 μM FAD, freshly adding 60 mM phosphocreatine and 0.375 Unit Creatine phosphokinase) in

the presence or absence of isoproterenol (100 μM final) or FSK (1 μM final). Reactions were carried at 37°C for 30 min, stopped by mixing with 20 μl 2.2M HCl, and then neutralized by 20 μl 2.2M NaOH. Levels of cAMP were measured by ELISA kit (R&D) by following its manual. To exclude the possibility that isoproterenol or FSK interrupts cAMP ELISA assay, isoproterenol (100 μM final) or FSK (1 μM final) were added in the mixture of lysis, extraction, and reaction buffers alone for cAMP assay. For the pre-clearance of HA-CRY1, cytosol fractions from Ad-HA-CRY1 infected HEK293T cells were immunoprecipitated by anti-HA or mouse IgG beads at 4 °C for 1 hour.

2.5.5 GST Pull-down

GST-CRY1 and control GST-GFP proteins were prepared as described (19). Briefly, bacteria BL21 AI (Invitrogen) harboring either construct DNA were grown till O.D.600 = 0.7-1.0, subsequently induced by Isopropyl β-D-1-thiogalacto-pyranoside (IPTG, 1mM) and Arabinose (0.2% w:v) for 5 h at room temperature with 250 rpm shaking. After sonication, cell lysates were incubated with Glutathione-Sepharose beads (GE Healthcare) for purification. For the pull-down assay, 1 x 10^7 293T cells were prepared for membrane fractionation as described above. Membrane pellets were dissolved in pull-down buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 2mM Dithiothreitol (DTT), 100 μM PMSF and proteinase inhibitors), and mixed with

10 μg GST proteins as determined by Coomassie Brilliant Blue staining. After 2 h incubation at 4 °C and washing 5 times with pull-down buffer, pellets were suspended in SDS loading buffer. For each loading, 1% input samples were applied for quantification.

2.5.6 Statistical Analyses

All studies were performed on at least three independent occasions. Results are reported as mean and s.e.m. Except for described, the comparison of different groups was carried out using a two-tailed unpaired Student's *t*-test. Differences were considered statistically significant at $P < 0.05$.

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2.7 Figures and Supplementary Figures

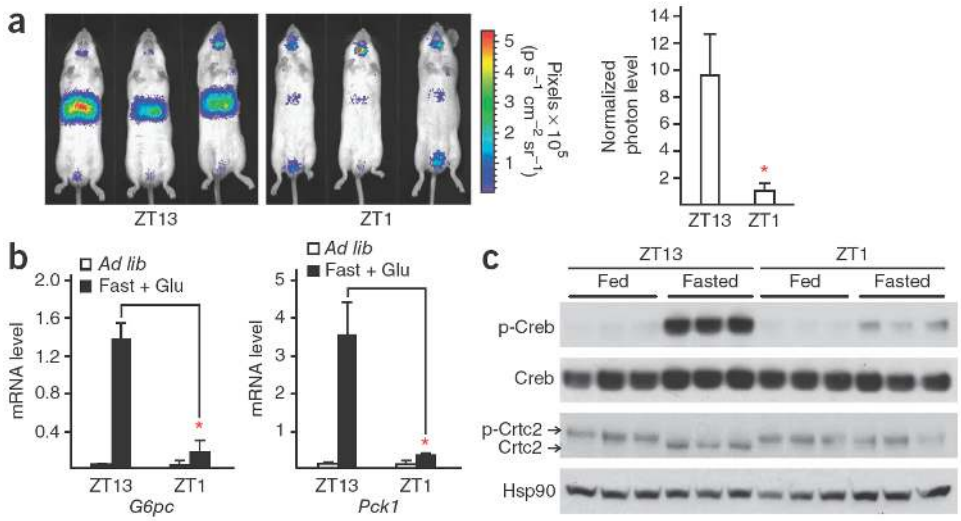


Figure 2-1. Hepatic Creb-Crtc2 activity is modulated by the circadian clock. (a) Left, CRE-luc reporter activity in mice fasted for 3 h followed by i.p. glucagon administration. The relative effect of fasting at ZT10-13 (ZT13) and ZT22-1 (ZT1) is indicated. Right, bar graph showing CRE-luc activity from hepatic lysates normalized to β-galactosidase activity from co-infected Ad-RSV-β-gal adenovirus. **P* < 0.01, *n* = 5. (b) Quantitative PCR analysis of gluconeogenic gene expression in mice fasted at ZT10–13 or ZT22-1 and then injected i.p. with glucagon (Glu). **P* < 0.01, *n* = 5. (c) Immunoblot of Creb and Crtc2 proteins in liver extracts from mice fasted ZT10-13 (ZT13) or ZT22-1 (ZT1) followed by i.p. injection with glucagon. The relative effects ZT13 and ZT1 fasting on hepatic amounts of phospho-Crtc2 (p-Crtc2) and phospho-Creb (Ser133; p-Creb) are also shown. Dephosphorylated Crtc2 (active) runs faster than the p-Crtc2 (inactive) in ZT13 fasting mice. The loading control is heat shock protein 90 (Hsp90). Data represent means ± s.e.m.

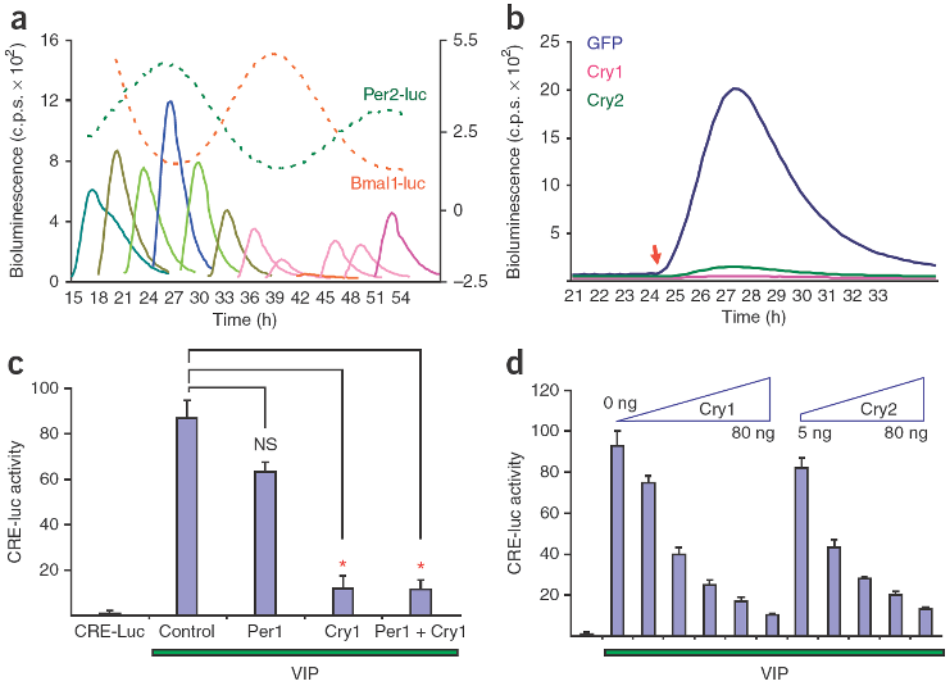


Figure 2-2. Cry inhibits Creb activity. (a) CRE-luc reporter activity in Vipr2-expressing mouse fibroblasts synchronized by serum shock and then exposed to VIP at indicated times. Replicate samples (a total of 24) received VIP treatment every 3 h after synchronization from 15–48 h. For clarity, one responsive curve per time point is shown in a different color. Mouse fibroblasts harboring Per2-luc or Bmal1-luc reporters were used to report the two distinct phases of clock gene expression (scale at right); units are the same as at left. Data are representative of three experiments. c.p.s., count per second. (b) Effect of Cry overexpression on CRE-luc reporter activity. Vipr2–CRE-luc cells were infected with lentiviral particles expressing either GFP, Cry1 or Cry2. Cells were treated with VIP 24 h after medium change (red arrow). (c,d) CRE-luc activity in transfected cells expressing Vipr2. CRE-luc and Vipr2 expression plasmids were co-transfected into HEK 293T cells along with Per1, Cry1 or Cry2 as indicated. Twenty-four hours after transfection, cells were treated with VIP to induce CRE-luc activity. NS, not significant. $^{*}P < 0.01$, Student's *t* test. Data represent means ± s.e.m.

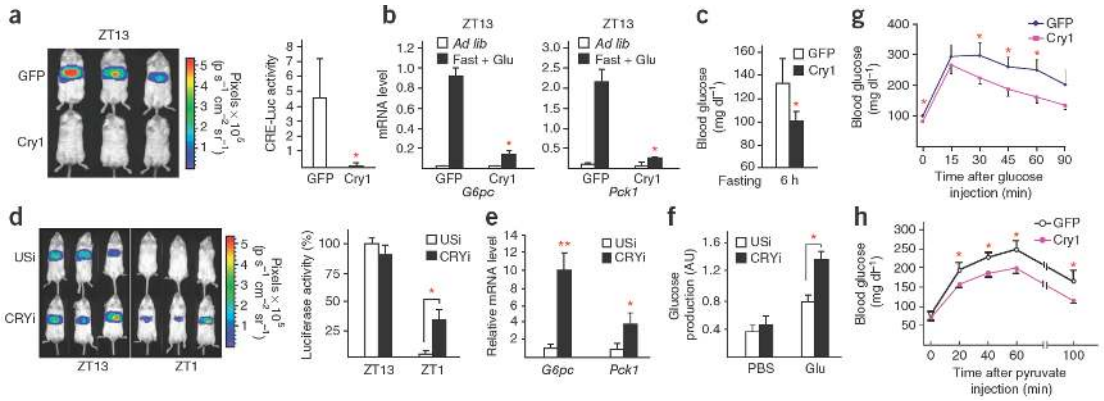


Figure 2-3. Cry blocks induction of the gluconeogenic genetic program by Creb and Crtc2. (a) Left, live imaging analysis of hepatic CRE-luc reporter activity in control (Ad-GFP) and Ad-Cry1 expressing mice. CRE-luc activity in fasted mice (ZT10-13) injected with glucagon is shown. Right, quantitative analysis of CRE-luc activity. Asterisk indicates $P < 0.01$, $n = 5$. (b) Quantitative PCR analysis of *G6pc* and *Pck1* gene expression in control and Cry1-expressing fasted (after i.p. injection of glucagon) and fed mice. *$P < 0.05$, $n = 5$. (c) Effect of Cry1 overexpression on fasting blood glucose concentrations relative to control GFP expressing mice. *$P < 0.05$, $n = 3$. (d) Left, live imaging analysis of CRE-luc reporter activity in mice expressing adenovirally encoded Cry1- and Cry2- specific shRNA (CRYi) or nonspecific shRNA (USi) in liver. Right, quantitative analysis of CRE-luc activity. *$P < 0.01$, $n = 4$. (e) Quantitative PCR analysis of hepatic *G6pc* and *Pck1* mRNA amounts in control (USi) and CRYi-expressing mice. **$P < 0.001$ for *G6pc* and *$P < 0.01$ for *Pck1*, $n = 3$. (f) Glucose production assay in primary hepatocytes infected with Ad-CRYi or control. *$P < 0.01$, $n = 3$. (g) Glucose tolerance testing of *db/db* mice expressing adenovirus encoded Cry1 or GFP. *$P < 0.01$, $n = 5$. (h) Pyruvate tolerance testing of wild-type mice expressing adenovirus-encoded Cry1 or GFP. Fasted mice were injected with pyruvate and blood glucose concentrations were measured at indicated times. *$P < 0.05$, $n = 5$. Data represent means ± s.e.m.

