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Genetic and Biochemical Dissection of Differential Functions of Cryptochrome 1 and 2 in the Mammalian Circadian Clock

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GENETIC AND BIOCHEMICAL DISSECTION OF DIFFERENTIAL FUNCTIONS
OF CRYPTOCHROME 1 AND 2 IN THE MAMMALIAN CIRCADIAN CLOCK

by

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DEDICATION

I would like to dedicate my dissertation to my parents, my wife and my mentor Dr. Andrew Liu. Without their help, inspiration and support it would not have been possible for me to achieve this degree.

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ABSTRACT

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Circadian clocks in mammals are based on a negative feedback loop in
which transcriptional repression by the Cryptochromes, CRY1 and CRY2, lies at
the heart of the mechanism. Despite similarities in their sequence, domain
structure and biochemical activity, they play distinct roles in the mammalian clock
function. However, detailed biochemical studies have not been straightforward
and function of *Cryptochrome* (*Cry*) has not been examined in real clock cells
using kinetic measurements. In this study, we demonstrate, through cell-based
genetic complementation and real-time molecular recording, that *Cry1* alone is
able to maintain cell-autonomous circadian rhythms, while *Cry2* cannot. Using
this novel functional assay, we identify a Cryptochrome differentiating α-helical
domain within the photolyase homology region (PHR) of CRY1 protein,
designated as CRY1-PHR(313-426), that is required for clock function and
distinguishes CRY1 from CRY2. Further, in contrast to speculation, we
demonstrate that the divergent carboxyl-terminal tail domain (CTD) is
dispensable for circadian clock function, but it serves to modulate rhythm
amplitude and period length. Finally, we identify the biochemical basis of their
distinct function; CRY1 is a much more potent repressor of BMAL1/CLOCK
transcriptional activity than CRY2, and the strength of repression by various
forms of CRY proteins significantly correlates with rhythm amplitude. Taken
together, our results demonstrate that the CRY1-PHR(313-426), not the

divergent CTD, is critical for clock function. These findings provide novel insights into the evolution of the diverse functions of the photolyase/cryptochrome family of flavoproteins and offer new opportunities for mechanistic studies of CRY function.

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LIST OF ABBREVIATIONS AND SYMBOLS

ARNT	aryl hydrocarbon receptor nuclear translocator
bHLH	basic helix-loop-helix
BMAL	brain and muscle ARNT-like protein
bZIP	basic leucine zipper
CC2	coiled-coil 2
CK1	casein kinase 1
CLK	CLOCK
CLOCK	circadian locomotor output cycle kaput
CMV	cytomegalovirus
COP1	constitutively photomorphogenic1
CPD	cyclobutane-pyrimidine-dimers
Cry	Cryptochrome
CTD	carboxy terminal tail domain
CYC	CYCLE
DBP	D-box binding protein
dCRY	Drosophila cryptochrome
DMEM	Dulbecco's modified Eagle's medium
E.coli	Escherichia coli
E3	ubiquitin ligase enzyme
EST	expressed sequence tag
FAD	flavin adenine dinucleotide
FBS	fetal bovine serum
HEK	human embryonic kidney cell line

HEPES	hydroxyethyl piperazineethanesulfonic acid
HLF	hepatic leukemia factor
HRP	horseradish peroxidase
HTS	high-throughput screening
kDa	kilodaltons
KD	knock down
LD	light:dark
ml	milliliter
mM	millimolar
MTHF	methenyltetrahydofolate
ng	nanogram
NPAS2	neuronal PAS domain-containing protein 2
NR1D1	nuclear receptor subfamily 1, group D, member 1
NR1D2	nuclear receptor subfamily 1, group D, member 2
NS	non-specific
PAS	PER-ARNT-SIM domain
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDB	Protein Databank
Per	Period
PHL	photolyase
PHR	photolyase homology region
PMT	photomultiplier tube
PVDF	polyvinylidene fluoride
RHT	retino-hypothalamic tract

RIPA	radioimmunoprecipitation assay
RMSD	root mean square deviation
ROR	RAR-related orphan receptor
RRE	rev response element
SCN	suprachiasmatic nuclei
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	short hairpin RNA
SIM	single-minded protein
STH	salt tolerance-like protein
STO	salt tolerance protein
TEF	thyrotroph embryonic factor
TIM	timeless
UV	ultraviolet
WB	western blot
Δ	deleted

INTRODUCTION

Rotation of the earth around its axis and around the sun brings about daily and seasonal changes in our natural environment. To cope with these changes in the environment, natural selection has favored the evolution of endogenous time-keeping system or biological clocks. From unicellular organisms like cyanobacteria to complex organisms like fungi, plants, insects and vertebrates, including humans, endogenous clocks with a period length of ~24 hr have been evolved to regulate different physiology and behavior according to time of the day. These clocks are thought to provide an adaptive advantage by enabling organisms to *anticipate* daily environmental changes for their survival by enabling them to tailor their behavior and physiology to the specific time of the day (1,2). In mammals, daily behavioral and physiological rhythms such as sleep/wake cycle, hormone production, body temperature, blood pressure, metabolism etc. are driven by their endogenous time-keeping system (3,4). Genetic and environmental factors that affect this internal time-keeping system can cause clock-related disorders such as sleep disorders (delayed or advanced sleep phase syndrome), metabolic disorders, and cancer (5-7).

This internal time-keeping system is synchronized everyday with the light-dark cycle of the natural environment. This daily synchronization allows organisms to keep track of not only the daily cycles of day and night but also the change in duration of light-dark periods. This daily time-keeping system is known as circadian clock. (In Latin, circa means about, and diem means day; and circadian means about a day.) Interestingly, the circadian time-keeping system

that carries out daily adjustment is also used as a seasonal timer. The external cues are so-called "zeitgeber" or time-giver. For almost all living organisms on earth, light is the primary zeitgeber to the clock. As a result, specific photoreception and transduction mechanisms have evolved in these organisms (8,9). However, circadian clocks are self-sustainable oscillators; they run even in the absence of external zeitgebers. Circadian clocks are also temperature compensated (10); therefore, organisms can maintain their circadian rhythms over a range of environmental temperatures.

My dissertation focuses on mammalian circadian clocks. Here, I will first discuss the mammalian circadian time-keeping system followed by its underlying molecular mechanisms. Then, I will present the most current understanding of the structure and function of cryptochromes, CRY1 and CRY2, the core clock components of the mammalian clock. I will present *in vivo* and *in vitro* genetic evidences that suggested their differential functions in the clock mechanism. Prior to my study, the underlying basis of their differential functions was elusive. My dissertation aims at the understanding of the genetic and biochemical basis of the differential functions of CRY 1 and 2.

Hierarchy of Mammalian Circadian Time-keeping System–The mammalian circadian time-keeping system consists of mainly three components; input signals (environmental cues), a multi-oscillator network (circadian rhythm generator) and output signals (circadian overt rhythms) (11). The multi-oscillator network is a hierarchical system in mammals. At the organismal level, the suprachiasmatic nucleus (SCN) of the anterior hypothalamus is the master

oscillator that regulates circadian behavioral activities. Ablation of SCN resulted in complete loss of circadian rhythms whereas transplantation of intact SCN from a wild type animal to a mutant resulted in reestablishment of circadian rhythms (12,13). Therefore, SCN is accepted as the master oscillator.

The molecular clock machinery of this master oscillator is contained in individual neurons. Intercellular coupling among these neurons serves to synchronize their rhythms and also confers system robustness to the SCN, a special attribute of the SCN network (14). Remarkably, cell- or tissue-autonomous peripheral oscillators (slave oscillators) are present in all the tissues of the body. The presence of peripheral oscillators was demonstrated by rhythmic gene expression in different tissue explants and in cultured fibroblasts (15,16). Unlike SCN, these peripheral oscillators cannot be entrained by light cues; rather, the SCN, the master clock, functions to synchronize all the peripheral slave oscillators throughout the body (17,18). The SCN synchronizes peripheral oscillators using systemic, neuronal and humoral routes (19). Upon synchronization, these peripheral oscillators generate overt circadian rhythms such as sleep-wake cycle, rhythms in hormone secretion, blood pressure, metabolism etc as their output (Fig. 1). Genetic studies have demonstrated that the molecular composition of the timing mechanism in the SCN clock and peripheral clocks is very similar (14,20). Thus, cultured cells are the functional units for rhythm generation and maintenance and provide an experimentally more tractable *in vitro* system for mechanistic studies and gene discovery related to circadian clocks.

Transcriptional Feedback Loops Underlie The Molecular Clock

Mechanism–Circadian rhythms are emanated ultimately from the cell-autonomous clocks. In general, all circadian systems described so far are composed of an autoregulatory negative feedback loop that contains both positive and negative transcriptional components (17,21-23). In the mammalian system, the molecular clock consists of two inter-connected transcription/translation feedback loops. The positive elements (activators) in the core loop include the two basic helix-loop-helix (bHLH)/PAS domain-containing transcription factors, BMAL1 (brain and muscle ARNT-like protein or ARNTL1) and CLOCK (circadian locomotor output cycle kaput). They form a heterodimer which binds to the E-box element within the promoter region and activates the transcription of many genes including Periods (*Per1, 2* and *3*), Cyrptochromes (*Cry 1* and *2*) and *Rev-Erbα*. The repressors PER and CRY translocate to the nucleus in which they interact with BMAL1/CLOCK heterodimer and inhibit their own transcription.

In a separate auxiliary loop (interconnected with major loop), circadian oscillation of *Bmal1* expression is regulated by a balanced act of transcription activator ROR-α and repressor *Rev-Erb-α* (23) (Fig. 2). Thus, in the auto-regulatory negative feedback loops, positive components drive the expression of negative components, that in turn repress the activity of the activators and therefore the expression of the repressors; this regulation thus constitutes the second negative feedback loop. Post-translational mechanisms play critical roles
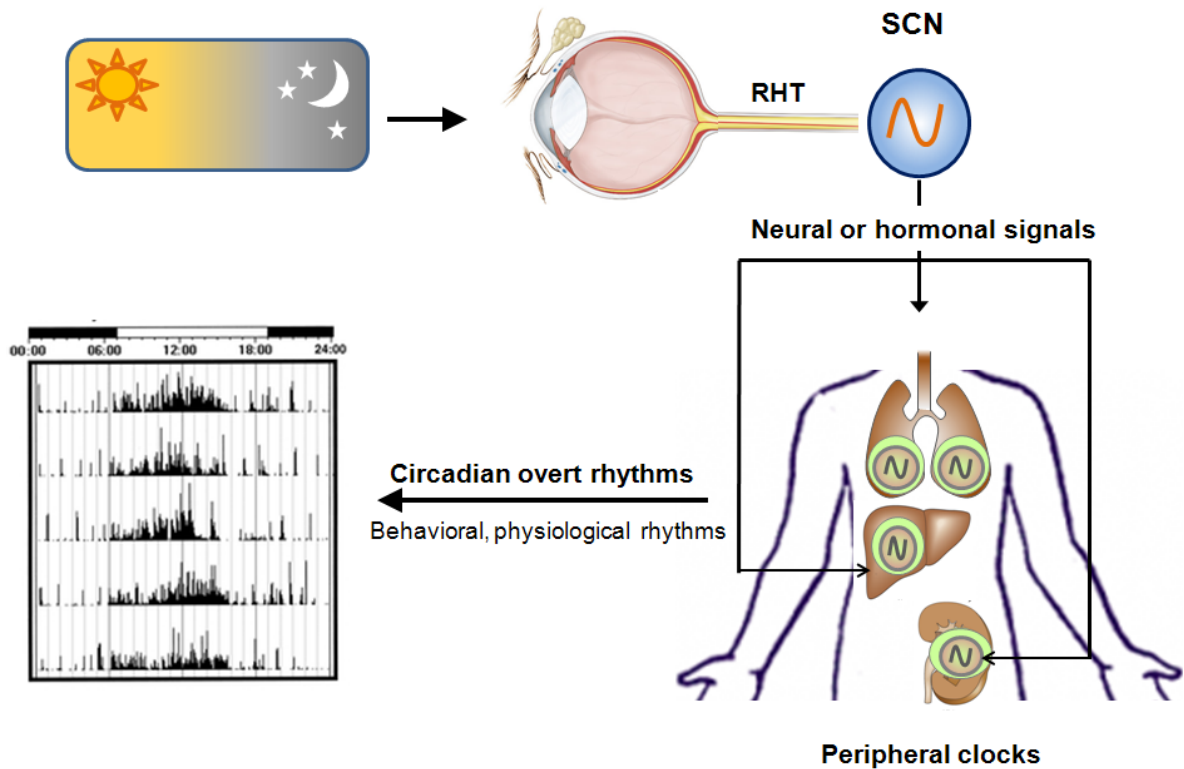
FIGURE 1**. Hierarchy of mammalian circadian time-keeping system.** Daily oscillation of light/dark cycle is directly perceived by eyes. The light cue is then transported through retinohypothalamic tract (RHT) to the master oscillator suprachiasmatic nucleus (SCN) located in the anterior hypothalamus. The SCN acts as the master oscillator, which is entrained by the light/dark cycle and generates concerted rhythms in its output. The output signals from SCN entrain the peripheral oscillators located in different organs in the body. Upon synchronization, the peripheral oscillators generate overt rhythms in different physiology and metabolisms of the organism. The external cues for entrainment, the master and slave oscillators, and the output rhythms are the three basic components of mammalian circadian time-keeping system.

in regulating protein turnover of the key components, thus temporally separating the positive and negative components and preventing the clock from reaching equilibrium, thereby causing rhythmic gene expression with a period length of ~24 hr (24-27).

Circadian rhythms in all living organisms consist of several basic characteristics and parameters (Fig. 3). Circadian rhythms are entrainable to environmental cues known as zeitgebers. The most dominating zeitgeber for most of the organisms is the daily light:dark (LD) cycle. Circadian rhythms are self-sustainable; they persist even in absence of any external cues and these rhythms are known as free-running rhythms. In mammals, free-running rhythms are generally referred to as behavioral rhythms under constant darkness. Similarly, cell- or tissue-autonomous rhythms are also observed when they are cultured under constant conditions. The phase of a circadian rhythm is defined as the timing of peaks relative to a fixed event (e.g., beginning of the night phase). The time-lapse between two phase reference points (e.g., two peaks) is known as period length of the rhythms. The difference between the peak (or trough) and the mean value is called amplitude (Fig. 3).

In mammalian clocks, the transcriptional mechanism is regulated by at least three DNA elements; morning time element E-box/E'-box (18,28-30), day-time element D-box (18,31), and night-time element RRE (18,32-34). E-box/E'-box-regulated transcription is mediated by at least 11 transcription factors, including four bHLH-PAS domain containing transcription activators CLOCK, NPAS2, BMAL1 and BMAL2 (35-38), two transcription repressors, CRY1 and
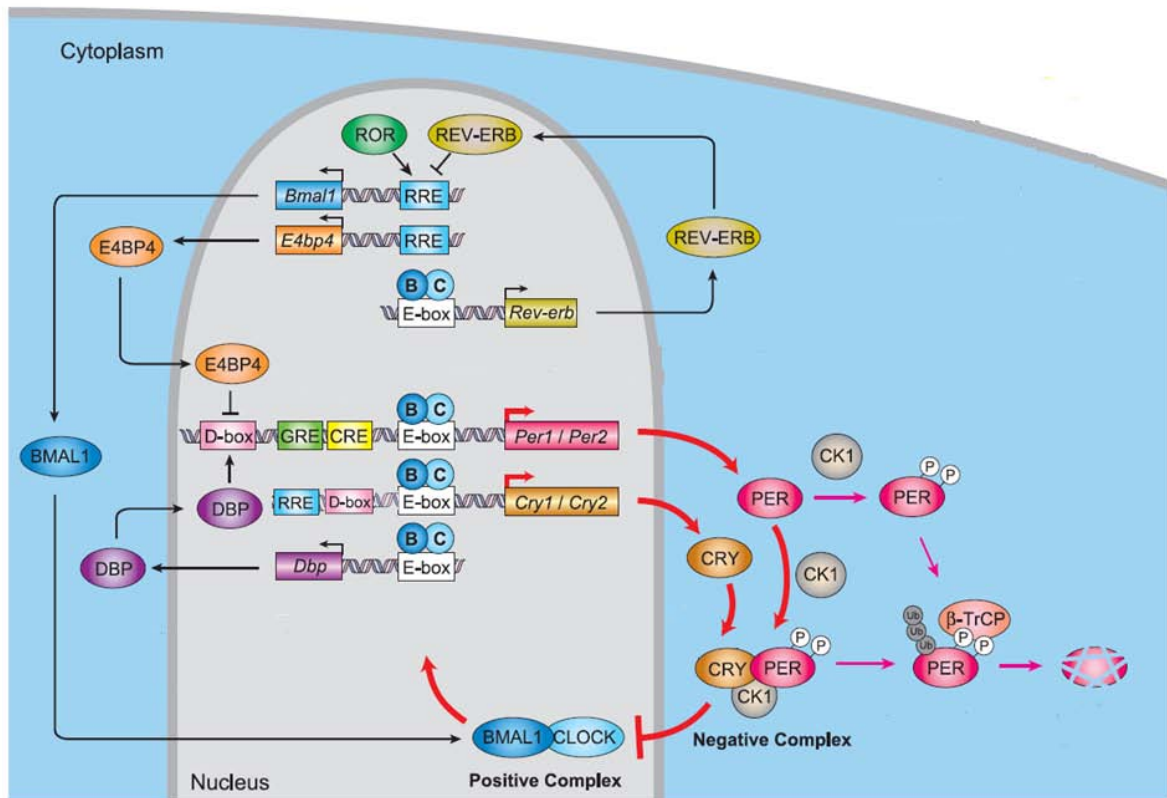
FIGURE 2. **Mammalian circadian clock network consisting of two interlocking negative feedback loops.** The heterodimer BMAL1/CLOCK acts as transcription activators by interacting with the E-box located in the promoter region of genes including *Per* and *Cry.* PER and CRY in turn suppress their own transcription by inhibiting BMAL1/CLOCK transcriptional activity. Upon phosphorylation by CK1, PER is targeted for proteosomal degradation which results in starting of next cycle. Assisting this core loop, DBP and E4BP4 regulate *Per* expression by acting as transcriptional activator and repressor of D-box element, respectively. In the interconnected Bmal1 feedback loop, rhythmic expression of Bmal1 is regulated by the action of transcriptional activator ROR and repressor REV-ERB on circadian element, RRE. These two interlocking loops together regulate circadian rhythms of additional genes with additional phases. Adapted with modifications from Doi, M. 2012 (39).

CRY2 (24) and three period proteins, PER1, PER2 and PER3 (40-42). The D-box-mediated transcription is regulated by four bZIP transcription factors, DBP, HLF, and TEF as activators, and E4BP4 as a repressor (43-46). The RRE-mediated transcription is controlled by five orphan nuclear receptors, REV-ERBα and REV-ERBβ repressors (also known as NR1D1 and NR1D2) and ROR-α, ROR-β and ROR-γ activators (23,33,34). All these circadian elements, in combination or individually, regulate the expression phases of the clock genes resulting in a complex clock network which generates circadian rhythms in mammals.

Among all clock genes, interestingly, *Cry1* has a unique expression pattern that peaks at the evening phase (~CT16-20), which is distinct from day or night phase and is not accounted for by any one of the circadian elements. *Cry1* peak expression is at an intermediate phase in between D-box- and RRE-regulated gene expression (18,34). The *Cry1* expression pattern (i.e., rhythm and phase) plays an important role in the core regulatory loop of the circadian clock network.

Previous studies identified an E/E'-box within *Cry1*'s regulatory region (18,47) and two RREs within the first intron of the *Cry1* gene. Recently we identified additional D-boxes in the promoter region of *Cry1* and showed that a synthetic promoter contacting all three types of circadian elements conferred evening-time expression of a luciferase reporter, likely through combinatorial regulation. Further, this delayed *Cry1* expression was required for circadian clock function (48). Among the PERs and CRYs in mammals, CRYs are much more
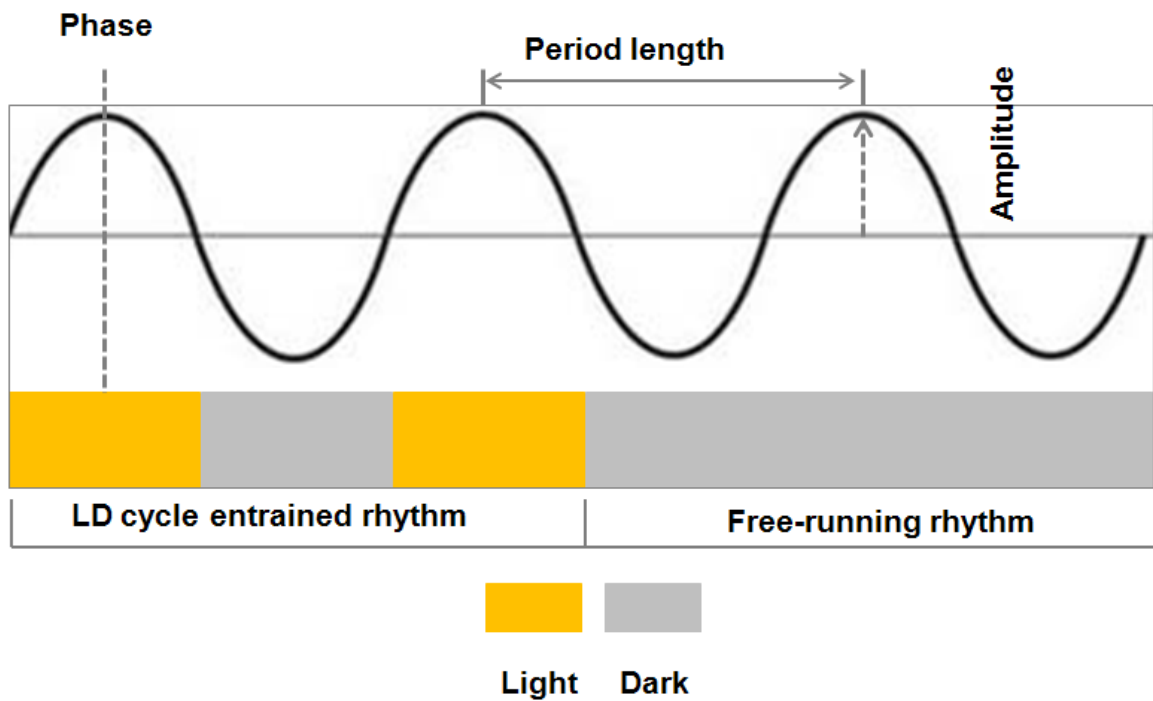
FIGURE 3. **Parameters of circadian rhythms.** Circadian rhythms in all living organisms are entrained by LD cycle in the natural environment. They exhibit free-running rhythms under constant darkness. The phase is the timing of peaks with relative to a fixed event (e.g. beginning of dark phase). The time lapse between two peaks is called period length of the rhythm. The difference between peak (or trough) and midline is called amplitude.

potent repressors than PERs (24); however, the underlying mechanism of repression by the CRYs is still largely elusive.