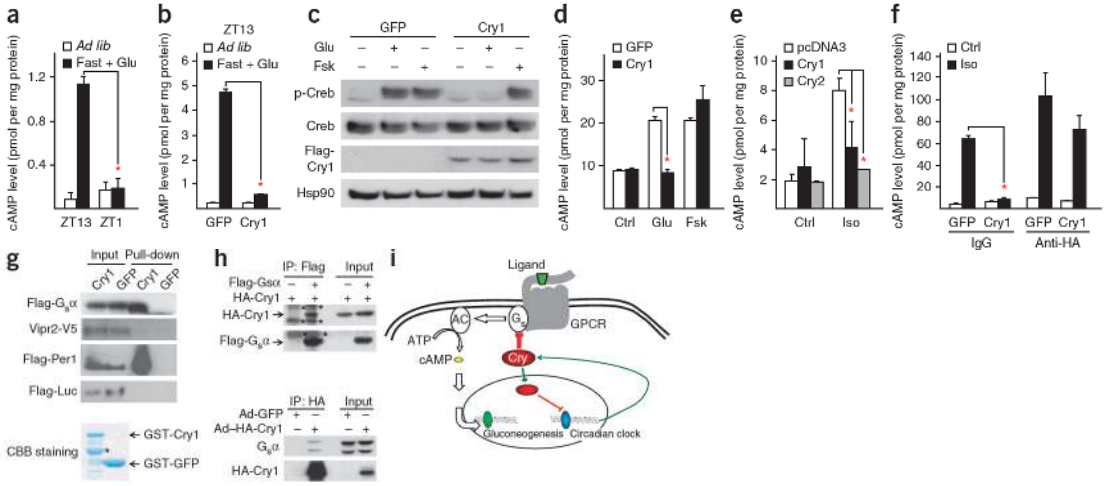
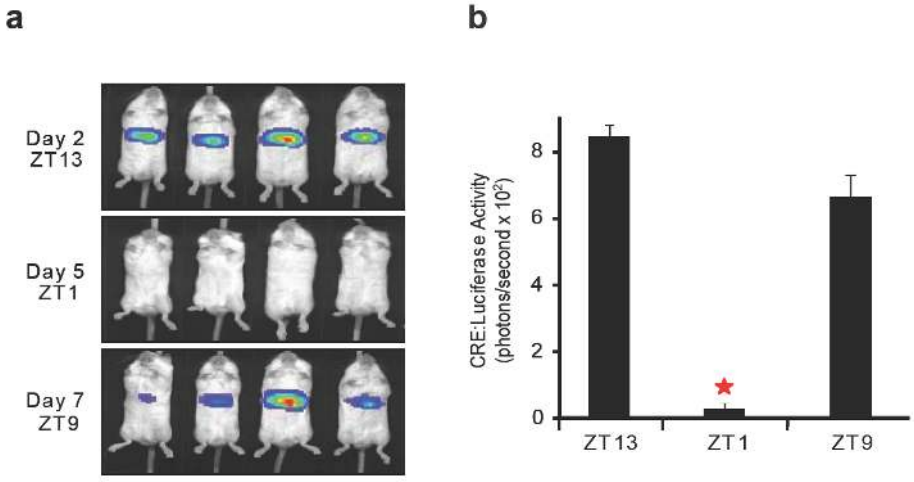
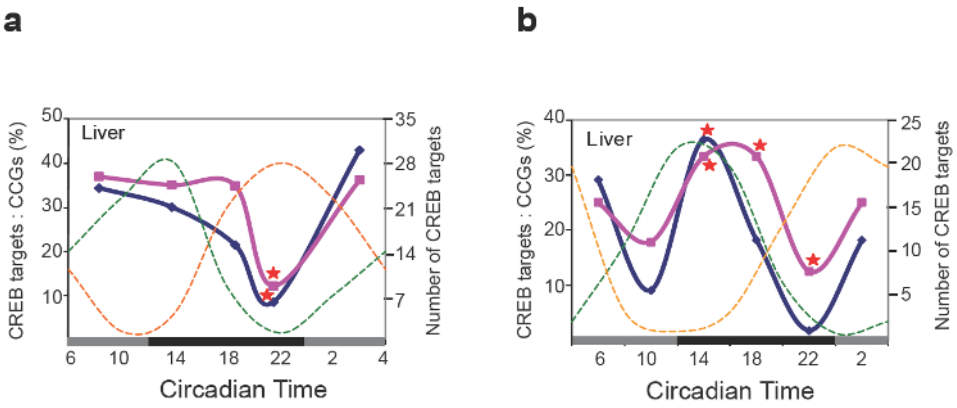


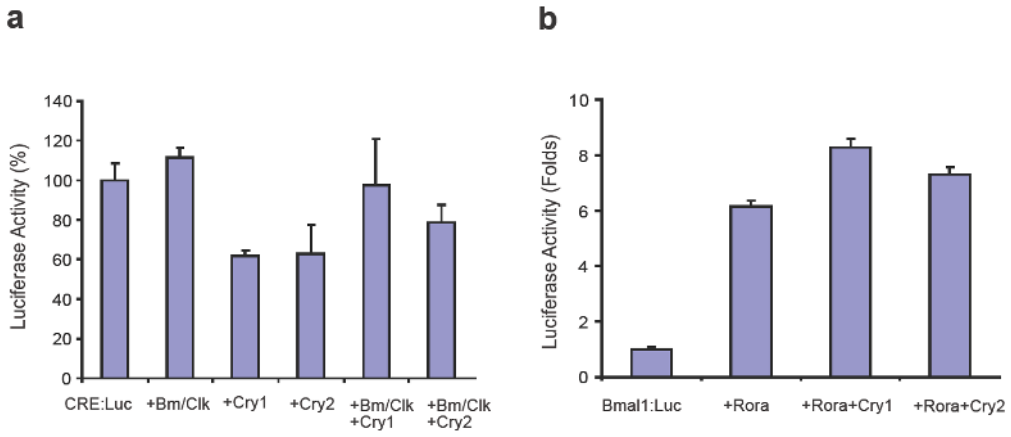
Figure 2-4. Cry inhibits GPCR-dependent increases in adenyl cyclase activity. (a) Hepatic cAMP concentrations in fed or fasted mice after i.p. injection of glucagon. (b) Effect of Cry1 overexpression on hepatic cAMP levels at ZT13 in fasted mice after i.p. glucagon administration and in fed mice. (c) Immunoblot showing the effect of Ad-Cry1 expression on amounts of phospho-Creb (Ser133) in primary hepatocytes treated with glucagon or forskolin (Fsk). (d) Effect of Adenoviral Cry1 expression on intracellular cAMP concentrations in hepatocytes exposed to forskolin or glucagon. Ctrl, control. (e) *In vitro* reconstitution studies showing effect of cytosolic fractions from control (GFP) and Cry1-expressing cells on cAMP accumulation in reactions containing plasma membrane fractions from HEK293T cells. Iso, isoproterenol. (f) Immunodepletion assays showing effect of antibody presence on cAMP production. Anti-HA, HA-specific antibody. (g) Immunoblot showing recovery of Gsα and Vipr2 from membrane fractions of transfected HEK293T cells incubated with recombinant GST-Cry1 or GST-GFP control. Top, input and pull-down signals from 293T cells overexpressing Flag G_sα, Vipr2-V5, Flag-Per1 and Flag-Luc. Bottom, GST-tagged proteins were purified and stained with Coomassie brilliant blue (CBB). Asterisk indicates truncated GST-Cry1 polypeptide. (h) Co-immunoprecipitation of G_sα and Cry1. Top, immunoblot showing recovery of HA-Cry1 from immunoprecipitates of Flag-G_sα prepared from HEK293T cells. Asterisks indicate nonspecific signals. IP, immunoprecipitation. Bottom, immunoblot showing recovery of endogenous Gsα from immunoprecipitates of HA-Cry1 prepared from primary mouse hepatocytes. (i) Schematic diagram indicating that the circadian regulation of cAMP signaling in liver is Cry dependent. Blue oval represents E-box–bound transcription factors CLOCK-BMAL1, and green oval represents Cre-mediated transcription activators or coactivators such as Creb, p300/Cbp and Crtc2. AC, adenyl cyclase. $^*P < 0.05$.



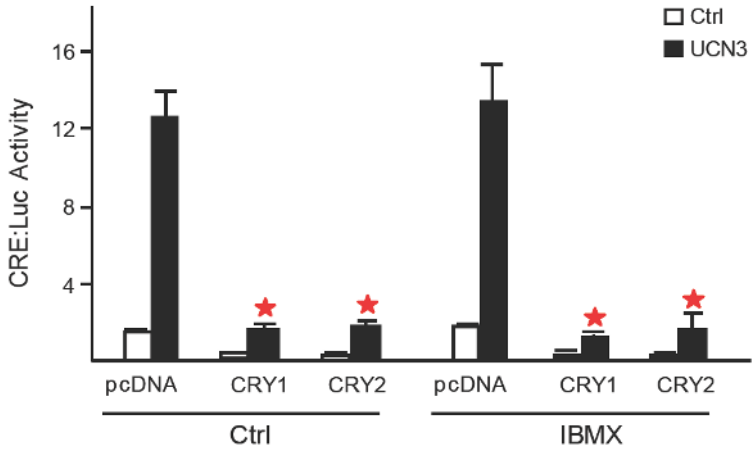
Supplementary Figure 2-1. Circadian gating of CRE:Luc induction by glucagon stimulation. Adenoviruses carrying a CRE:Luc reporter were injected into mice via tail vein at ZT3-8 on day 0. Mice were then fasted for three hours prior to glucagon injection, followed by injection of luciferin 1 hr later. Luciferase activity was monitored through bioluminescence imaging at the indicated time points (ZT13, ZT1 and ZT9; Left panel) and normalized with β-galactosidase activity (right panel). To minimize variation among individual animals, imaging was conducted in the same mouse at different time-points, i.e., the same mice were placed at the same order in each panel. Note that the CRE:Luc activity was repressed at ZT1.



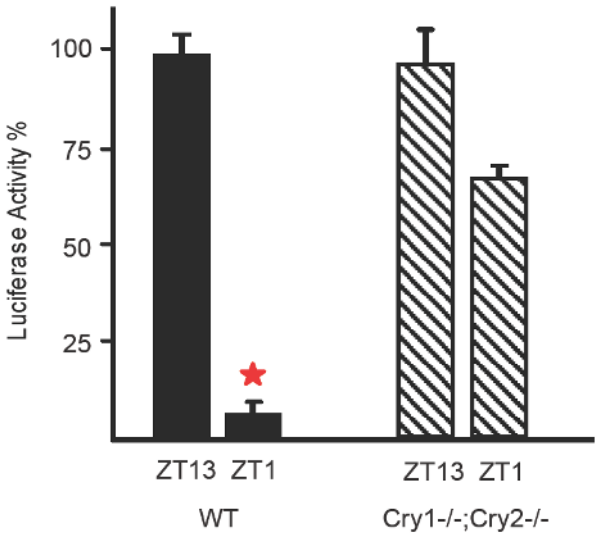
Supplementary Figure 2-2. Bioinformatics analysis of CREB targets among CCGs. The sum (blue solid) and ratio (pink solid) of CREB target genes are plotted against the circadian time. Analyses were based on the CCG data sets reported by Ueda et al (21) (**a**) and by Panda et al (9) (**b**). The standard used to define a CREB target was described before (ref. S3). For comparison of gene expression, Per2 (green dot) and Bmal1 (red dot) expression patterns versus circadian time were also shown. Note that both CREB targets numbers and the ratios are significantly less at CT22, compared to average CREB targets vs non-CREB target CCGs. Asterisks indicate $P < 0.05$ as analyzed by contingency tables, two-tailed Fisher's exact test.



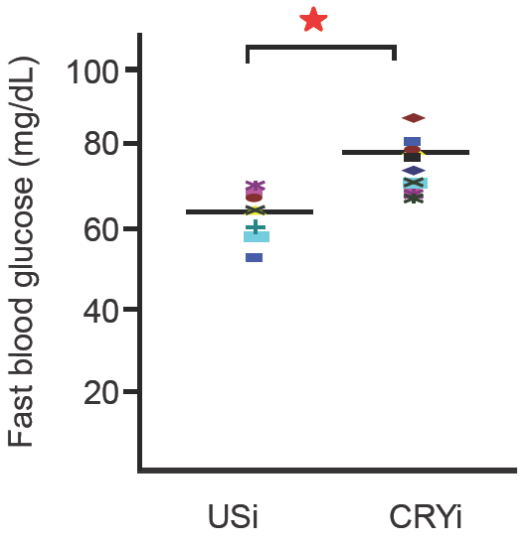
Supplementary Figure 2-3. Specificity of CRY repression on CREB-dependent signaling. a, CRE:Luc reporter activity is not modulated by BMAL1/CLOCK, suggesting that inhibition by CRY is not E-box mediated. **b**, CRY did not inhibit Bmal1:Luc expression activated by RORa.



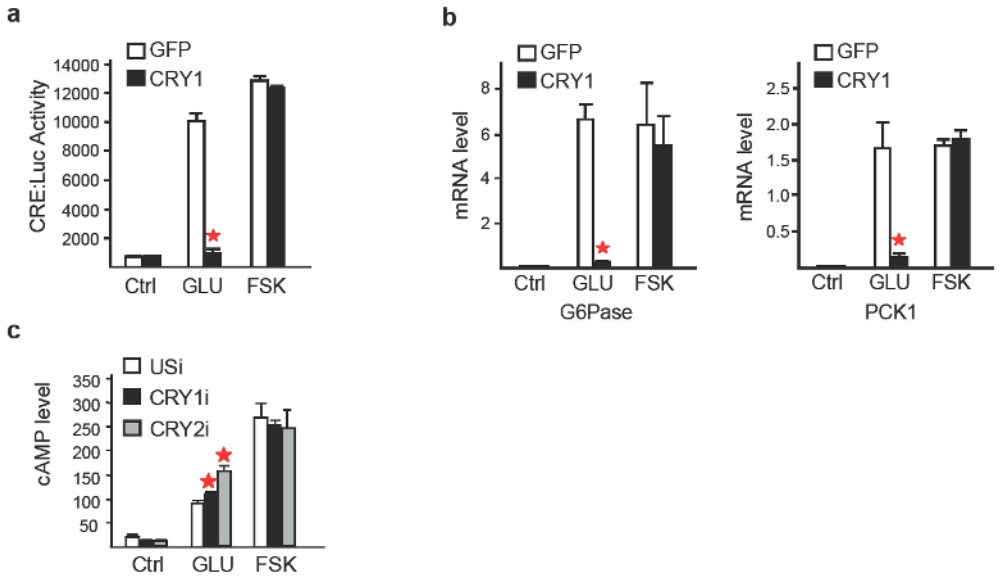
Supplementary Figure 2-4. CRE:Luc reporter activity in HEK293T cells transfected with CRFR2B and exposed to UCN3. Co-treatment with phosphodiesterase inhibitor IBMX indicated. Asterisks indicate $P < 0.05$, n=3.



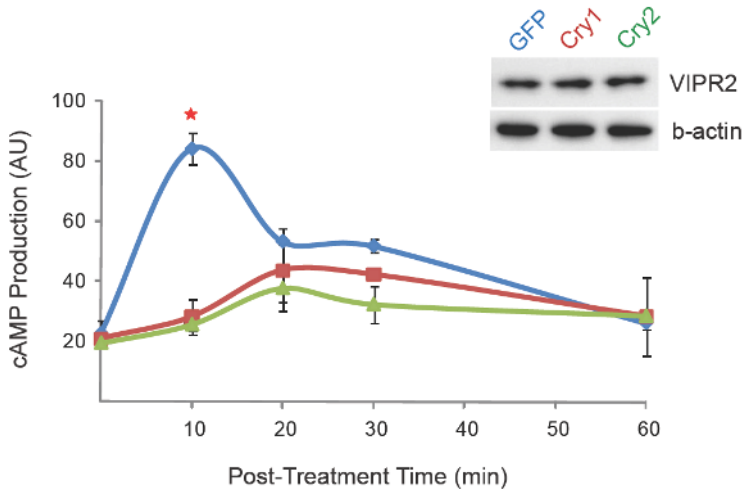
Supplementary Figure 2-5. Loss of circadian gating in the liver of Cry1/2 knockouts. In the absence of CRY function in $Cry1^{-/-};Cry2^{-/-}$ mice, CRE:Luc expression at ZT1 was significantly higher than those in WT ($p < 0.001$ for WT and $p = 0.291$ for $Cry1^{-/-};Cry2^{-/-}$ mice; $n = 3$).



Supplementary Figure 2-6. Circulating blood glucose concentrations is higher in fasted CRY-deficient mice. Mice were fasted for overnight and blood glucose was measured at ZT1. Asterisk indicates $P < 0.02$; $n = 8$ for each genotype. All data are shown in individual dots.



Supplementary Figure 2-7. CRY1 inhibits CRE:Luc reporter activity and gluconeogenic gene expression in cultured primary hepatocytes. a. Primary hepatocytes from CRE:Luc transgenic mice were infected with Ad-GFP or Ad-Cry1 for 24 hr, then stimulated with glucagon or adenyl cyclase activator FSK indicated. **b.** Gluconeogenic gene expression in primary hepatocytes stimulated with glucagon or FSK. **c**. RNAi-mediated knockdown of CRY1 and CRY2 increases cAMP accumulation. Intracellular cAMP levels were measured in primary hepatocytes exposed to glucagon (100nM) or FSK (10uM). All data are shown in mean and s.e.m representing at least three independent experiments. Asterisks indicate $P < 0.05$, $n = 3$.



Supplementary Figure 2-8. Inhibition of cAMP production by CRY overexpression in fibroblasts. VIPR2/CRE:Luc fibroblast cells infected with GFP control (blue), Cry1 (red), or Cry2 (green) were used for cAMP production assay after 2 nM VIP treatment. $P < 0.001$ at time point of 10 min post-treatment. All data are shown in mean and s.e.m representing three independent experiments. Insert shows equal amount of VIPR2 (V5-tagged) gene expression among these cell lines.

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P. Pongsawakul performed an experiment that contributed to Fig. 2-4g.

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Chapter 3:

The Role of Cytoplasmic Cryptochrome in Regulating cAMP and Ca^{2+} Signaling Pathways

3.1 ABSTRACT

The circadian clock anticipates daily changes in environmental cycles to coordinate cellular, physiological, and behavioral rhythms. Rhythms in feeding-fasting and metabolic pathways are controlled by the clock, which at the molecular level comprises circuitries of activators and repressors. In the core clock loop, CLOCK and BMAL1 activate Period (*Per*) and Cryptochrome (*Cry*) genes. After translation in the cytoplasm, PER and CRY translocate back to the nucleus and repress the activity of CLOCK/BMAL1. CRYs (CRY1 and CRY2) function as potent repressors of CLOCK/BMAL1 and the glucocorticoid receptor. In addition, CRYs also regulate fasting gluconeogenesis by interacting with $G_s\alpha$ subunit of the G protein in the cytoplasm and inhibiting glucagon-mediated stimulation of cAMP signaling. Here, we investigate the novel role of cytoplasmic CRYs in cAMP signaling using cytoplasmic mutants Cry1-ΔCCm and Cry2-ΔCCm. We found that cytoplasmic CRY inhibits cAMP signaling by interacting with $G_s\alpha$. We also revealed a CRY inhibitory effect on $G_q\alpha$-mediated Ca^{2+} signaling by

interacting with the $G_q\alpha$ subunit. Our study provides evidence that CRY is a link between core transcription-translation feedback loop and cytosolic oscillations, which are integral components of circadian pacemaker in the suprachiasmatic nucleus and other tissues.

3.2 INTRODUCTION

The intrinsic daily biological rhythms, named circadian rhythms, are found ubiquitously in organisms from cyanobacteria to plants and animals (1, 2). Many behavioral, physiological, and cellular processes are controlled by the circadian clock present in cells and tissues throughout organisms. In mammals, such processes include the sleep-wake cycle, feeding cycle, hormonal secretion, cellular metabolism, cell cycle, and gene expression (3-5). These oscillations are endogenously driven by molecular clocks, which provide an adaptive advantage through synchronizing biological processes with the day-night cycles (6, 7). The hypothalamic suprachiasmatic nucleus (SCN) represents the master circadian pacemaker in mammals; it coordinates and synchronizes oscillators in the peripheral tissues such as heart, liver, kidney, and lung (8, 9). Disease severity also varies depending on the time of the day: the incidence of myocardial infarction peaks in the morning while asthma symptoms are most severe in the middle of the night (10). Conversely, many studies have suggested that people with misalignment between their

endogenous circadian clocks and behavior such as shift workers have higher risk of diabetes, obesity, cardiovascular disease, and cancers (11-13).

The eukaryotic circadian clocks are driven by interactions of interlocking transcription/translation negative feedback loops. In mammals, the core loop comprises transcription factors CLOCK and BMAL1 that activate Period (*Per1* and *Per2*) and Cryptochrome (*Cry1* and *Cry2*) genes. Once translated, PER and CRY proteins form complexes and translocate back to the nucleus to repress CLOCK/BMAL1 activity, forming a negative feedback loop (14, 15). REV-ERB repressors (α and β) and ROR activators (α, β, and γ) form an additional interlocking loop necessary for normal rhythmicity (16, 17).

Many G protein-coupled receptors (GPCRs) mediate synchronization and rhythmicity of the SCN neurons via intercellular paracrine signaling, in addition to neuronal synapses (8, 18). GPCRs regulate cytosolic signals including cAMP (via $G_s\alpha$ and $G_i\alpha$) and Ca^{2+} (via $G_q\alpha$) pathways through the heterotrimeric G protein that consists of α, β, and ϒ subunits (19). Once a ligand such as a hormone or neurotransmitter binds to GPCR, it activates the Gα subunit by replacing GDP with GTP. The active GTP-bound Gα then dissociates from the GβϒY subunits and interacts with downstream effector proteins. $G_s\alpha$ stimulates adenylyl cyclase activity, increasing the intracellular cAMP production. $G_i\alpha$, on the other hand, inhibits adenylyl cyclase activity,

thereby reducing cAMP production. $G_q\alpha$ activates phospholipase C (PLC) that triggers intracellular release of Ca^{2+} via inositol trisphosphate (IP_3). These signals can be terminated by hydrolysis of GTP to GDP, returning Gα subunit to the inactive GDP-bound state. Gα subunits possess an intrinsic GTPase activity, which can be accelerated by other factors including regulator of G protein signaling (RGS) proteins.

In the SCN, cAMP and Ca^{2+} signaling pathways have been implicated as integral components of the core molecular clock (20, 21). Vasoactive intestinal peptide (VIP) and its cognate G_s-coupled $VPAC_2$ receptor are necessary for rhythmic locomotor behavior and SCN neuron synchronization (22, 23). Gastrin-releasing peptide (GRP) acts through G_q-coupled bombesin BB_2 receptor, also known as GRP receptor (GRPR), and regulates phase resetting of SCN similar to the photic input (24). Arginine vasopressin (AVP) functions as rhythmic SCN output and regulates circadian rhythms of locomotor activity (25, 26).

Phosphorylation of cAMP-responsive element (CRE)-binding protein (CREB) is believed to transmit converging cAMP and Ca^{2+} signals to the core clock by activating *Per1* and *Per2* expression (27, 28). Conversely, the core clock also regulates circadian rhythms of Ca^{2+} and cAMP in the SCN. Ca^{2+} and cAMP rhythms were altered in circadian mutant SCNs in accordance with shortening or lengthening of locomotor period of the mutant animals (29).

We have recently shown that CRY interacts with the $G_s\alpha$ subunit of the G protein in the cytoplasm and inhibits glucagon-mediated stimulation of cAMP signaling during fasting hepatic gluconeogenesis (30). This is the first report of mammalian CRY function in the cytoplasm, linking the core clock protein to G protein regulation. Here, we employed molecular and pharmacological approaches to elucidate the effects of CRY on cytosolic signals mediated by the G proteins. We used the cytoplasmic CRY mutants to investigate the effects of CRY in the cytoplasm on signaling pathways that are mediated by $G_s\alpha$, $G_i\alpha$, or $G_q\alpha$ subunit. We found that CRY inhibits $G_s\alpha$-mediated cAMP signaling and $G_q\alpha$-mediated Ca^{2+} signaling by interacting with the $G_s\alpha$ and $G_q\alpha$ subunit, respectively.

3.3 RESULTS

The cytoplasmic mutants Cry1-ΔCCm and Cry2-ΔCCm are tools to study CRY functions in the cytoplasm

The dynamic change in cytoplasmic-nuclear translocation of CRY *in vivo* has made it difficult to study the functions of CRY in the cytoplasm (15). We chose to study CRY functions in cultured cells, which are isolated from rhythmic systemic cues. Overexpressing Cry1 and Cry2 results in translated proteins to be predominantly localized in the nucleus of commonly used cell lines HEK293T (100% for CRY1; 100% for CRY2), COS7 (93.33%; 98.89%),

and NIH3T3 (91.11%; 96.11%) (Fig. 3-1A and Supplementary Fig. 3-1 A and B). In *Per2::Luciferase* (*Per2*^{Luc}) mouse adult fibroblasts (MAFs), Cry1 or Cry2 overexpression display nuclear distribution in half of the cells (56.11% and 56.67% respectively) (Fig. 3-1 A and D) (9, 31). Because overexpressing wild-type Cry1 and Cry2 did not exhibit cytoplasmic localization, we used an approach that successfully separated nuclear and cytoplasmic functions of *Arabidopsis* cryptochrome 1 (32). We inserted a nuclear export signal (NES) to the coding sequence of *Cry1* and *Cry2* in order to manipulate CRY subcellular localization. Overexpressing CRY1-NES-YFP and CRY2-NES-YFP, however, did not shift the localization from nucleus to cytoplasm (Supplementary Fig. 3-2A). In addition, cells expressing Cry1-NES-YFP and Cry2-NES-YFP were able to inhibit CLOCK/BMAL1-mediated transcriptional activation similar to the wild-type counterparts in HEK293T cells cotransfected with *Per2* promoter-driven Luciferase (Luc) reporter. This result confirms the intact nuclear localization of the NES mutants (Supplementary Fig. 3-2B). Then, we utilized the previously characterized cytoplasmic mutant Cry1-ΔCCmutNLSc (hereafter called Cry1-ΔCCm) (33). An additional mutant Cry2-ΔCCm that also lacks the coiled-coil domain and contains mutations at the carboxy-terminal (C-terminal) nuclear localization signal was also generated (Supplementary Fig. 3-3). Overexpression of CRY1-ΔCCm exhibits cytoplasmic localization in HEK293T (100%), COS7 (88.89%), and NIH3T3

(67.22%) cells (Fig. 3-1 and Supplementary Fig. 3-3). Similarly, CRY2-ΔCCm overexpression also display cytoplasmic expression patterns in the majority of HEK293T, COS7, and NIH3T3 cells (87.22%, 90.56%, and 66.67%, respectively). Interestingly, *Per2*^{Luc} MAFs overexpressing CRY1-ΔCCm and CRY2-ΔCCm display either cytoplasmic localization (41.11%; 35.00%) or both nuclear and cytoplasmic localization (58.89%; 65.00%) (Fig. 3-1) similar to subcellular distributions seen in *Cry1*$^{-/-}$*;Cry2*$^{-/-}$ *Per2*^{Luc} MAFs (Supplementary Fig. 3-4).