Cryptochromes: origin and classification– CRYs belong to the photolyase/ cryptochrome super-family of flavoproteins (49). These two classes of flavoproteins use the same two cofactors, flavin and pterin methenyltetrahydofolate (MTHF), to carry out distinct biological functions (50,51). Photolyases are DNA repair enzymes that revert UV-induced photoproduct inside DNA – cyclobutane-pyrimidine-dimers (CPD) and (6-4) pryrimidine-pyrimidone photolesions – back to the original pyrimidine-pyrimidine dinucleotides to maintain genetic integrity of the organisms (52). Though there are controversies about the evolution of the cryptochromes, it is largely accepted that they are evolved from photolyases.

Cryptochrome was first discovered in *Arabidopsis thaliana* in 1993 when the HY4 gene was isolated (53). *Arabidopsis* seedlings that grow under light exhibit different photomorphogenic responses from those that grow under dark, such as inhibited stem elongation and stimulated leaf expansion. Seedlings under light grow significantly shorter stems (hypocotyl) than those grown in darkness; this response is governed by blue (400-490 nm), red (600-700 nm) and far-red (700-750 nm) light. Genetic screen for hypocotyl mutants identified several mutants (hy) in which mutations result in inability to respond to one or more of these monochromatic lights (54). Among all the mutants, the hy4 mutant lost its ability to respond to blue light selectively, indicating a disruption of a blue-light photoreceptor or a component in blue-light signaling pathway.

Subsequently, cloning of the HY4 gene revealed significant sequence homology to *E.coli* photolyase (55). However, unlike photolyases, the recombinant protein did not display any DNA repair activity (56,57). These biochemical studies of Hy led to identification of the first member in a novel class of blue-light photoreceptors. Since they share a high percentage of sequence homology to photolyase yet without DNA repair activity, they were named accordingly cryptochromes (CRY) ever since. The second Cryptochrome gene, *Cry2*, was cloned in 1996 and was shown to have effect only under dim light conditions (58,59).

Many CRYs in other species have since been discovered. On the basis of their sequence similarities, CRYs can be classified into three categories; plant cryptochromes, animal cryptochromes and cryptochrome-DASH (DASH= *D*rosophila, *A*rabidopsis, *S*ynechocystis, *H*omo) reviewed by (60).

Plant Cryptochrome– Since cryptochrome was discovered first in *Arabidopsis thaliana*, it has been the most used plant species for studying cryptochrome function in plants. *Arabidopsis* cryptochromes (*Cry1* and *2*) have the greatest effect on the photomorphogenic response. They have complementary functions in regulating photmorphogenic responses, as *Cry1* mainly functions under bright light and *Cry2* functions under dim light conditions. *Arabidopsis* cryptochromes exert their photomorphogenic effects by regulating gene expression. Approximately, one-third of *Arabidopsis* genes have light dependent change in their expression. It has been shown that cryptochromes acting as blue-light photoreceptors in *Arabidopsis* regulate light-dependent gene

expression and thus, photomorphogenic responses (61,62). Regulation of light-dependent gene expression by cryptochrome is thought to be mediated by its direct interaction with E3 ubiquitin ligase COP1. In the dark, COP1, a zinc-finger protein, degrades bZIP transcription factors including HY5, STO, STH, and HFR (63-65). In the presence of light, COP1 is translocated out of the nucleus and thus its ligase activity on transcription factors is inhibited (64,66-68), thus allowing for accumulation of transcription factors and subsequent regulation of gene expression. CRY1 and CRY2 have been shown to directly interact with COP1 through their C-terminal tail domain in a light-dependent fashion (69,70). Therefore, light-dependent, CRY-mediated nuclear exclusion of COP1 (66-68) is the key for photomorphogenic effect of cryptochromes in plants.

However, many of the cryptochrome-regulated genes in *Arabidopsis* are also regulated by phytochromes, a red-light photopigment. Therefore, cryptochromes function in conjunction with phytochromes to regulate photomorphogenic responses in plants such as growth and development, photoperiodic flowering, and circadian clock entrainment (71).

Animal Cryptochromes– Animal cryptochromes possess more sequence similarity with (6-4) photolyases than with plant cryptochromes (50). The first mammalian cryptochrome was identified when a photolyase ortholog was identified as an expressed sequence tag (EST) in the human genome database (72). A second photolyase ortholog was subsequently identified (73). It was further shown that these two homologs do not possess any photolyase activity. Therefore, they were classified as cryptochromes (73). Thereafter, cryptochrome

homologs were identified in many other animals, including insects, amphibians, fish, birds, and other mammals (74).

Among all animal cryptochromes, Drosophila cryptochrome represents one of the best characterized animal cryptochromes thus far. Fly cryptochrome was discovered in a genetic screen for circadian rhythm mutants in Drosophila (75). In their screen, Stanewsky and his colleagues identified a loss-of-function mutant *Cry^b* or *Cry^{baby}* that was caused by a substitution mutation of Asp→Asn at amino acid 410. *Cry^b* was shown to have lost its sensitivity to phase-shifting light pulses in the dark even though their molecular clock was still functional, indicating the loss of photoentrainment of the clock (75). Interestingly, the *Cry^b* mutant was shown to maintain normal circadian rhythms under constant light which would otherwise results in arrhythmia in wild-type flies (75,76), suggesting a role of cryptochrome as a photoreceptor required for circadian clock entrainment in flies. One important aspect of the Drosophila time-keeping system is that almost every cell in their body is light sensitive, and clocks in these cells can be reset by light cues (77,78). The photo-entrainment of fly clocks has been shown to be mediated via interaction of cryptochrome with Timelss (TIM). Drosophila CRY (dCRY) was found to interact with TIM only in presence of light (79,80). The resetting of the clock most likely occurs through cryptochrome-mediated induction of light-dependent, ubiquitin-mediated degradation of TIM (81-83). TIM degradation then causes degradation of PER, the repressor of CYCLE/CLOCK transcriptional activator activity, and thus allows for transcription

to occur (84). Therefore, light dependent degradation of TIM by dCRY is the key for photo-entrainment of the fly clock.

Cryptochrome-DASH– Recently, a new class of cryptochromes has been discovered, first in the cyanobacterium *Synechocystis* sp. PCC 6803 (85,86) and subsequently in many different species such as plants (87), prokaryotes (88), aquatic vertebrates and fungi (89). This class of cryptochromes is called DASH as it bears more homology to <u>D</u>rosophila, <u>A</u>rabidopsis, <u>S</u>ynechocystis and <u>H</u>uman than bacterial photolyases (90). Initial characterization of CRY-DASH demonstrated no photoreception activity for this class of proteins, which led to its classification as a cryptochrome. More recently, CRY-DASH has been shown to be single strand-specific CPD-photolyases (91,92).

Cryptochrome Structure and Functions– Photolyases are DNA repair enzymes that use blue light to repair UV-induced DNA damage in bacteria by removing adjacent pyrimidine dimers from DNA and thus maintain the genetic integrity. Two major types of pyrimidine dimers are formed; cyclobutane pyrimidine dimer (CPD) and (6-4) pyrimidine-pyrimidone dimer. CPD photolyses repair cyclobutane pyrimidine dimers and 6-4 photolyses repair (6-4) pyrimidine-pyrimidone dimers. These two types of photolyases, along with the cryptochromes, constitute the photolyase/ cryptochrome superfamily.

In general, the crystal structure of photolyases contains two modular domains: an amino-terminal α/β domain and a carboxy-terminal α-helical domain (Fig. 4). This family of proteins contains two non-covalently bound cofactors/ chromophores in their structures. The catalytic chromophore FAD binds within

the α-helical domain whereas pterin (MTHF) binds at the interface between these two domains. The pterin harvests energy from light and transfers the excitation energy to FAD, which subsequently transfers an electron to repair damaged DNA (93). The crystal structure of the PHR domain of *Arabidopsis* CRY1 (AtCRY1) was determined in 2004 in which a non-covalently bound FAD cofactor is present within the PHR (94). Recently, the crystal structure of *Drosophila* Cryptochrome has been determined that contained the FAD cofactor (95). However, so far there are no crystal structures available for mammalian cryptochromes.

Thus, cryptochromes also seem to utilize the same two (FAD and MTHF) chromophores, though formal proof of the presence of MTHF is lacking for cryptochromes. The main structural difference between cryptochromes and photolyases lies in the carboxy terminal tail domain (CTD); while all the CRYs contain an extended CTD, photolyases lack this tail region. The extended CTDs of cryptochromes range from 30 to 350 amino acids in length (96), and their sequences have diverged significantly across species as well as between mammalian CRY1 and CRY2.

Biochemical and biophysical studies demonstrated that the CTDs of cryptochromes are highly unstructured when expressed alone (97,98). However, they form a rigid conformation by interacting with the PHR domain (71,97). Even though mouse CRY1 and CRY2 share 70-80% sequence identity/similarity, the least conserved domain is located at the CTD (Fig. 5). Since there is no crystal
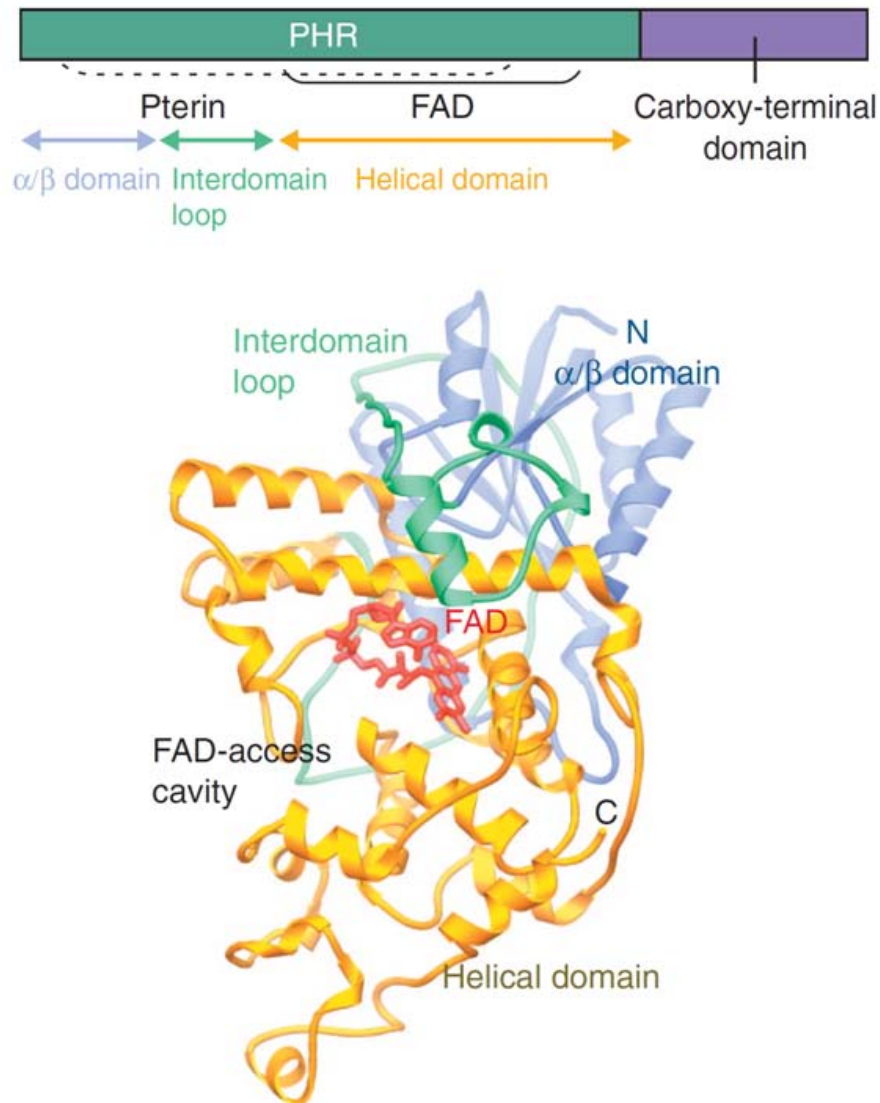
FIGURE 4. **Typical structural features of photolyases.** N-terminal α/β domain is shown in blue and C-terminal α-helical domain in yellow. These two domains together are called photolyase homology region (PHR) and connected by an inter-domain loop (green). The binding pockets for two cofactors, FAD and Pterin lie within the photolyase homology region. Adapted with modifications from Lin and Todo, 2005 (60).

structure of mammalian cryptochromes available, it is difficult to understand the structure-function relationship for this variable CTD of cryptochromes. It would be of immense interest to identify the functional significance of this variable carboxy-terminal domain which could provide clues to the differential functions of cryptochromes.

In spite of having high sequence and domain similarities, cryptochromes function differently across species. They act as photoreceptors to regulate growth and development in plants (53,99) or to reset or entrain the circadian clocks in plants and insects including Drosophila (75,100,101). Evidences seem to suggest that the Drosophila cryptochrome (dCRY) may be involved in core clock function in the peripheral tissues (75,101). Intriguingly, however, CRYs in mammals do not have photolyase activity and do not function as photoreceptors (102-105); rather, they function as potent repressors that inhibit E-box-mediated transcription of clock controlled genes. Therefore, they are core clock components in mammals (25,106-108).

Differential Functions of Mammalian Cryptochromes: Loss-of-Function Studies– The Drosophila dPER is a potent repressor and required for regulation of circadian rhythms (109). Mutations at the *Per* locus can alter clock function: compared to WT, *per^S* and *per^L* mutants show shorter and longer periods, respectively, while *per^0* null mutants are arrhythmic under free-running conditions (110,111). In contrast, the potent repressors in the mammalian clock are CRYs, not PERs (24). Although both CRYs are repressors, experimental data suggest that CRY1 and CRY2 have overlapping but differential functions in the clock

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mechanism. It has been shown that *Cry1* and *Cry2* play opposite roles in regulating animal behavior: *Cry1*$^{-/-}$ and *Cry2*$^{-/-}$ mice display shorter and longer free-running period length of locomotor activity rhythms, respectively, compared to wild type mice (25,108). Similarly, SCN explants from *Cry1*$^{-/-}$ mice exhibit shorter period length than wild type, whereas *Cry2*$^{-/-}$ SCN explants exhibit longer period (14).

Interestingly, *Cry1* and *Cry2* play distinct roles in generating and maintaining cell-autonomous circadian rhythms. For example, dissociated individual SCN neurons derived from *Cry1*$^{-/-}$ mice are arrhythmic or only transiently rhythmic, whereas neurons from *Cry2*$^{-/-}$ SCN show persistent rhythms of higher amplitude with longer period lengths than in wild type (14). Similarly, peripheral tissue explants and cells from *Cry1*$^{-/-}$ mice are arrhythmic whereas from *Cry2*$^{-/-}$ mice are rhythmic with longer period length than wild type (14,20). The more prominent role of *Cry1* is also supported by behavioral phenotypes of compound knockouts; *Cry1*$^{+/-}$:*Cry2*$^{-/-}$ mice show more persistent rhythms than *Cry1*$^{-/-}$:*Cry2*$^{+/-}$ mice, and while *Per2*$^{-/-}$:*Cry2*$^{-/-}$ mice are rhythmic, *Per2*$^{-/-}$:*Cry1*$^{-/-}$ mice are arrhythmic (108,112). Taken together, these studies indicate that *Cry1* is required for cellular rhythmicity and plays a more prominent role than *Cry2* in the clock mechanism.
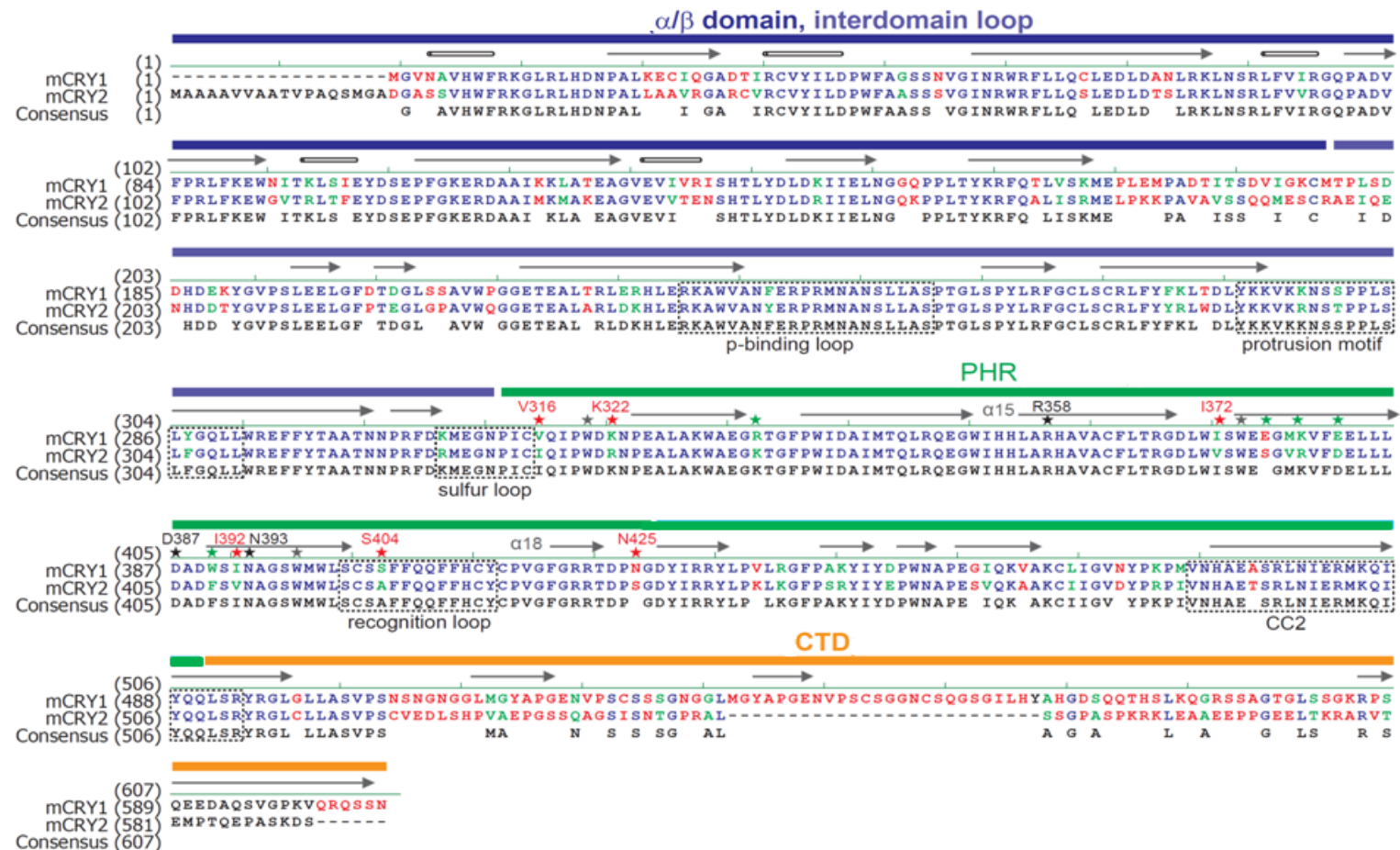
FIGURE 5. **Sequence alignment and key structural features of mCRY1 and mCRY2.** mCRY1 and mCRY2 share ~70/80% sequence identity/similarity. Amino acid sequence alignment was generated using Vector NTI (Invitrogen). Amino acid sequence alignment: blue, identical; green, similar; red, divergent. Secondary structures: arrow, alpha helix (numbers are assigned based on Arabidopsis UVR3); barrel, beta strand; CC2: coiled coil 2. FAD-binding residues; gray star, Trp triad; red star.

Specific Research Objectives–

Aim1: Identification of Sequences That Differentiate Cry1 from Cry2. The above mentioned knockout studies demonstrated that CRY1 and CRY2 play different roles in circadian clock function, both at behavioral and at cellular levels. However, molecular details underlying the functional distinction between the two mammalian cryptochromes are not well understood. Though CRY1 and CRY2 share 70% identity and 80% homology in amino acid sequences (Fig. 4) and possess similar biochemical function as a repressor, their genetic perturbation has opposite effects on clock function. Specifically, CRY1 functions to lengthen period length of rhythms and is required for persistent cellular rhythms, whereas CRY2 functions to shorten the period and reduce the amplitude of the rhythm.

A hallmark of circadian clock function is the rhythmic expression of clock genes, the functional importance of which has been revealed by recent studies. For example, while the *Bmal1* gene is essential, its rhythmic expression is dispensable for core clock function (113). In contrast, rhythmic expression of *Cry1* is required for cell-autonomous circadian oscillation (48). In addition to the E/E'-box (responsible for morning-time phase of gene expression, e.g., *Rev-erbα*) at the core of the clock mechanism, at least two other circadian cis-elements are involved: the DBP/E4BP4 binding element (D-box; day-time phase, e.g., *Per3*) and the ROR/REV-ERB binding element (RRE; night-time phase, e.g., *Bmal1*). In a recent study, we showed that *Cry1* transcription is mediated by all three circadian elements (i.e., E/E'-box and D-box elements in the promoter and RREs in the first intron of the *Cry1* gene), giving rise to the distinct *Cry1*

evening-time phase (48). Further, through genetic complementation, we showed

that this distinctive delayed phase of *Cry1* expression is required to restore

circadian rhythmicity in the arrhythmic *Cry1$^{-/-}$*:*Cry2$^{-/-}$* fibroblasts (48). It is now

known that *Cry1* is regulated by a combinatorial transcription mechanism and

strongly rhythmic in most tissues including the SCN, while *Cry2* has only weak

rhythms (24,34,48,106,107,114,115).Their differential expression patterns may

partially explain the differential roles in clock function in vivo. Alternatively, CRY1

protein level may be higher than CRY2, or CRY1 may be a stronger repressor

than CRY2.

Based on experimental evidences as summarized above, we hypothesize

that the differential functions of CRY1 and CRY2 lie at the protein and

biochemical levels. In order to understand the genetic and biochemical basis of

their differential functions in the circadian clock, I examined CRY1 and CRY2

function in a genetic complementation assay in which their transcription is under

control of the same promoter and proteins are expressed to similar levels. I took

advantage of the *Cry* rescue assay to dissect the differential functions of *Cry1*

and *Cry2*. First, I confirmed that *Cry1* can restore cell-autonomous circadian

rhythms in *Cry1$^{-/-}$*:*Cry2$^{-/-}$* cells, while *Cry2* cannot. Through systematic analyses

of protein domain structure-function relationships, I identified a highly conserved

α-helical domain within the PHR that distinguishes CRY1 from CRY2.