Cytoplasmic mutants CRY1-ΔCCm and CRY2-ΔCCm have less inhibitory effects on CLOCK/BMAL1 activity

CRYs function as potent transcriptional repressors that inhibit the activity of CLOCK/BMAL1 (34, 35). Nuclear translocation of CRYs and the repressor complexes is an important mechanism to achieve this inhibitory effect (33, 36). We then tested the CRY1-ΔCCm and CRY2-ΔCCm inhibitory effects on CLOCK/BMAL1-mediated activation. HEK293T cells overexpressing wild-type CRY1 or CRY2 efficiently repress CLOCK/BMAL1 activity in a dosage dependent manner. However, cells overexpressing Cry1-ΔCCm or Cry2-ΔCCm show less potent repression than that of the wild-type Cry1 or Cry2, respectively (Fig. 3-2A). These inefficient repression effects of CRY1-ΔCCm or CRY2-ΔCCm are in agreement with other Cry1 mutants that lack the coiled-coil domain or the C-terminal domain (33). The partial

repression of Cry1-ΔCCm and Cry2-ΔCCm can be attributed to trace amounts of proteins present in the nuclear fractions (Fig. 3-2B). With this cell-based reporter assay together with the subcellular localization images, we have established Cry1-ΔCCm and Cry2-ΔCCm as tools to study CRY functions in the cytoplasm in mammalian tissue cultures.

Cytoplasmic mutant CRY1-ΔCCm is a potent inhibitor of cAMP signaling

We have previously shown in an *in vitro* reconstitution assay that cytoplasmic lysate expressing Cry1 was able to reduce the production of cAMP in response to isoproterenol (ISO) in a mixture with plasma membrane (30). Because expressing Cry1-ΔCCm alters CRY1 localization to the cytoplasm predominantly, we hypothesize that CRY1-ΔCCm would have a stronger effect than wild-type CRY1 on cAMP signaling. Indeed, we observed a strong repression in cells expressing Cry1-ΔCCm upon treatment with VIP, and that inhibition is significantly stronger than that of the wild-type Cry1 (Fig. 3-3A). Similarly, overexpression of Cry1-ΔCCm significantly repressed the CRE-Luc activity in cells stimulated with glucagon-like peptide 1 (GLP1) (Fig. 3-3B). Since both VIP and GLP1 activate adenylyl cyclase through $G_s\alpha$, we then investigated the interaction of CRY1-ΔCCm and CRY2-ΔCCm with $G_s\alpha$ by co-immunoprecipitation. HEK293T cells were co-transfected with Gnas, a gene that encodes $G_s\alpha$ subunit, with Cry1, Cry1-ΔCCm, Cry2, Cry2-ΔCCm, or GFP control. After immunoprecipiation of $G_s\alpha$, we detected co-

immunoprecipitated CRY proteins as previously reported (30). Interestingly, we found that there was greater abundance of CRY1-ΔCCm and CRY2-ΔCCm co-immuprecipitated with $G_s\alpha$ than CRY1, and CRY2, respectively (Fig. 3-3C). The strong interaction of CRY1-ΔCCm and $G_s\alpha$ supports the notion that cytoplasmic mutant protein CRY1-ΔCCm has more suppressive effect on $G_s\alpha$-mediated cAMP/CREB activity than that of the wild-type CRY1 (Fig. 3-3 A and B).

CRY inhibits $G_q\alpha$-mediated Ca^{2+} signaling

Because cytoplasmic CRY interacts with the $G_s\alpha$ subunit and strongly inhibits cAMP signaling as described above, we then investigated whether CRY can regulate signaling pathways mediated by other Gα subunits. Since activation of $G_q\alpha$-mediated GPCRs induces Ca^{2+} release from intracellular storage, we tested the effects of CRY1-ΔCCm on $G_q\alpha$-coupled receptors. We used calmodulin/calcineurin-mediated activity of NFAT (nuclear factor of activated T cells) as a cell-based reporter (NFAT-Luc) for intracellular Ca^{2+} (37, 38). In HEK293T cells cotransfected with NFAT-Luc and Vipr2 (a gene that encodes $VPAC_2$ receptor), we observed activation NFAT-Luc when stimulated with VIP, but not with the adenylyl cyclase agonist forskolin (FSK). Unexpectedly, we saw inhibitory effect of CRY1-ΔCCm on Ca^{2+}-dependent NFAT-Luc activation that is significantly stronger than that of the wild-type CRY1 (Fig. 3-4A). As predicted, we saw potent repression by regulator of G

protein signaling 2 (RGS2), which has an ability to enhance GTPase activity of the $G_q\alpha$ subunit (39). In addition, cotreatment with the PLC inhibitor U-73122 blunts the activation by VIP, suggesting that CRY1-ΔCCm affects intracellular Ca^{2+} signaling mediated by $G_q\alpha$ (Supplementary Fig. 3-5A). Using this cell-based system, we further characterized CRY-ΔCCm with $G_q\alpha$-mediated GPCRs that are important for SCN synchronization. We observed similar pattern of repression by CRY1-ΔCCm and, to lesser extent, by CRY1 in HEK293T cells coexpressed with BB_2 receptor and AVPR (Fig. 3-4B and Supplementary Fig. 3-5B). Moreover, we also observed CRY1-ΔCCm-dependent repression in cells coexpressed with other $G_q\alpha$-mediated GPCRs such as gonadotropin-releasing hormone (GnRH) receptor and ghrelin receptor (Supplementary Fig. 3-5 C and D). These results demonstrate that cytoplasmic CRY is able to repress $G_q\alpha$-mediated Ca^{2+} signaling.

CRYs interact with $G_s\alpha$ and $G_q\alpha$ subunits of the G protein

Because CRY can inhibit $G_q\alpha$-mediated GPCR, we hypothesized that cytoplasmic CRY interacts with the $G_q\alpha$ subunit. We carried out a co-immunoprecipitation assay to test interaction of CRY1-ΔCCm and CRY2-ΔCCm with $G_q\alpha$ as well as $G_s\alpha$ and $G_i\alpha$. CRY1-ΔCCm and CRY2-ΔCCm was co-immunoprecipitated with $G_s\alpha$ and $G_q\alpha$ subunits (Fig. 3-4C). However, we did not observe the interaction of CRY1-ΔCCm or CRY2-ΔCCm with $G_i\alpha$ to the same extent as $G_s\alpha$ and $G_q\alpha$. Then, we used a $G_i\alpha$ inhibitor pertussis

toxin (PTX) to test the hypothesis that CRY1-ΔCCm may inhibit cAMP signaling by activating $G_i\alpha$. However, we did not observe changes in CRY1-ΔCCm repression upon treatment with PTX (Fig. 3-4C). These results suggest a mechanism in which CRY interacts with $G_s\alpha$ and $G_q\alpha$ and thereby inhibits $G_s\alpha$-mediated cAMP signaling and $G_q\alpha$-mediate Ca^{2+} signaling, respectively.

3.4 DISCUSSION

In this study, we investigated novel roles of cytoplasmic CRY in regulating G protein-mediated pathways. Because overexpressed CRY1 and CRY2 are predominantly localized in the nucleus of cultured cells, and NES was not sufficient to alter CRY localization, we employed cytoplasmic mutants Cry1-ΔCCm and Cry2-ΔCCm as tools to further investigate CRY functions in the cytoplasm. We comprehensively compared subcellular localizations of wild-type Cry1/2 and Cry1/2-ΔCCm in different cultured cells and found distinct nuclear and cytoplasmic localization, respectively, in HEK293T and COS7 cells. However, overexpression in NIH3T3 cells and *Per2*^{Luc} MAFs cause less distinct subcellular localization patterns (Fig. 3-1 and Supplementary Fig. 3-1). Because NIH3T3 cells and *Per2*^{Luc} MAFs can drive rhythmic expression of a circadian reporter, it is likely that endogenous clock proteins such as CLOCK and PERs are able to facilitate nuclear localization

of CRY1-ΔCCm and CRY2-ΔCCm (36, 40, 41). When we compared subcellular localizations in MAFs derived from wild-type and *Cry1*$^{-/-}$*;Cry2*$^{-/-}$ background, we did not observe significant differences in subcellular distributions. This result verifies that other partners, besides CRYs themselves, mediate CRY nuclear translocation.

Since CRYs are historically regarded as potent repressors of CLOCK/BMAL1 in the nucleus, the weak inhibition of CLOCK/BMAL1-mediated transcription activation by CRY1-ΔCCm and CRY2-ΔCCm functionally confirms their cytoplasmic localizations shown by confocal microscopy analyses (Figs. 3-1 and 3-2A). Some repression by CRY1-ΔCCm and CRY2-ΔCCm may be contributed by traces amount of proteins expressed in the nuclear fractions (Fig. 3-2B). It was previously reported that CLOCK can mediate CRY1-ΔCCm nuclear localization, and that may contribute to partial inhibition of CLOCK/BMAL1 by CRY1-ΔCCm and CRY2-ΔCCm (33).

Previous *in vitro* assay suggested that cytoplasmic CRY lysate is able to inhibit cAMP production upon stimulation with β-adrenergic receptor agonist ISO (30). In line with this evidence, we found that cytoplasmic mutant CRY1-ΔCCm has a stronger inhibitory effect than the predominantly nuclear wild-type CRY1 on $G_s\alpha$-mediated stimulation of the cAMP signaling pathway. Furthermore, we detected more CRY1-ΔCCm and CRY2-ΔCCm co-immunoprecipitated with $G_s\alpha$ subunit than CRY1 and CRY2, and the amount

of CRY1 and CRY2 reflects protein expression of CRY1 and CRY2 in the cytoplasmic fractions of HEK293T lysates (Figs. 3-2B and 3-3C). These results indicate that cytoplasmic CRY interacts with $G_s\alpha$ and thereby inhibits $G_s\alpha$-mediated cAMP signaling. A recent report has demonstrated that CRY1 inhibits cAMP production by binding to and inhibiting the activity of adenylyl cyclase (42). It is possible that cytoplasmic CRY can interact with both $G_s\alpha$ subunit of the G protein and adenylyl cyclase to inhibit cAMP production, an inhibition mechanism that was proposed for an RGS protein (43). Additional *in vitro* binding experiments will be needed to elucidate the direct interaction and affinity of CRY with $G_s\alpha$ and/or adenylyl cyclase.

Growing evidence from SCN studies highlights important roles of cAMP and Ca^{2+} for intercellular communication and sustainment of transcription rhythms (18, 20, 22). Intracellular cAMP production is induced by $G_s\alpha$ and inhibited by $G_i\alpha$; whereas intracellular Ca^{2+} is mediated by $G_q\alpha$. We then used PTX to test the hypothesis that CRY1-ΔCCm may inhibit cAMP signaling by activating $G_i\alpha$. Upon pre-treatment or co-treatment with PTX, we did not observe de-repression of CRY1-ΔCCm when stimulating with VIP (Fig. 3-4C). We also did not observe CRY1-ΔCCm and CRY2-ΔCCm co-immunoprecipitated with $G_i\alpha$ with the same affinity as $G_s\alpha$ (Fig. 3-4D). These findings support our hypothesis that cytoplasmic CRY regulates cAMP

signaling by inhibiting $G_s\alpha$-mediated cAMP increase, rather than activating $G_i\alpha$-mediated cAMP decrease.

Because cAMP and Ca^{2+} can activate CREB/CRE-activity, we took advantage of calmodulin/calcineurin-mediated NFAT-Luc reporter that is responsive to changes in intracellular Ca^{2+} (29, 37, 44). We observed activation of NFAT-Luc activity in cells stimulated with GRP and AVP, known $G_q\alpha$-mediated SCN neuropeptides. Inhibition by CRY1-ΔCCm and, to a lesser extent, by CRY1 on NFAT-Luc activity in HEK293T cells stimulated with GRP or AVP was noted (Fig. 3-4B and Supplementary Fig. 3-5B). We also detected inhibition by CRY1-ΔCCm of NFAT-Luc activation with other $G_q\alpha$-mediated receptors, including GnRH receptor and ghrelin receptor (Supplementary Fig. 3-5 C and D).