Subsequently, I demonstrated that, contrary to previous speculation, the least

conserved CTD is dispensable for circadian oscillation, but serves to modulate

rhythm amplitude and period length.

Aim2: Identification of the Biochemical Basis for Differential Functions of CRY1 and 2. The basic molecular mechanism underlying the circadian negative feedback loop in mammals was established in the late 1990s (22,116,117), in which PERs and CRYs were the transcriptional repressors of BMAL1/CLOCK transcriptional activity. Later on, it was further demonstrated that the feedback repression is mediated primarily by CRYs, not PERs (24,118). Mutation studies on *Bmal1* and *Clock* demonstrated that CRY-mediated repression of BMAL1/CLOCK activity is required for clock function and maintenance of circadian rhythmicity (119). Although *Cry1* and *Cry2* have differential transcription regulation and expression pattern in vivo, which may play roles in their differential functions, they localize to the nucleus in a synchronous manner through posttranslational regulation (114). Thus, regulation of subcellular localization is similar between the two proteins. Therefore, we confirm that the basis for their differential function lies at the difference in their intrinsic biochemical activity and not their differential expression pattern.

To understand the biochemical basis of their differential functions, we performed transcriptional repression assay of CRY1 and CRY2 in *Cry* deficient cells under our genetic rescue condition. In these studies, we demonstrated that CRY1 is a much stronger repressor than CRY2, and repression strength positively correlates with rhythm amplitude. Thus, our data demonstrate that CRY1-specific repression is necessary for normal clock function.

In summary, these findings provide, for the first time, the biochemical basis for differential functions of CRY1 and 2 in the mammalian circadian clock.

We identified a novel domain within the CRY1's PHR that is critical for circadian rhythmicity, and we demonstrated that this domain functionally differentiates CRY1 from CRY2. We also demonstrated that, the least conserved CTD, which was speculated to be the differential domain responsible for CRY functions, is not required for circadian rhythmicity, but plays a key role in regulating the period length of circadian rhythmicity in mammals. Finally, we identified that differential transcriptional repression activity is the biochemical basis underlying differential function, and CRY1-specific repression is critical for circadian rhythmicity. Overall, our findings shed new light on the functional importance of CRY1-PHR(313-426) and the CTD in the clock mechanism.

MATERIALS AND METHODS

Plasmid Constructions–

Cloning of Cry genes in pMU vector. The *Cry1* expression vector, pMU2-P(*Cry1*)-*intron-Cry1*, was made in a previous study (48). To generate pMU2-P(*Cry1*)-*intron-Cry2*, the full-length coding region of mouse *Cry2* was amplified using HiFi-DNA polymerase (Invitrogen) with forward primer (5'-<u>TCTAGA</u>TGG C AAACAGCTATTATGGGTATTATGGGTGCGGCGGCTGCTGTGGTG-3'; underline, *Xba*I restriction site) and reverse primer (5'-<u>GTCGAC</u>TGCCATTTCA TTACCTCTTTCTCCGCACCCGACATAGATTCAGGAG TCCTTGCT-3'; underline, *Sal*I). The PCR product was cloned into pCR2.1-TOPO vector (Invitrogen) and the digested *Xba*I/*Sal*I fragment was then sub-cloned into pMU2 vector in place of the *Cry1* gene (Fig. 6). For construction of pMU2-P(CMV)-*Cry2*, the full-length coding sequence of *Cry2* was digested from pMU2-P(SV40)-*Cry2* (48) with *PI-Psp*I and *PI-Sce*I, and the *Cry2* fragment was cloned into the *PI-Psp*I-*PI-Sce*I sites immediately downstream of the CMV promoter.

Domain swapping chimeric constructs. Domain swapping constructs were generated by overlapping PCR. The primers (Table 1) were designed so that swap junctions reside in highly conserved or identical sequences, so as to minimize major structural changes and protein folding problems. Similar to *Cry2* cloning, the PCR products were first cloned into pCR2.1-TOPO vector according to manufacturer's protocol (Invitrogen). Following cloning into pCR2.1-TOPO vector, the cloned genes were digested with *Xba*I/*Sal*I and gel-purified. Subsequently, the purified fragments were cloned into the *Xba*I/*Sal*I digested

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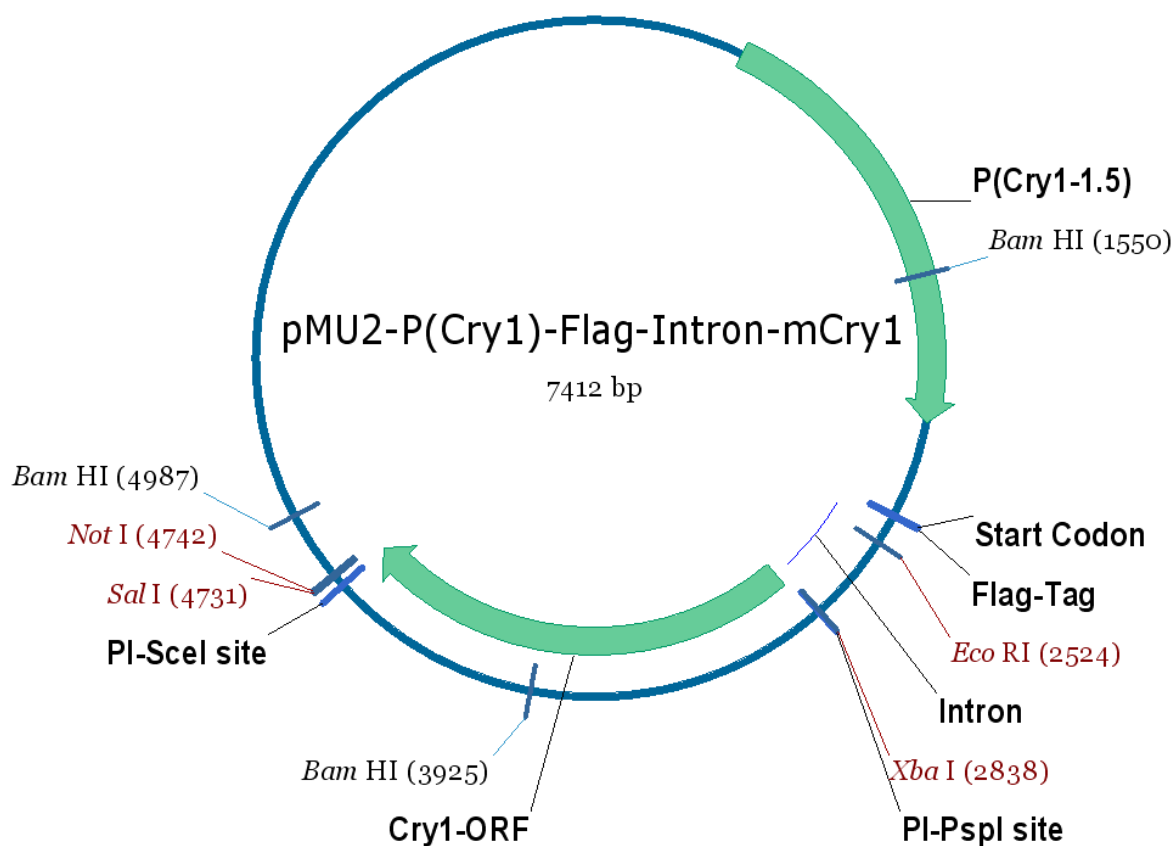
FIGURE 6. **pMU2-P(*Cry1*)-Flag-*Intron*-m*Cry1* construct.** *Cry1* gene expression is driven by *Cry1* promoter element (P(*Cry1*)) and first intron of *Cry1* gene (48). To clone chimeric and mutant constructs into pMU vector, pMU2-P(*Cry1*)-Flag-*Intron*-m*Cry1* construct was digested with *Xba*I and *Sal*I. Digested vector was ligated with chimeric or mutant sequences digested with *Xba*I and *Sal*I from pCR2.1-TOPO vector (Invitrogen).

pMU2 vector.

　　Mutant constructs. Site-directed mutagenesis using overlapping PCR was performed to generate single mutations within the CRY1-PHR(313-426) of CRY*. The primers (Table 2) were designed in such a way that they already incorporated the corresponding nucleotide substitutions to achieve expected mutation. To generate deletion mutant, same overlapping PCR strategy was employed.

　　As describe above, all the PCR products were first cloned in pCR2.1-TOPO followed by digestion with *Xba*I/*Sal*I. Subsequently, the digested fragments were cloned into the pMU2 vector.

　　Fibroblasts Culture and Real-time Circadian Reporter Assay– Mouse embryonic fibroblasts (MEF) from $Cry1^{-/-}$:$Cry2^{-/-}$ double knockout mice; $Cry1^{-/-}$:$Cry2^{-/-}$ cells were cultured in DMEM (Hyclone) supplemented with 10% FBS (Hyclone) and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin, Hyclone). Real-time circadian reporter assays were performed using a Lumicycle luminometer (Actimetrics, Inc.) as previously described (11,48) (Fig. 7). One day prior to transfection, 4×10^5 cells were plated onto 35 mm culture dishes. Cells were co-transfected using transfection reagent FuGene6 (120) with 3.95 µg of pGL3-P(*Per2*)-d*Luc* reporter plasmid (119) and 0.075 µg of a *Cry* expression plasmid. For the *Cry1* dose response experiment, the amount of plasmid was adjusted to 5.45 µg with empty vector. Three days post-transcription, the medium was replaced with 1.5 ml of HEPES-buffered (pH 7.2) recording medium supplemented with B-27 and containing 0.1 mM luciferin, antibiotics and10 µM

forskolin as previously described (48). Bioluminescence from each dish was continuously recorded with a photomultiplier tube (PMT) for ~70 sec at intervals of 10 min at 36°C. Raw data (counts/sec) were plotted against time (days) in culture and are presented in the figures.

Analysis of Circadian Rhythm Parameters– For analysis of rhythm parameters, we used the LumiCycle Analysis program (version 2.31, Actimetrics, Inc.). Raw data were baseline fitted, and the baseline-subtracted data were fitted to a sine wave (damped), from which the period was determined. For samples that showed persistent rhythms, goodness-of-fit of >80% was usually achieved. Due to high transient luminescence upon medium change, the first cycle was usually excluded from rhythm analysis. Amplitude of bioluminescence rhythms was determined as described previously (48). First, a moving average of the linearly detrended bioluminescence was calculated. The window size of the moving average was set to half of the estimated period. The moving average was smoothed by the smoothing spline method, resulting in an amplitude trend, which was then removed by dividing by the trend curve of the original time series.

Table 1. Primer list for generation of domain swapping chimeric constructs.

Name of the construct	Round of PCR		Primer sequence
pMU2-P(*Cry1*)-*Cry1 intron*-A1B2C2D2	First	Reaction 1	f: ggtagagctgtcaagattac r: ctggatgcagatgggggttcccttccattttgtcaaa
		Reaction 2	f: tttgacaaaatggaagggaaccccatctgcatccag r: gtatcttatcatgtctgctcg
	Second		f: ggtagagctgtcaagattac r: gtatcttatcatgtctgctcg
pMU2-P(*Cry1*)-*Cry1 intron*-A1B1C2D2	First	Reaction 1	f: ggtagagctgtcaagattac r: gcctttcagtttgggcagataacgcctaatatagtc
		Reaction 2	f: gactatattaggcgttatctgcccaaactgaaaggc r: gtatcttatcatgtctgctcg
	Second		f: ggtagagctgtcaagattac r: gtatcttatcatgtctgctcg
pMU2-P(*Cry1*)-*Cry1 intron*-A1B1C1D2	First	Reaction 1	f: ggtagagctgtcaagattac r: caatagacagagtccccggtaccgggaaagctgctgatagat
		Reaction 2	f: atctatcagcagctttcccggtaccggggactctgtctattg r: gtatcttatcatgtctgctcg
	Second		f: ggtagagctgtcaagattac r: gtatcttatcatgtctgctcg
pMU2-P(*Cry1*)-*Cry1 intron*-A2B1C1D1	First	Reaction 1	f: ggtagagctgtcaagattac r: ctgaacacagatgggggttcccctccattcggtcaaa
		Reaction 2	f: tttgaccgaatggaggggaaccccatctgtgttcag r: gtatcttatcatgtctgctcg
	Second		f: ggtagagctgtcaagattac r: gtatcttatcatgtctgctcg

Table 1. Primer list for generation of domain swapping chimeric constructs (contd).

Name of the construct	Round of PCR		Primer sequence
pMU2-P(*Cry1*)-*Cry1* intron-A2B2C1D1	First	Reaction 1	f: ggtagagctgtcaagattac
			r: gcctcttaggacaggtaagtaccgccggatgtagtc
		Reaction 2	f: gactacatccggcggtacttacctgtcctaagaggc
			r: gtatcttatcatgtctgctcg
	Second		f: ggtagagctgtcaagattac
			r: gtatcttatcatgtctgctcg
pMU2-P(*Cry1*)-*Cry1* intron-A2B1C2D2	First	Reaction 1	f: ggtagagctgtcaagattac
			r: ctgaacacagatggggttcccctccattcggtcaaa
		Reaction 2	f: tttgaccgaatggaggggaaccccatctgtgttcag
			r: gcctttcagtttgggcagataacgcctaatatagtc
		Reaction 3	f: gactatattaggcgttatctgcccaaactgaaaggc
			r: gtatcttatcatgtctgctcg
	Second		f: ggtagagctgtcaagattac
			r: gtatcttatcatgtctgctcg
pMU2-P(*Cry1*)-*Cry1* intron-A1B2C1D1	First	Reaction 1	f: ggtagagctgtcaagattac
			r: ctggatgcagatggggttcccttccattttgtcaaa
		Reaction 2	f: tttgacaaaatggaagggaaccccatctgcatccag
			r: gcctcttaggacaggtaagtaccgccggatgtagtc
		Reaction 3	f: gactacatccggcggtacttacctgtcctaagaggc
			r: gtatcttatcatgtctgctcg
	Second		f: ggtagagctgtcaagattac
			r: gtatcttatcatgtctgctcg

Table 2. Primer list for generation of site-directed mutations.

Name of the construct	Round of PCR		Primer sequence
pMU2-P(*Cry1*)-*Cry1 intron*-Cry*-V316I	First	Reaction 1	f: ggtagagctgtcaagattac
			r: tgtcccaagggatctggatacagatggggttcccctc
		Reaction 2	f: gaggggaaccccatctgtatccagatcccttgggaca
			r: gtatcttatcatgtctgctcg
	Second		f: ggtagagctgtcaagattac
			r: gtatcttatcatgtctgctcg
pMU2-P(*Cry1*)-*Cry1 intron*- Cry*-K322R	First	Reaction 1	f: ggtagagctgtcaagattac
			r: gccagagcctcggggttgcggtcccaagggatctgaac
		Reaction 2	f: gttcagatcccttgggaccgcaaccccgaggctctggc
			r: gtatcttatcatgtctgctcg
	Second		f: ggtagagctgtcaagattac
			r: gtatcttatcatgtctgctcg
pMU2-P(*Cry1*)-*Cry1 intron*- Cry*-R334K	First	Reaction 1	f: ggtagagctgtcaagattac
			r: atccacgggaagcctgtcttgccttctgcccatttggc
		Reaction 2	f: gccaaatgggcagaaggcaagacaggcttcccgtggat
			r: gtatcttatcatgtctgctcg
	Second		f: ggtagagctgtcaagattac
			r: gtatcttatcatgtctgctcg
pMU2-P(*Cry1*)-*Cry1 intron*- Cry*-I372V	First	Reaction 1	f: ggtagagctgtcaagattac
			r: tcccttcttcccagctgacccacaggtcaccacgagt
		Reaction 2	f: actcgtggtgacctgtgggtcagctgggaagaaggga
			r: gtatcttatcatgtctgctcg
	Second		f: ggtagagctgtcaagattac
			r: gtatcttatcatgtctgctcg

Table 2. Primer list for generation of site-directed mutations (contd).

Name of the construct	Round of PCR		Primer sequence
pMU2-P(*Cry1*)-*Cry1 intron*- *Cry**-E376S	First	Reaction 1	f: ggtagagctgtcaagattac
			r: tcccttcttcccagctgacccacaggtcaccacgagt
		Reaction 2	f: actcgtggtgacctgtgggtcagctgggaagaaggga
			r: gtatcttatcatgtctgctcg
	Second		f: ggtagagctgtcaagattac
			r: gtatcttatcatgtctgctcg
pMU2-P(*Cry1*)-*Cry1 intron*- *Cry**-E382D	First	Reaction 1	f: ggtagagctgtcaagattac
			r: tgcatcaagcagtaactcatcaaagaccttcatccct
		Reaction 2	f: agggatgaaggtctttgatgagttactgcttgatgca
			r: gtatcttatcatgtctgctcg
	Second		f: ggtagagctgtcaagattac
			r: gtatcttatcatgtctgctcg
pMU2-P(*Cry1*)-*Cry1 intron*- *Cry**-W390F	First	Reaction 1	f: ggtagagctgtcaagattac
			r: acttccagcatttatgctaaaatctgcatcaagcagtaa
		Reaction 2	f: ttactgcttgatgcagatttttagcataaatgctggaagt
			r: gtatcttatcatgtctgctcg
	Second		f: ggtagagctgtcaagattac
			r: gtatcttatcatgtctgctcg
pMU2-P(*Cry1*)-*Cry1 intron*- *Cry**-I392V	First	Reaction 1	f: ggtagagctgtcaagattac
			r: catccaacttccagcattcacgctccaatctgcatcaag
		Reaction 2	f: cttgatgcagattggagcgtgaatgctggaagttggatg
			r: gtatcttatcatgtctgctcg
	Second		f: ggtagagctgtcaagattac
			r: gtatcttatcatgtctgctcg

Table 2. Primer list for generation of site-directed mutations (contd).

Name of the construct	Round of PCR		Primer sequence
pMU2-P(*Cry1*)-*Cry1 intron*- *Cry**-S404A	First	Reaction 1	f: ggtagagctgtcaagattac
			r: aaaattgctgaaaaaaggcactgcaggacagccacat
		Reaction 2	f: atgtggctgtcctgcagtgccttttttcagcaatttt
			r: gtatcttatcatgtctgctcg
	Second		f: ggtagagctgtcaagattac
			r: gtatcttatcatgtctgctcg
pMU2-P(*Cry1*)-*Cry1 intron*- *Cry**-N425S	First	Reaction 1	f: ggtagagctgtcaagattac
			r: cgcctaatatagtctccactgggatctgtcctcctac
		Reaction 2	f: gtaggaggacagatcccagtggagactatattaggcg
			r: gtatcttatcatgtctgctcg
	Second		f: ggtagagctgtcaagattac
			r: gtatcttatcatgtctgctcg
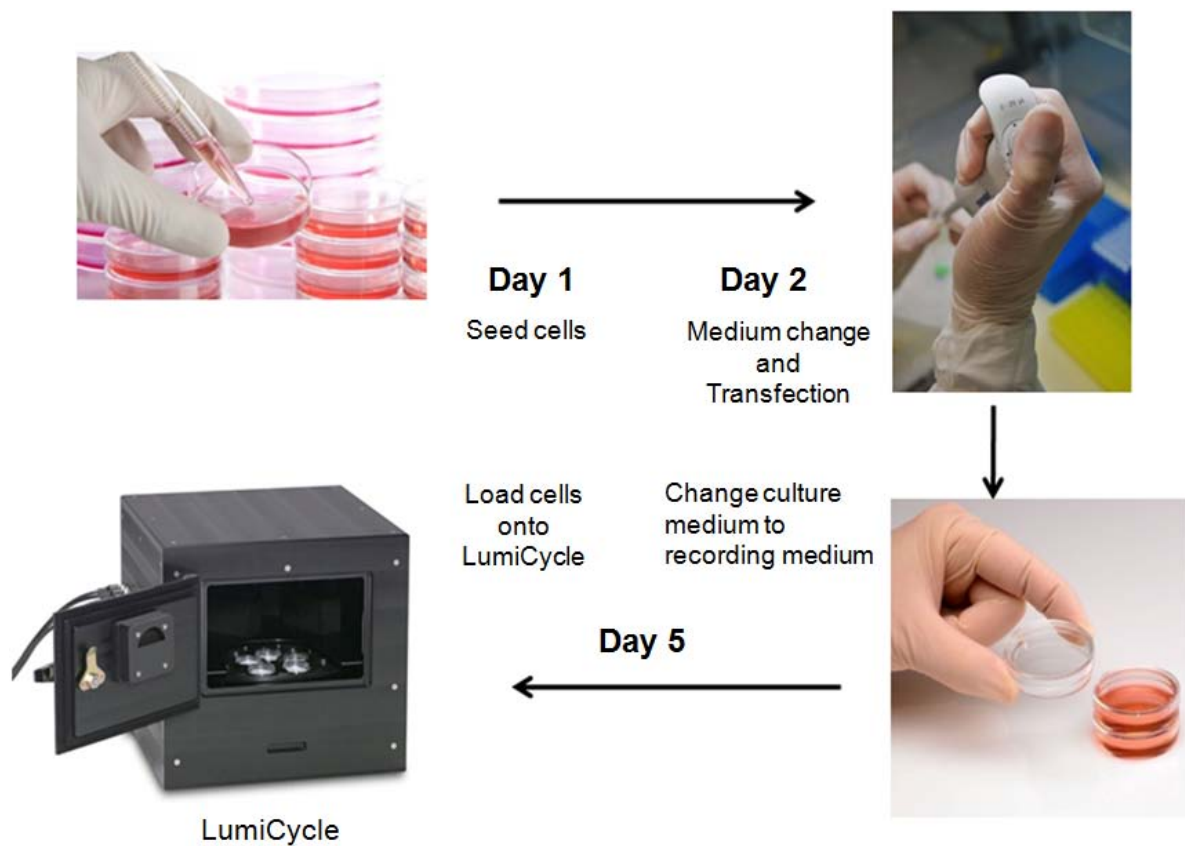
FIGURE 7. **Work flow for real-time circadian reporter assay.** *Cry1$^{-/-}$:Cry2$^{-/-}$*
cells are seeded at a density of 4 x 10^5 cells / dish, on 35-mm dish, one day prior
to transfection. On day 2, cells are cotransfected with reporter plasmid and
plasmid containing gene of interest. Two days post-transfection, cells are washed
with PBS and freshly prepared recording medium is added before loading them
onto the real-time bioluminescence recording device called LumiCycle.