We saw an induction of NFAT activity in cells coexpressed with Vipr2 upon treatment with VIP, and, similar to the other $G_q\alpha$-mediated receptors, the activation can be repressed strongly by CRY1-ΔCCm and moderately by CRY1 (Fig. 4A). There is no substantial activation of NFAT-Luc activation with FSK treatment, and the activation is blunted by a PLC inhibitor U-73122 (Fig. 3-4A and Supplementary Fig. 3-5A). These results indicate that VIP elicits $G_q\alpha$-mediated Ca^{2+} pathway that is dependent on PLC/IP_3 (21, 29). These findings with the co-immunoprecipitation assay of CRY1-ΔCCm and CRY2-ΔCCm with $G_q\alpha$ subunit (Fig. 3-4D) demonstrate that cytoplasmic CRY also

regulates Ca^{2+} signaling by interacting with $G_q\alpha$ and thereby inhibiting $G_q\alpha$-mediated activity.

In summary, we employed cytoplasmic mutants Cry1-ΔCCm and Cry2-ΔCCm to investigate the roles of CRY in cytosolic signaling. Our results indicate that cytoplasmic CRY can regulate cAMP and Ca^{2+} pathways by interacting with $G_s\alpha$ and $G_q\alpha$, respectively. As cAMP and Ca^{2+} are regarded as integral components of the transcriptional oscillator in the SCN, we provide reciprocal evidence of a core clock protein that regulates the cytosolic signals. Our study places CRY as a molecular link connecting core transcription-translation feedback and cytosolic loops. With a majority of drugs targeting GPCRs, understanding temporal regulation of cytoplasmic CRY in physiological context would provide rationale for chronotherapy as an important principle of personalized medicine.

3.5 METHODS

3.5.1 Plasmid Constructs. Mouse Cry1-ΔCCm and Cry2-ΔCCm constructs were generated by PCR-based site-directed mutagenesis using p3xFLAG-CMV14-mCry1 and p3xFLAG-CMV14-mCry2 as templates. C-terminally FLAG-tagged pCMV14 mCry1, mCry2, and GFP constructs were described previously (30). Deletion mutation and point mutations of Cry1-ΔCCm and Cry2-ΔCCm were generated analogously to the Cry1ΔCCmutNLSc construct,

previously described in (33). Cry1-YFP and Cry1-ΔCCm-YFP constructs were subcloned by PCR-generated YFP inserted between Kpn1 and Xba1 sites at the 3' end of the respective Cry1 constructs. Cry2-YFP and Cry2-ΔCCm-YFP were subcloned by PCR-generated YFP inserted between Xba1 site at the 3' end of the respective constructs. C-terminally hemagglutinin (HA)-tagged hGnas, hGnai, and hGnaq (encodes $G_s\alpha$, $G_i\alpha$, and $G_q\alpha$, respectively) were generated by PCR method and subcloned between NotI and EcoRI of p3xFLAG-CMV14 vector (Sigma).

3.5.2 Cell Culture. We cultured HEK293T cells, HEK293T-GLP1-1R-CRE-Luc cells, COS7 cells, NIH3T3 cells, and mouse adult fibroblasts (MAFs) harvested from the *Per2*^{Luc} knock-in mice tail fibroblasts in DMEM supplemented with 10% FBS (9, 31). Lipofectamine 2000 (Life Technologies) was used for plasmid transfection in HEK293T and HEK293-GLP1R-CRE-Luc cells. FuGENE6 (Promega) was used for plasmid transfection in COS7 cells. PolyFect (Qiagen) was used for plasmid transfection in NIH3T3 cells and *Per2*^{Luc} MAFs.

3.5.3 Fluorescence Microscopy and Subcellular Localization Quantification. COS7 cells, NIH3T3 cells, and *Per2*^{Luc} MAFs, seeded on 12 mm circular microscope cover glasses (Fisher Scientific), were transfected with YFP-tagged Cry1, Cry1-ΔCCm, Cry2, and Cry2-ΔCCm constructs. After 48 hours, cells were fixed (3 min in iced cold methanol for COS7; 5 min in 4%

PFA for NIH3T3 and *Per2*^{Luc} MAFs), washed twice with PBS, and mounted in VECTASHIELD mounting media with DAPI (Vector Labs). Sixty YFP-positive cells of each transfected condition were counted and scored for subcellular localizations using DAPI as nuclear marker. The data are presented as an averaged percentage of the total number of cells from three independent experiments. Confocal microscopy pictures of representative cells were imaged using Leica SP2 and Leica SP5 confocal microscopes.

3.5.3 Cell Fractionation. HEK293T cells were collected with PBS and resuspended in Buffer A (10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.05% NP40), and centrifuged at 2500 rpm for 5 min at 4°C. The supernatants were collected as cytosolic fractions. After washing the nuclei pellets twice with Buffer A, the pellets were resuspended in Buffer B (5 mM Hepes, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 20% glycerol (v/v), and 0.3 M $NaCl_2$) and incubated at 4°C for 1 hour. After centrifugation at 15,000 rpm for 30 min, the supernatants were collected as nuclear fractions.

3.5.4 Luciferase Reporter Assay. Per2-Luc and CRE-Luc reporter assays were performed as previously described (30). For Per2-Luc reporter assay, HEK293T cells were co-transfected with pGL3-Per2 promoter (25 ng), mClock (100 ng) and mBmal1 (50 ng) with Cry1, Cry1-ΔCCm, Cry2, Cry2- ΔCCm, or GFP control (1 ng and 10 ng) in the total of 200 ng plasmid DNA per one 96-

well. Luciferase signals were measured 48 hours post-transfection using Dual-Luciferase Reporter Assay System (Promega).

For CRE-Luc reporter assay, HEK293T cells were co-transfected with pGL3-*CRE* (25 ng), mVipr2 (50 ng) and Cry1, Cry1-ΔCCm, or GFP (50 ng or 100 ng). Cells were treated with 10 nM VIP 18 hours post-transfection, and the Luciferase signals were measured 5 hours afterward. HEK293-GLP1R-CRE-Luc cells were transfected with 150 ng of Cry1, Cry1-ΔCCm, YFP, and empty vector control. Twenty-four hours after transfection, 10 nM and subsequent serial dilutions of glucagon-like peptide (GLP1) were added together with Luciferin substrate previously described (41).

For NFAT-Luc reporter assay, HEK293T cells were co-transfected with NFAT-Luc (25 ng); hGRPR or mVipr2 (25 ng); and Rgs2, Cry1, Cry1-ΔCCm, or GFP (50 ng or 100 ng). Sixteen hours after transfection, the medium was washed with PBS and changed into Optimem (Life Technologies) supplemented with 0.5% FBS and 1% non-essential amino acids. Two hours after the media change, cells were stimulated with respective ligand VIP (10 nM), GRP (100 nM), or FSK (10 μM), and the Luciferase signals were measured 5 hours afterward. Data were average values of three technical replicates and normalized by the non-treated conditions. Graphs are representatives from three independent experiments.

3.5.5 Co-immunoprecipitation. Co-transfected HEK293T cells were collected in PBS and resuspended in TGED buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 5% glycerol, 0.5 mM DTT, 0.5% Triton X-100) (45). supplemented with 0.5 mg/mL n-dodecyl β-maltoside (Sigma), 0.5 mg/mL cholesteryl hemisuccinate (Sigma), 1 mM PMSF, and protease inhibitor cocktail (Roche). Cell lysates were incubated for 1 hour at 4°C and centrifuged at 13000-15000 rpm for 30 min. The supernatants were then mixed with anti-HA conjugated with Dynabeads (Life Technologies). After 4°C overnight incubation with gentle rocking, the magnetic beads were washed twice with TGED supplemented with 0.5% sodium deoxycholate (Sigma) and eluted with Laemmli sample buffer for 20 min at 65°C.

3.5.6 Statistical Analyses

All studies were performed on at least three independent occasions. Results are reported as mean and standard error of the mean (s.e.m). The comparison of different groups was carried out using a two-tailed unpaired Student's *t*-test. Differences were considered statistically significant at $P < 0.05$.

3.6 SUPPLEMENTARY METHODS

3.6.1 Plasmid Constructs. Nuclear export signal (NES)-YFP was added into Cry1 and Cry2 by PCR method similar to (32). The NES-YFP was inserted

into pCMV14-mCry1 and pCMV14-mCry2 between Kpn1 and Xba1 sites and Xba1 site, respectively.

3.6.2 Cell Culture and Real-Time Luciferase Assay. MAFs harvested from the tail fibroblasts of *Cry1*$^{-/-}$*;Cry2*$^{-/-}$ *Per2*^{Luc} mouse were cultured in DMEM supplemented with 10% FBS.

3.6.3 Fluorescence Microscopy. YFP-tagged Cry1 and Cry2 constructs were transfected to HEK293T cells and *Per2*^{Luc} *Cry1*$^{-/-}$*;Cry2*$^{-/-}$ MAFs seeded on 12 mm circular microscope cover glasses (Fisher Scientific). The cover glasses were coated with poly-lysine prior to HEK293T cell transfection. Eighteen to forty-eight hours post-transfection, cells were fixed (10 min in 1% paraformaldehyde (PFA) for HEK293T and 4% PFA for *Per2*^{Luc} *Cry1*$^{-/-}$*;Cry2*$^{-/-}$ MAFs), washed twice with PBS, and mounted in VECTASHIELD mounting media with DAPI (Vector Labs). Confocal microscopy pictures of representative cells were imaged using Leica SP2 and Leica SP5 confocal microscopes.

3.6.4 Luciferase Reporter Assay. Per2-Luc assay was performed as described in section 3.5.3 above for Cry1-YFP, Cry1-NES-YFP, Cry2-YFP, Cry2-NES-YFP, and YFP. For GnRH and Ghrelin NFAT-Luc assay, HEK293T cells were transfected with hAVPR1a, mGnrhr and mGhsr1a, respectively. Cells were then stimulated with AVP (100 nM), GnRH agonist (100 nM) and

ghrelin (100 nM), respectively (46). Cells were treated with 10 μM of U-73211 (Sigma) 30 min before VIP stimulation.

3.7 ACKNOWLEDGMENTS

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3.8 FIGURES AND SUPPLEMENTARY FIGURES

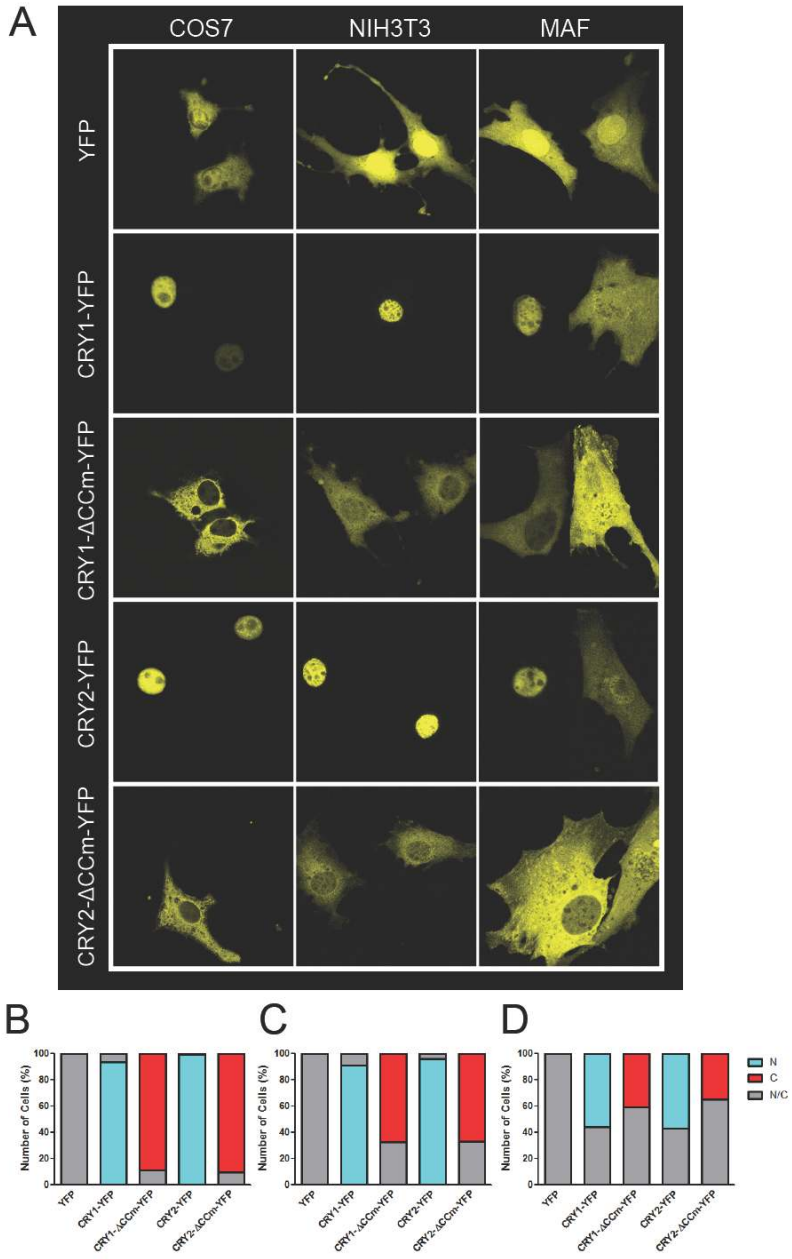


Figure 3-1. CRY1-ΔCCm and CRY2-ΔCCm are predominantly localized in the cytoplasm. (A) Confocal fluorescence images of COS7 cells, NIH3T3 cells, and *Per2*^{Luc} mouse adult fibroblasts (MAFs) overexpressing YFP, CRY1-YFP, CRY1-ΔCCm-YFP, CRY2-YFP, and CRY2-ΔCCm-YFP. (B-D) Quantification of the subcellular localization of the overexpressed proteins in HEK293T cells (B), NIH3T3 cells (C), and MAFs (D). N, nuclear, blue. C, cytoplasmic, red. N/C, nucleo-cytoplasmic, grey.