Transcription Repression Assay– Cry1$^{-/-}$:Cry2$^{-/-}$ fibroblasts were grown

and transfected as described above with the following modifications. In

transfection, 1 µg of reporter plasmid, pGL3-P(*Per2*)-d*Luc* (119), pGL3-3xE'-box-

P(SV40)-d*Luc*, pGL3-3xE-box-P(SV40)-d*Luc*, or pGL3-P(SV40)-d*Luc* (18), was

used together with 2 µg of a *Cry* expression plasmid. In some assays as

presented in Figures 9 and 16, 0.5 µg each of *Bmal1* and Clock plasmid DNA

(121) was also included. Empty vector was used to make up the total amount of

DNA to 4.1 µg per well. As an internal control, 50 ng of a phRL-SV40 plasmid

expressing Renilla Luciferase (R*Luc*) (Promega) was added in each transfection.

Forty-eight hours after transfection, cells were harvested and assayed with Dual-

Luciferase Reporter Assay System (Promega). Luciferase activity was

normalized by R*Luc* activity.

For evaluation of correlation between rhythm amplitude and repression

activity, linear fit of a first-order-polynomial was performed by the least square

method. Statistical significance was evaluated by Pearson's correlation. Analysis

was performed using Microsoft Excel or R version 2.8.1.

Immunoblotting– Each *Cry* construct (1 µg) was co-transfected with either

empty vector (0.4 µg) or *Bmal1*/*Clock* (0.2 µg each) in 293T cells or in *Cry1*$^{-/-}$

:*Cry2*$^{-/-}$ fibroblasts in a 12-well plate. Forty-eight hours after transfection, cells

were trypsinized and washed twice with phosphate buffer saline (PBS). Next,

trypsinized cells were collected in 1.5 ml microcentrifuge tubes and lysed in 100

ul RIPA buffer containing 150 mM sodium chloride, 1.0% Triton X-100, 0.5%

sodium deoxycholate, 0.1% SDS (sodium dodecyl sulphate), 50 mM Tris, pH 8.0

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and protease inhibitors. The lysates were cleared by centrifugation at 4°C, 10,000 rpm for 20 min and supernatants were collected in new tubes for protein sample preparation. Samples for SDS-PAGE were prepared by adding 6X loading sample loading dye and boiling for 5 min at 100 °C. Proteins samples were then separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After separation, proteins were transferred to polyvinylidene fluoride (PVDF) membrane for immunoblotting. Western blot analysis was performed with guinea pig polyclonal antibodies against CRY1 or CRY2 as described previously (24,34,114,122) or against *Flag* tag according to manufacturer's protocol (Sigma). Antibodies used for this study are listed in Table 3.

Protein structure homology modeling– Homology models for full length mCRY1 and mCRY2 were generated using the I-TASSER protein structure prediction server (123-125). This server first threads fragments of the target sequence to representative PDB structure templates with matched sequence identity greater than 70%. The fragments are then assembled into a full-length model while the unmatched regions are built via *ab initio* modeling. Hence, unlike other homology modeling software, this server predicts the structure even when there are no matched sequences in known PDB structures. The quality of predicted structure was assessed with a scoring method, and five atomistic models with highest scores were obtained for each input protein sequence. Images of predicted structures were created using PyMOL software, version 1.2r3pre (Schrödinger, LLC.).

Table 3. Antibodies used for immunoblotting.

	Primary antibody				**Secondary antibody**		
	Catalog number	Source	Dilution		Catalog number	Source	Dilution
Mouse anti-Flag	F3165	Sigma	1:5000	Goat anti-mouse	1858413	Pierce	1:200
Guinea pig anti-mCry1	—	Lee lab (Lee et al., Cell, 2001)	1:1000	Rabbit anti-guinea pig	—	Lee lab (Lee et al., Cell, 2001)	1:1000
Guinea pig anti-mCry2	—	Lee lab (Lee et al., Cell, 2001)	1:1000	Rabbit anti-guinea pig	—	Lee lab (Lee et al., Cell, 2001)	1:1000
Goat anti-β-actin	sc-1615	Santa Cruz Biotech Inc.	1:1000	Donkey anti-goat	sc-2033	Santa Cruz Biotech Inc.	1:1000

RESULTS

Cry1, But Not Cry2, Can Restore Circadian Clock Function in Cry1$^{-/-}$:Cry2$^{-/-}$ Fibroblasts– To confirm the differential functions of *Cry1* and *Cry2* in clock function, we first tested their ability to restore circadian rhythms in otherwise arrhythmic *Cry1$^{-/-}$:Cry2$^{-/-}$* fibroblasts through genetic complementation and kinetic bioluminescence recording. In this assay, expression of *Cry* is under control of a composite *Cry1*-phase promoter containing E/E'-box and D-box elements in the promoter and RREs in the first intron of the *Cry1* gene (Fig. 8A).

As expected, *Cry1* was able to restore rhythms in these arrhythmic *Cry1$^{-/-}$:Cry2$^{-/-}$* cells (Fig. 8B), consistent with previous results (48), and the rescued cells showed longer period lengths than wild type, characteristic of *Cry2$^{-/-}$* cells (14). In contrast, however, *Cry2* was unable to restore circadian oscillation to *Cry1$^{-/-}$:Cry2$^{-/-}$* fibroblasts, confirming results found for cells from *Cry1$^{-/-}$* mice (14) (Fig. 8B). Interestingly, rescue of rhythmicity is largely independent of the dose of *Cry1*, ranging from nanograms to micrograms of DNA used in the transfection (Fig. 8C, left panel). On the other hand, *Cry2* of any amount failed to rescue circadian rhythmicity in these cells (Fig. 8C, right panel). Thus, our data establish that *Cry1* and *2* play differential roles at the level of core clock function: while *Cry1* is essential for generation of cell-autonomous circadian clock function, *Cry2* is dispensable.

FIGURE 8. **Cry1, but not Cry2, restores circadian rhythmicity in arrhythmic Cry1$^{-/-}$:Cry2$^{-/-}$ fibroblasts.** (A) Schematic representation of expression vectors and general experimental design. In the *Cry* expression vector, *Cry* is under control of a composite *Cry1*-phase promoter that contains all three circadian elements: E-box, D-box from the *Cry1* promoter, and RRE from a *Cry1 intron*. The reporter vector contains the destabilized *Luciferase* (d*Luc*) gene driven by the *Per2* promoter. Transfected *Cry1$^{-/-}$:Cry2$^{-/-}$* fibroblasts are either harvested for a transcription repression assay, or synchronized for kinetic bioluminescence recording. (B) Representative bioluminescence records from *Cry1$^{-/-}$:Cry2$^{-/-}$* fibroblasts expressing *Cry1* or *Cry2*. Genetic complementation of *Cry1* (*red*), butnot *Cry2* (*blue*), restored circadian rhythms in these cells. Each expression
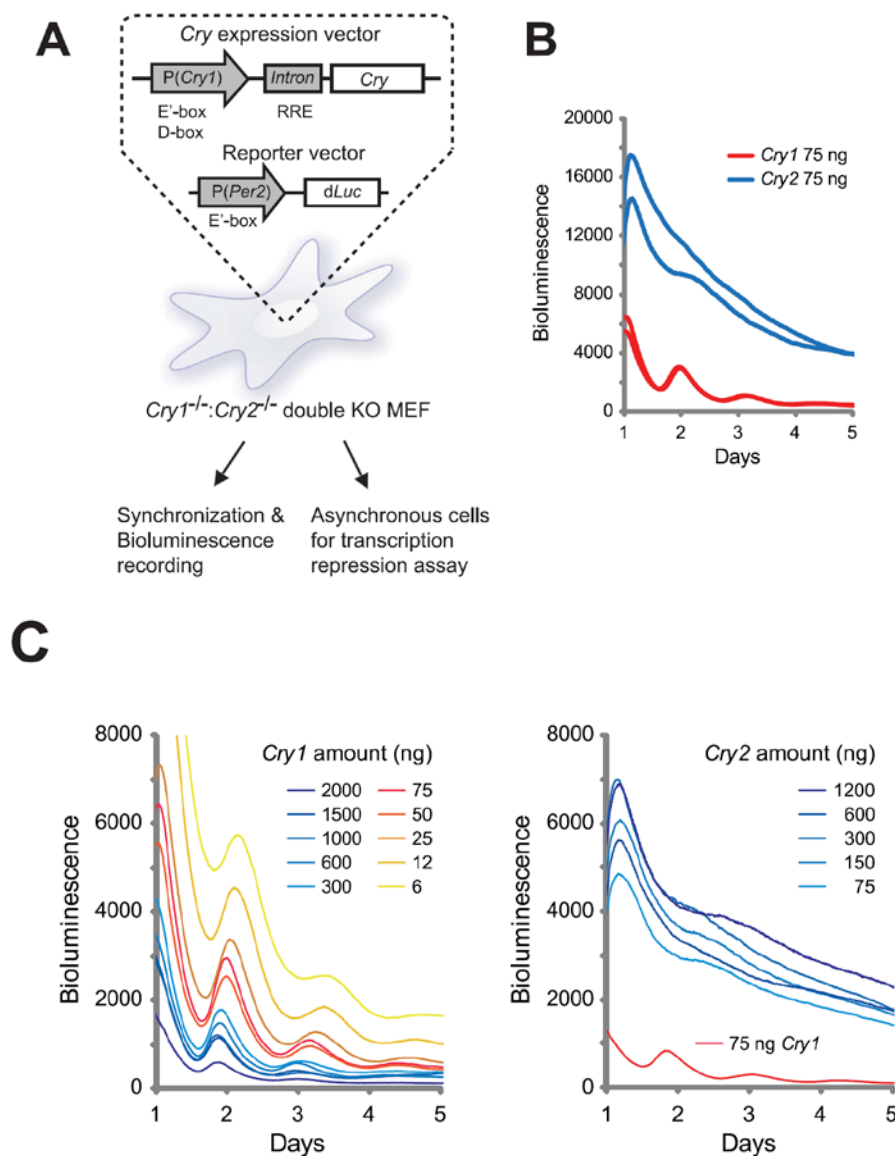
FIGURE 8. ***Cry1*, but not *Cry2*, restores circadian rhythmicity in arrhythmic *Cry1*$^{-/-}$:*Cry2*$^{-/-}$ fibroblasts (contd.).**

construct was cotransfected with the P(*Per2*)-d*Luc* into the cells. Three days posttransfection, the cells were synchronized by forskolin treatment and followed by bioluminescence recording for 5–6 days. (C) *Cry1* of different amounts of plasmid DNA restored circadian rhythms in *Cry1*$^{-/-}$:*Cry2*$^{-/-}$ fibroblasts. Experiments were done as in *B*.

As the *Cry* expression level in these fibroblasts was below detection limit, the ability of P(*Cry1*)-*Intron-Cry* constructs to express CRY proteins was tested by Western blot in transfected 293T cells (Fig. 9A). Additionally, to compare their relative expression in *Cry1*$^{-/-}$:*Cry2*$^{-/-}$ fibroblasts, we determined that 3xFlag-*Cry1* and 3xFlag-*Cry2* (functionally comparable to *Cry1* and *Cry2*, respectively, in the rescue assay; Fig. 9B, left panel) are expressed to similar levels in these cells (Fig. 9B, right panel).