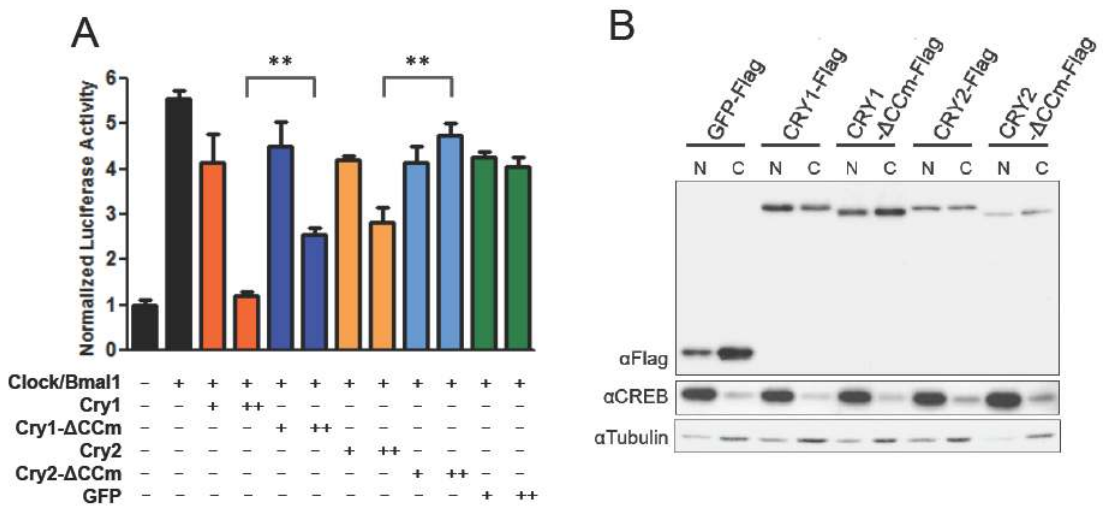


Figure 3-2. CRY1-ΔCCm and CRY2-ΔCCm exhibit weak inhibition for CLOCK/BMAL1 activity. (A) CLOCK/BMAL1-mediated activation of *Per2* promoter driven-Luc reporter activity. HEK293T cells were expressing Cry1 (orange), Cry1-ΔCCm (blue), Cry2 (light orange), Cry2-ΔCCm (light blue), and GFP (green). (B) Fractionation assay of HEK293T cells expressing GFP, CRY1, CRY1-ΔCCm, CRY2, and CRY2-ΔCCm. Antibody against CREB or tubulin was used to detect nuclear or cytoplasmic protein, respectively. ***P* < 0.01; all values represent mean + s.e.m.

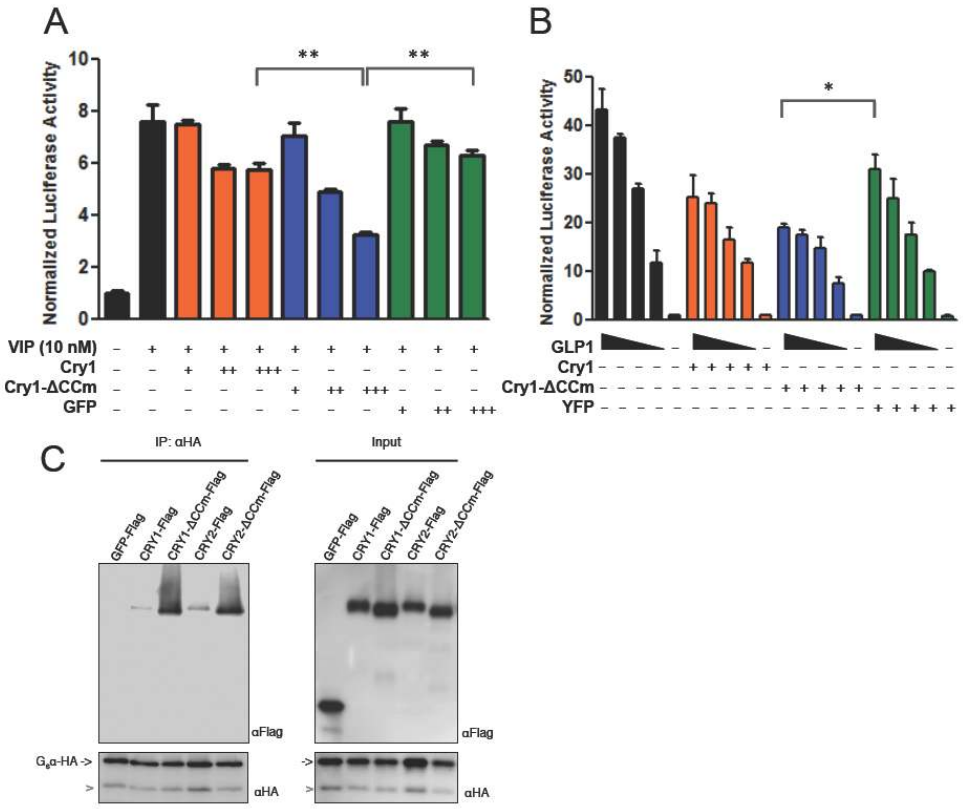


Figure 3-3. CRY1-ΔCCm inhibits CRE-Luc activity with higher potency than wild-type CRY1. (A) CRE-Luc activity in HEK293T cells expressing Vipr2. CRE-Luc and Vipr2 were co-transfected along with Cry1 (orange), Cry1-ΔCCm (blue), and GFP (green). Eighteen hours after transfection, cells were treated with 10 nM of VIP. (B) CRE-Luc activity of HEK293T-GLP1R-CRE-Luc transfected with Cry1 (orange), Cry1-ΔCCm (blue), and GFP (green). Twenty-four hours after transfection, cells were stimulated with 10 nM of GLP1 and subsequent serial dilutions together with Luciferin substrate. (C) Co-immunoprecipitation of CRY and $G_s\alpha$. Top, immunoblot showing recovery of CRY-Flag from immunoprecipitated $G_s\alpha$ (bottom). IP, immunoprecipitation. *$P < 0.05$; **$P < 0.01$; all values represent mean + s.e.m.

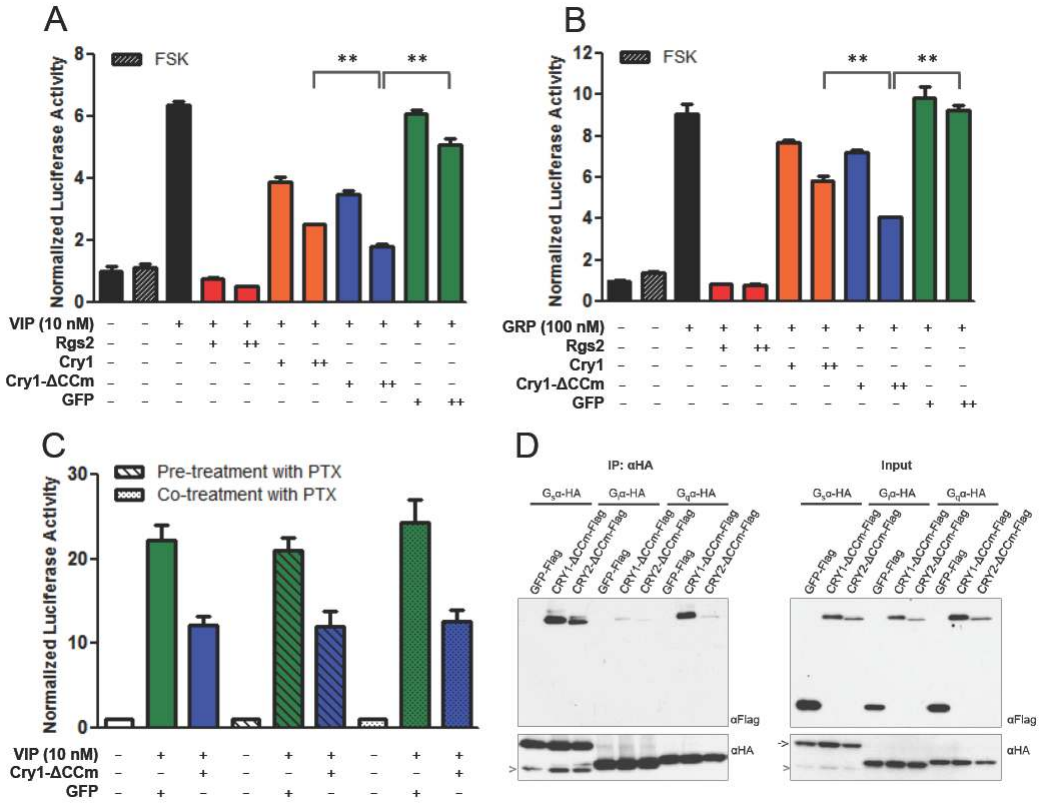
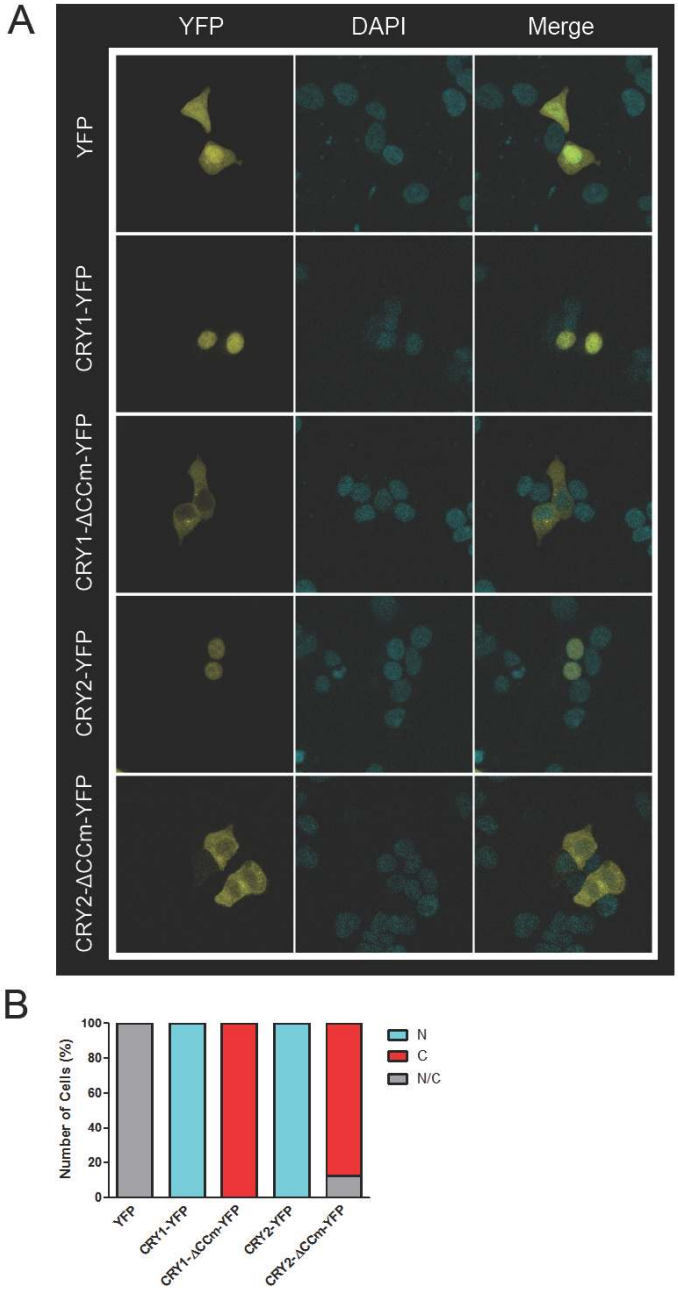
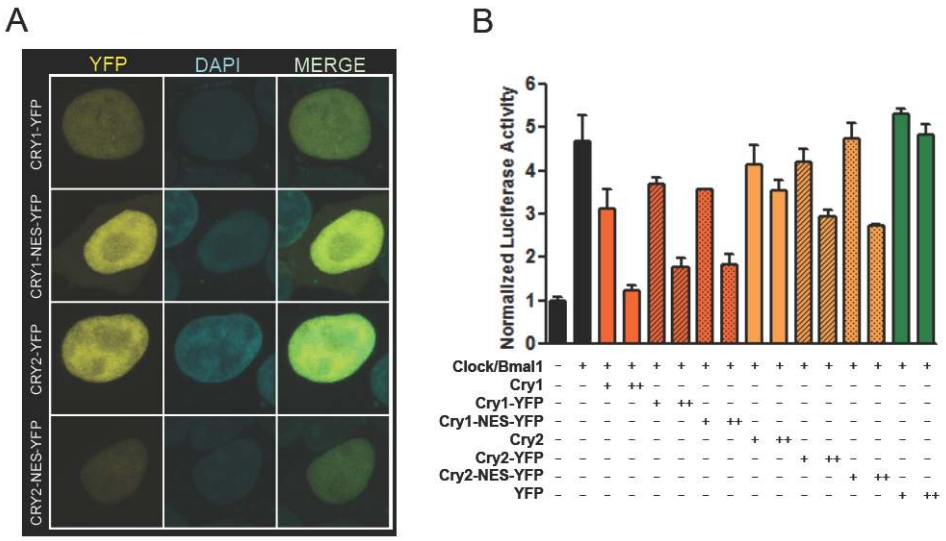


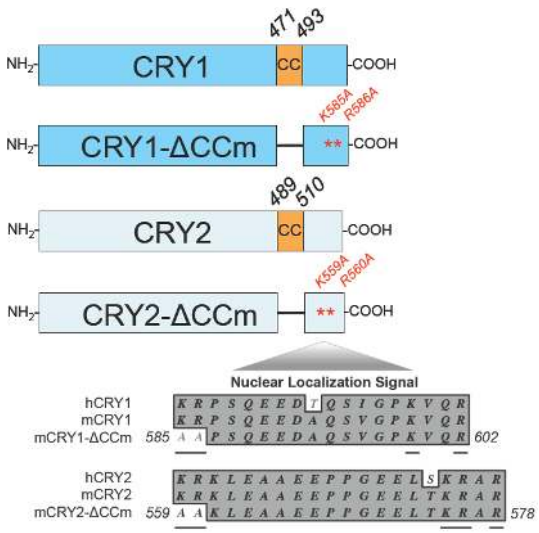
Figure 3-4. CRY1-ΔCCm inhibits NFAT-Luc activity with higher potency than wild-type CRY1. (A) NFAT-Luc activity in HEK293T cells expressing Vipr2. NFAT-Luc and Vipr2 were co-transfected along with Rgs2 (red), Cry1 (orange), Cry1-ΔCCm (blue), and GFP (green). Eighteen hours after transfection, cells were treated with 10 nM of VIP. (B) NFAT-Luc activity in HEK293T cells expressing GRPR in a similar setup as shown in (A). Eighteen hours after transfection, cells were treated with 100 nM of GRP. (C) CRE-Luc activity in HEK293T cells expressing Vipr2. CRE-Luc and Vipr2 were co-transfected along with Cry1-ΔCCm (blue) and GFP (green). Cells were treated with 10 μM of Pertussis toxin (PTX) for 5 hours prior to or concurrently with VIP stimulation. (D) Co-immunoprecipitation assay of CRY1-ΔCCm and CRY2-ΔCCm with G_sα, G_iα, or G_qα. Top, immunoblot showing recovery of CRY-Flag from immunoprecipitated Gα, G_iα, or G_qα (bottom). FSK, forskolin (10 μM). IP, immunoprecipitation. ***P* < 0.01; all values represent mean + s.e.m.



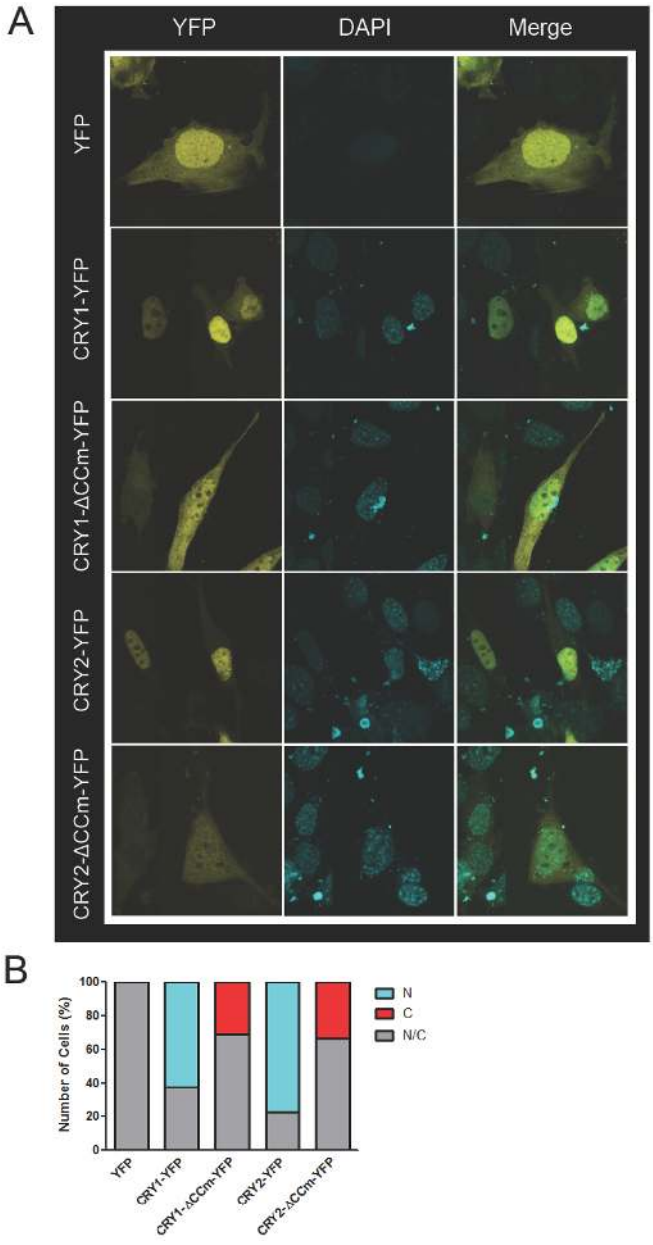
Supplementary Figure 3-1. CRY1/2 and CRY1/2-ΔCCm are distinctly localized in the nucleus and cytoplasm of HEK293T cells, respectively. (A) Confocal fluorescence images of HEK293T overexpressing CRY1-YFP, CRY1-ΔCCm-YFP, CRY2-YFP, CRY2-ΔCCm-YFP, and YFP. (B) Quantification of the subcellular localization of the overexpressed proteins in HEK293T cells. N, nuclear, blue. C, cytoplasmic, red. N/C, nucleo-cytoplasmic, grey.



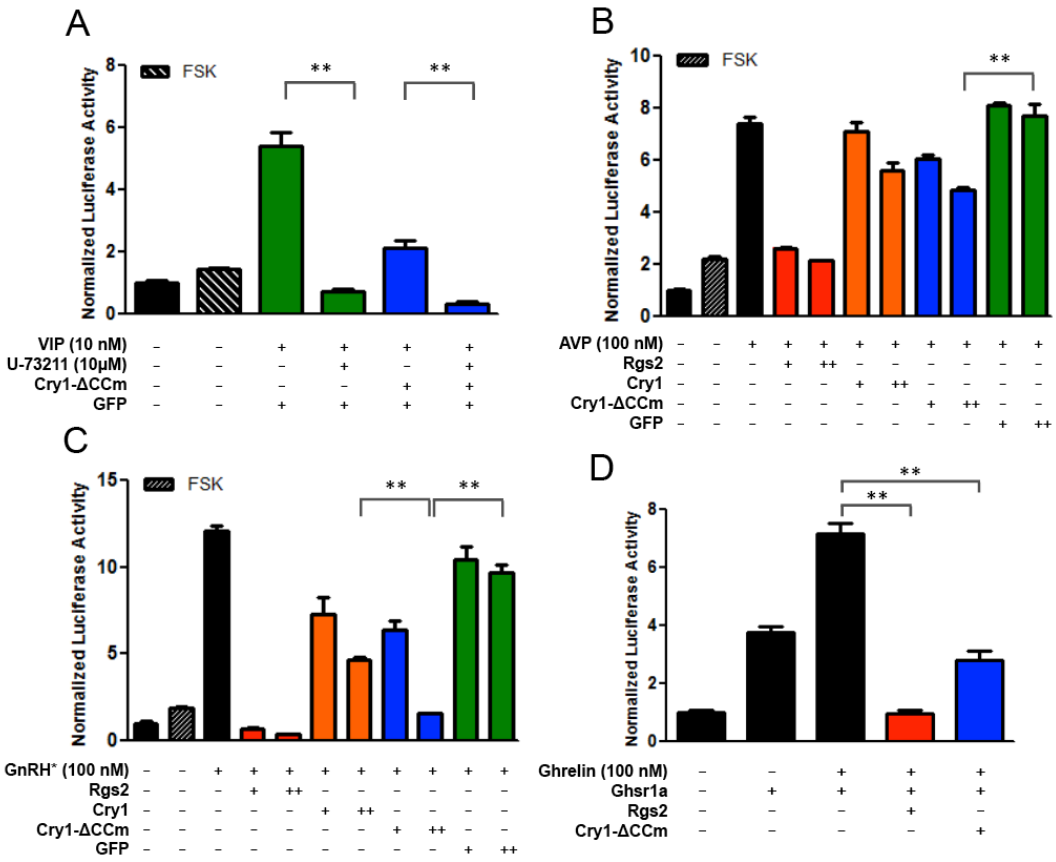
Supplementary Figure 3-2. Nuclear export signal (NES) is not sufficient for CRY cytoplasmic localization. (A) Confocal fluorescence images of HEK293T expressing CRY1-YFP, CRY1-NES-YFP, CRY2-YFP, and CRY2-NES-YFP. (B) CLOCK/BMAL1-mediated activation of *Per2* promoter driven-Luc reporter activity. HEK293T cells were co-transfected with Cry1, Cry1-YFP, Cry1-NES-YFP, Cry2, Cry2-YFP, Cry2-NES-YFP, and YFP.



Supplementary Figure 3-3. Schematic representation of CRY1-ΔCCm and CRY2-ΔCCm constructs. CRY1-ΔCCm contains a deletion at coiled-coil (CC) domain (amino acids 471-493) and substitution mutations at nuclear localization signal (K585A R586A) as previously described in Ref. (33). Analogously, CRY2-ΔCCm contains a CC domain deletion (amino acids 489-510) and substitution mutations at nuclear localization signal (K559A R560A).



Supplementary Figure 3-4. Subcellular localization of CRY1/2 and CRY1/2-ΔCCm in *Cry1*$^{-/-}$*;Cry2*$^{-/-}$ *Per2*^{Luc} mouse adult fibroblasts (MAFs). (A) Confocal fluorescence images of *Cry1*$^{-/-}$*;Cry2*$^{-/-}$ *Per2*^{Luc} MAFs overexpressing CRY1-YFP, CRY1-ΔCCm-YFP, CRY2-YFP, CRY2-ΔCCm-YFP, and YFP. (B) Quantification of the subcellular localization of the overexpressed proteins in *Cry1*$^{-/-}$*;Cry2*$^{-/-}$ *Per2*^{Luc} MAFs. N, nuclear, blue. C, cytoplasmic, red. N/C, nucleo-cytoplasmic, grey.



Supplementary Figure 3-5. CRY1 and CRY1-ΔCCm mediate NFAT-Luc activity of G_q-coupled receptors via phospholipase C/IP_3. (A) NFAT-Luc activity in HEK293T cells expressing Vipr2. NFAT-Luc and Vipr2 were co-transfected along with GFP (green) and Cry1-ΔCCm (blue). Cells were treated with 10 μM of U-73211 (a PLC inhibitor) 30 minutes before VIP stimulation. (B and C) NFAT-Luc activity assays were set up similar to that of Fig. 3A, and cells were stimulated with 100 nM of GnRH agonist (GnRH*) (A) and 100 nM of AVP (B) 18 hours post-transfection. (D) NFAT-Luc activity of Cry1-ΔCCm in ghrelin receptor assay. HEK293T cells were co-transfected with NFAT-Luc and Ghsr1a along with Rgs2 (red) or Cry1-ΔCCm (blue). Cells were stimulated with 100 nM of ghrelin 18 hours post-transfection. FSK, forskolin (10 μM). $^{**}P < 0.01$; all values represent mean + s.e.m.

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P. Pongsawakul performed experiments that contributed to Figs. 3-1 to 3-4 and Supplementary Figs. 3-1, 3-2, 3-4, and 3-5.

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Chapter 4: Discussion

4.1 Circadian Timing in Health and Disease

Virtually all mammalian tissues have endogenous circadian clocks that are self-sustainable (1). The master pacemaker SCN synchronizes these peripheral clocks throughout the body to appropriate phases. The SCN can synchronize peripheral oscillators by sympathetic output, hormonal signals, core body temperature, rest-activity cycle, and feeding-fasting cycle (2). It is believed that jet-lag is a symptom where different clock tissues are out of synchronization from each other (3).

Approximately 10-20% of genes in each peripheral tissue exhibit rhythmic expression, and the majority of them are tissue-specific. In the liver, many of these rhythmic clock-controlled genes encode enzymes that are rate-limiting factors of fundamental pathways such as glycolysis, fatty-acid metabolism, cholesterol biosynthesis, and xenobiotic. In addition, more than half of the nuclear receptor family members also display rhythmic expression in various tissues. Because many of the nuclear receptors are themselves directly involved in cellular metabolism, the circadian clock may impose temporal metabolic regulation via nuclear receptors (4).

Mounting evidence demonstrates the important regulation of circadian clocks in metabolism. By studying circadian clock mutant animals, researchers are

able to uncover metabolic disorders as a result of clock disruption. In *Clock*$^{\Delta 19}$ mutant mice, altered feeding behavior is observed even though the locomotor activity is similar to that of the wild-type mice. *Clock*$^{\Delta 19}$ mutant mice consume significantly more food during light period (resting period for nocturnal rodents), and the net energy intake and body weight are also higher than the wild-type mice (5). Comparing metabolic parameters, *Clock*$^{\Delta 19}$ mutant mice have hypercholesterolemia, hypertriglyceridemia, hyperglycemia, and hyperinsulinemia when fed a regular diet. These symptoms were exacerbated when the mutant mice were fed a high-fat diet. This is the first time that circadian mutant mice were used to demonstrate that disruption in molecular clock results in metabolic disorders.

In another study using *Clock*$^{\Delta 19}$ mutant mice, Bass and colleagues found that the mutant mice have elevated blood glucose during light and dark periods (6). The *Clock*$^{\Delta 19}$ mutant mice also display reduction in insulin release upon a glucose tolerance test, suggesting a defect in pancreatic function. In order to elucidate the specific function of pancreatic clock in metabolism, Bass and colleagues used pancreas-specific *Bmal1* deletion mice. These conditional knockout mice have disrupted circadian clock only in the pancreas, but display elevated glucose throughout the day, as well as impaired glucose tolerance and decreased insulin secretion (6). This study

and others signified functions of the peripheral circadian clocks in metabolism regulation (6-8).

In a human study performed in a laboratory, Scheer and colleagues found that circadian misalignment significantly reduces leptin levels and increases postprandial glucose and insulin (9). Furthermore, Buxton and colleagues studied the effect of circadian disruption in combination with sleep deprivation in a laboratory environment that mimics shift work. Researchers found that young and old participants exhibit elevated blood glucose and reduced insulin similar to prediabetic patients after 3-week exposure of circadian disruption and sleep restriction protocols (10). These results emphasize the adverse metabolic consequences of circadian disruption and sleep deprivation that shift workers commonly experience. Therefore, it is not surprising that many population studies find that night-shift work is associated with a higher body mass index and increases the likelihood of developing insulin resistance (11).

4.2 Cryptochrome in Metabolic Control

As core clock components, Cryptochromes (CRY1 and CRY2) negatively regulate their own genes and many other clock output genes (12). A recent study has shown that CRYs interact with the glucocorticoid hormone receptor and alter transcriptional response to glucocorticoids (13). One of the

targets of CRY:glucocorticoid is the phophoenolpyruvate carboxykinase 1 (Pck1) gene, that encodes a rate-limiting gluconeogenic enzyme in the liver. Upon chronic glucocorticoid treatment, mice lacking both *Cry1* and *Cry2* exhibit fasting hyperglycaemia due to an upregulation of Pck1. This study highlights a novel role of CRYs with the glucocorticoid nuclear receptor that regulates many physiological processes including inflammation and metabolism beyond the CLOCK/BMAL1 repression. Supporting the notion of a non-clock function of CRY, we found that cytoplasmic CRY inhibits hepatic gluconeogenesis via cAMP signaling. These rhythmic functional "outputs" of CRY in the nucleus and cytoplasm may result from the circadian rhythm of CRY protein levels that are directly regulated by the core feedback loops. As a result, gluconeogenesis is rhythmically regulated by the circadian clock via CLOCK/BMAL1 and by the clock-outputs nuclear CRY and cytoplasmic CRY via glucocorticoid pathway, and cAMP pathway, respectively (13-15).

Using cytoplasmic mutants Cry1-ΔCCm and Cry2-ΔCCm, we demonstrate that cytoplasmic CRY interacts with $G_s\alpha$ and $G_q\alpha$ subunits of the G protein and inhibits the cAMP and Ca^{2+} signaling, respectively (Fig. 4-1). The mechanism by which CRY inhibits the activity of $G_s\alpha$ and $G_q\alpha$ is still elusive. However, the ability of CRY to repress $G_s\alpha$ and $G_q\alpha$ functions is similar to that of a protein known as regulator of G protein signaling 2 (RGS2). RGS2 is a GTPase activating protein (GAP) of $G_q\alpha$, but not $G_s\alpha$ (16).

However, RGS2 can block cAMP production by interacting with $G_s\alpha$ and adenylyl cyclase in living cells (17, 18). A recent study reported that CRY also interacts with adenylyl cyclase (19). From these results, it is tempting to speculate that CRY may interact and co-regulate with RGS2 in repressing $G_s\alpha$ and $G_q\alpha$ signaling. *Rgs2* RNAi may be a useful tool in elucidating whether CRY inhibition is dependent on RGS2, and *Cry1* or *Cry2* RNAi can be used to test the reciprocal speculation. If RGS2 and CRY co-regulate, it is likely that they would interact. An *in vitro* binding assay or co-immunoprecipication may be performed to test this hypothesis.

In the SCN, paracrine signaling via GPCRs such as $VPAC_2$, BB_2, and AVPR1 is important for synchronization among individual SCN neurons, in addition to the synapses. Mice lacking VIP or $VPAC_2$ display arrhythmic locomotor activity under constant darkness, and individual SCN neurons are out of synchronization (20, 21). Furthermore, in a co-culture experiment, a graft wild-type SCN slice can restore the circadian gene expression of host SCN slices that lacks VIP or $VPAC_2$ even though the slices are separated by a semipermeable membrane (22). These studies suggest that SCN neurons communicate at the intercellular level via paracrine signaling, and this communication transduces inside the cell via G protein-mediate pathways such as cAMP or Ca^{2+} signaling.

cAMP signaling is shown to be an integral component of the transcriptional oscillator. Using pharmacological perturbations against components of the cAMP signaling pathway, Hastings and colleagues demonstrated that disruption of intracellular cAMP production alters the period and amplitude of the *Per2*^{Luc} reporter, suggesting that a cytosolic signal can drive the transcriptional oscillator (23). On the other hand, the core transcription oscillation also regulates the pace of cAMP and Ca^{2+} rhythms in the SCN. Using CRE-Luc and GCaMP3 as reporters for intracellular cAMP and Ca^{2+}, respectively, Hastings and colleagues found that the period of cAMP and Ca^{2+} rhythms in SCN slices are altered in accordance with the intrinsic locomotor period of the circadian mutant mice where the SCN are isolated from (24).

The interaction of both transcription-translation feedback loops and cytosolic loops are important to maintain robust circadian rhythmicity of the SCN (25). Since CRY is a core component of the circadian clock that directly regulates the intracellular signaling of the GPCR, CRY may be a molecular link for the transcription-translation loops that control the pace of cAMP and Ca^{2+} rhythms in the SCN neurons. Future experiments will be needed to elucidate the effects of cytoplasmic CRY in the SCN. We speculate that overexpression of cytoplasmic CRY protein using Cry1-ΔCCm or Cry1-ΔCCm

construct will disrupt the cAMP and Ca^{2+} rhythms in the SCN slices, and the rhythms will be more severely disrupted in isolated SCN neurons.

We have previously shown that overexpressing Cry1 in the liver reduces blood glucose and improves glucose tolerance test in diabetic *db/db* mice (15). Developing small molecules that activate CRY would potentially reduce blood glucose and be clinically beneficial. Indeed, a CRY-activating small molecule KL001 represses glucagon-mediated stimulation of glucose production in primary hepatocyte culture (26). With a recent identification of an ubiquitin ligase FBXL21 that stabilizes CRY in the cytoplasm, screening of small molecules that stabilize the interaction of FBXL21 and CRY would be beneficial for circadian biology and future therapeutics (27, 28). The cytoplasmic CRY-activating small molecules will be useful reagents to study the physiological functions of cytoplasmic CRY *in vivo* and tissues *ex vivo*. More importantly, these cytoplasmic CRY-activating small molecules could be developed as treatments to reduce hepatic gluconeogenesis via cAMP production inhibition in individuals with type 2 diabetes with a minimal disruption of the core transcription oscillator in the nucleus.

GPCRs are the most common target of therapeutic drugs, and many are regulated via cAMP and Ca^{2+} signaling pathways. Our findings suggest that CRY may temporally regulate intracellular responses to the therapeutic drugs, which may result in circadian variations of drug potency. In addition,

many drugs target biological processes that are directly controlled by canonical clock regulation including cell cycle (29). Clinical treatment that considers the internal timing of patients or chronotherapy may be a potential application for personalized medicine in the future.

4.3 Figure

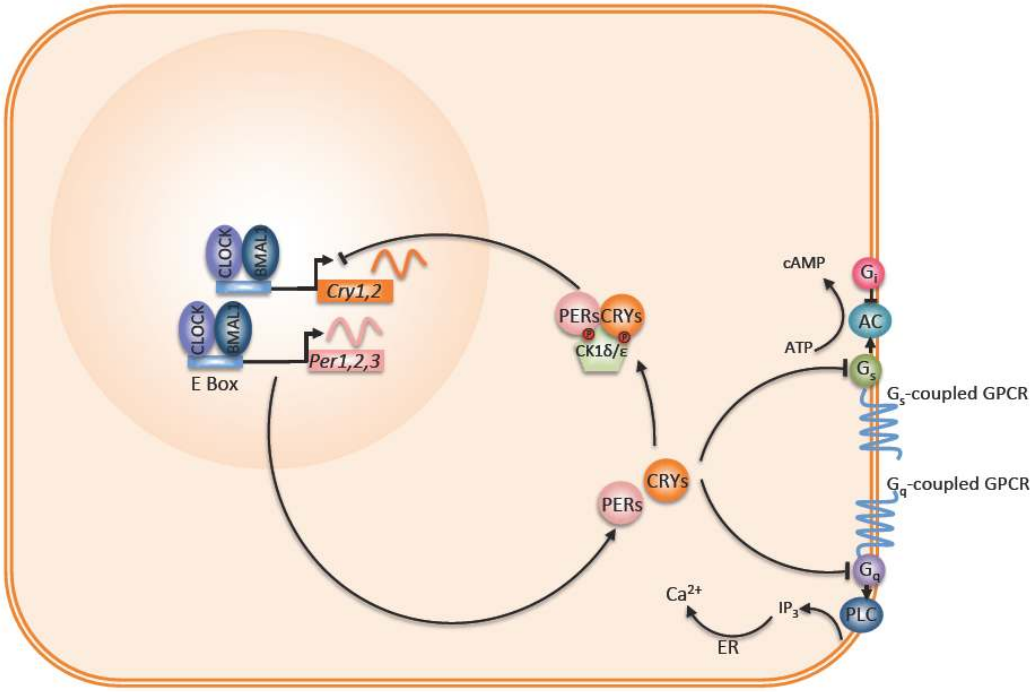

Figure 4-1. Cytoplasmic Cryptochrome inhibits cAMP signaling and Ca^{2+} signaling. Cytoplasmic Cryptochrome interacts with $G_s\alpha$ and $G_q\alpha$ subunits of the G protein and inhibits cAMP signaling and Ca^{2+} signaling, respectively.

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