Unlike the high-amplitude rhythmic expression of *Cry1* in various tissues and cells, *Cry2* expression is either not rhythmic or rhythmic at very low amplitude (24,34,114,122). It is thus possible that this differential rhythmic expression contributes to functional differences *in vivo*. In our *in vitro* rescue assay, the same *Cry1*-phase promoter is used to control both *Cry1* and *Cry2* expression, so this strategy eliminates confounding effects of differential transcriptional regulation. Thus, our data showing that *Cry1* (but not *Cry2*) restores circadian rhythms in *Cry1*$^{-/-}$: *Cry2*$^{-/-}$ fibroblasts suggest that CRY1 and 2 possess different intrinsic biochemical properties at the protein level that call for further investigation.
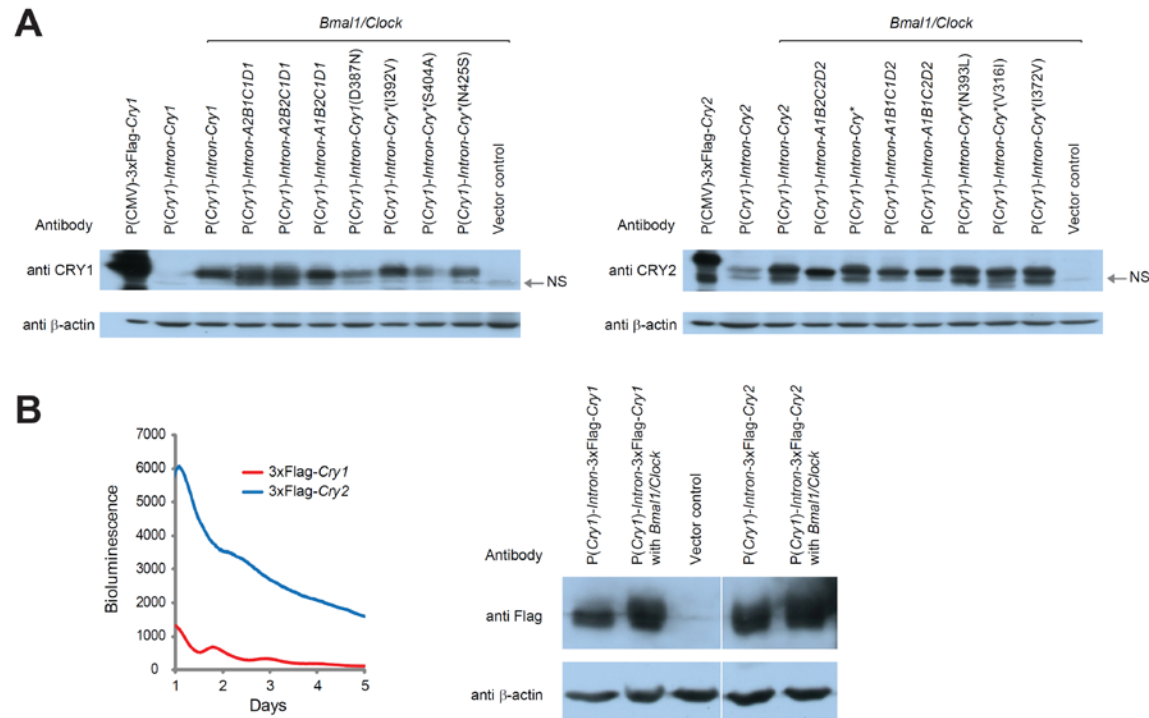
FIGURE 9. **Western blot analysis of CRY proteins.** (A) HEK-293T cells were transfected with different Cry constructs in the absence or presence of Bmal1/Clock as indicated. Total cell extracts were probed with either anti-CRY1 (left panel) or anti-CRY2 (right panel) antibody. Compared to CMV promoter, P(*Cry1*)-Intron drives low levels of protein expression, which can be further induced by cotransfected Bmal1/Clock to a higher level. Detection of CRY proteins indicates that all *Cry* constructs are able to express the proteins. Because the regulation of *Cry* expression in functional clock cells (e.g., those that are rescued by P(*Cry1*)-*Intron-Cry1*) is different from that in arrhythmic cells (e.g., those expressing *Cry2*), to directly compare Cry expression, transfected 293T cells provide a better means to test these constructs for their ability to express proteins. 3xFlag-CRY1 and CRY2, positive controls. Arrow, non-specific (NS) band. (B) *Cry1* and *Cry2* are expressed to similar levels in $Cry1^{-/-}:Cry2^{-/-}$ fibroblasts in the rescue assay.

CRY1-PHR(313-426) is Critical for CRY1 Function– To probe the

biochemical origin of the differential functions of CRY1 and 2, we set out to

identify the critical structural region that differentiates the two. Based on known

structure and domain function of PHL/CRY proteins (60,96,126), we divided

CRY1 and 2 proteins into four regions, namely A, B, C and D (Fig. 10). Using an

overlapping PCR strategy, we generated a series of *Cry* swapping chimeras by

systematically substituting different regions of *Cry1* with the corresponding

sequences from *Cry2* (Fig. 11A). To minimize major structural changes and

protein folding problems, we selected highly conserved or identical sequences at

swap junctions (Fig. 10). The ability of these chimeras and the mutant *Cry*

constructs to express CRY proteins was tested by Western blot (Fig. 9). These

chimeras were then tested for their ability to restore circadian rhythms in $Cry1^{-/-}$

$:Cry2^{-/-}$ fibroblasts. *Cry1* chimeras that harbor A, C, or D regions of *Cry2* were

able to generate cellular rhythms, suggesting that these regions of *Cry1* and *2*

have comparable clock function (Fig. 11B). However, when the B region in *Cry1*

(*Cry1*-B) is replaced with the corresponding B region of *Cry2* (*Cry2*-B), the

A1B2C1D1 chimera failed to restore rhythms, suggesting that *Cry1*-B is required

for circadian clock function (Fig. 11C)

To further confirm the role of *Cry1*-B, we generated a *Cry2* chimera,

A2B1C2D2, in which the B region of *Cry2* is replaced by the corresponding *Cry1*-

B, designated as *Cry**. Similar to *Cry1*, *Cry** was also able to generate rhythms,

indicating that the B region of *Cry1* is sufficient to render *Cry2* able to perform the

role of *Cry1* in clock function (Fig. 11C). In fact, all chimeras that harbor *Cry1-B*
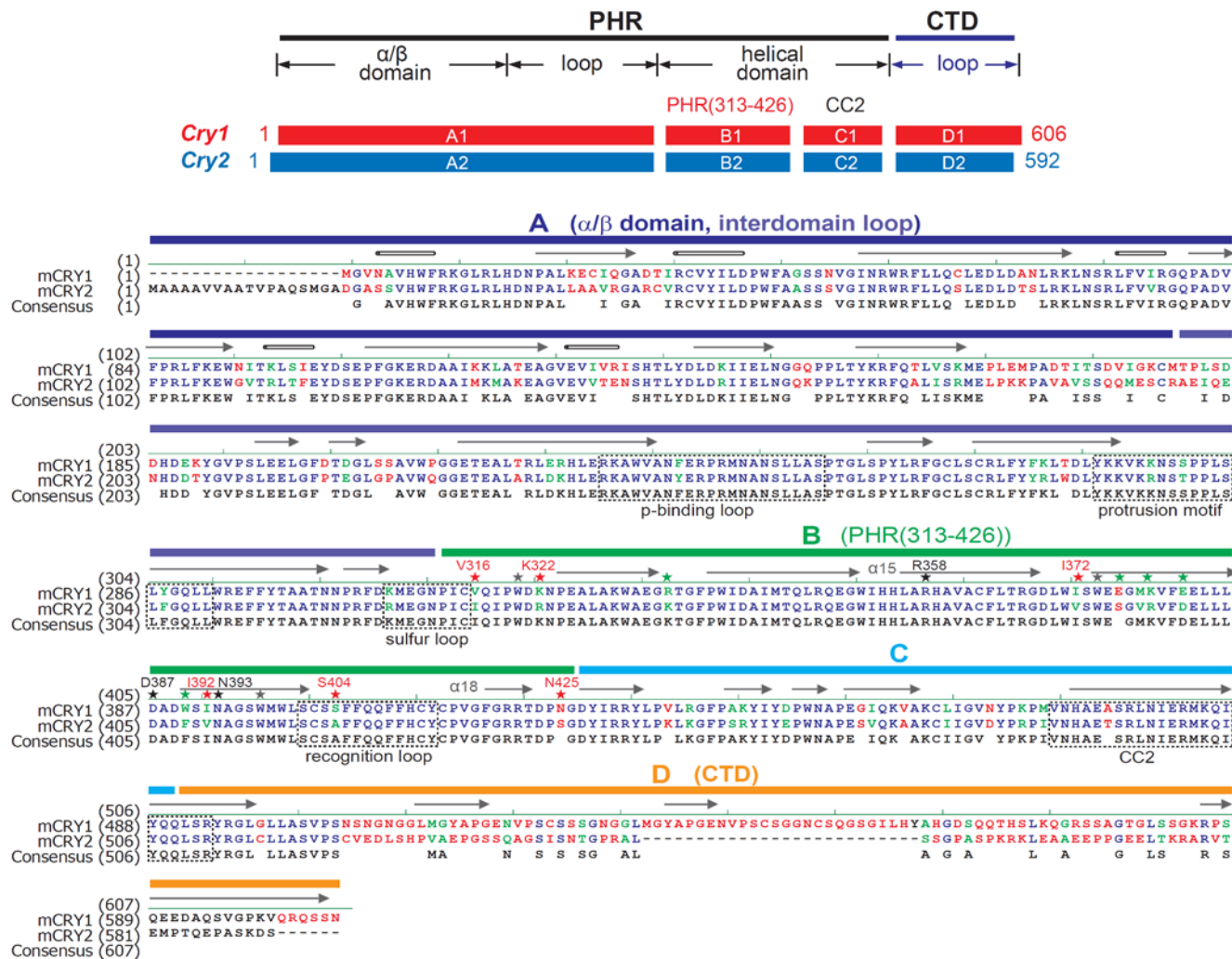
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FIGURE 10. **A schematic diagram of domain structure of CRY proteins.** To identify a region that differentiates CRY1 from CRY2, entire protein sequence was divided into four domains: domain A (blue), B (green), C (cyan), and D (yellow) based on the predicted their predicted structure (126).

were able to sustain circadian oscillation, while those containing *Cry2*-B failed to

do so (Fig. 11A). Interestingly, a previous mutagenesis study also hinted that this

region likely differentiates CRY1 and CRY2 (1). Thus, we have identified a critical

region within the highly conserved α-helical domain of CRY1 PHR (from amino

acid 313 to 426) that can differentiate CRY1 from CRY2 and is critically required

for *Cry1* function. We name this region as CRY1-PHR(313-426).

    Identification of Critical Amino Acid Residues within the CRY1-PHR(313-426)– Since the CRY1-PHR(313-426) underlies functional divergence of CRY1

and CRY2, we performed site-directed mutagenesis to identify the critical amino

acid residues. Among the ~100 residues within the CRY1-PHR(313-426), twelve

are divergent between CRY1 and 2 (Fig. 10). Each of the 12 amino acids in *Cry**

was mutated to the corresponding residue in *Cry2,* one or two at a time. Since

these amino acid residues exist naturally in *Cry2*, major structural changes are

unlikely to occur. We then tested individual mutants for their ability to rescue

rhythms in *Cry1$^{-/-}$:Cry2$^{-/-}$* fibroblasts. Among 12 mutants, six restored circadian

rhythms in these cells, similar to *Cry1 and Cry**, whereas the other 6 mutants

failed to do so: *Cry**-V316I, K322R, I372V, I392V, S404A (Fig. 12A), indicating

that these six residues within the CRY1-PHR(313-426) are critical for CRY

function in the clock mechanism.
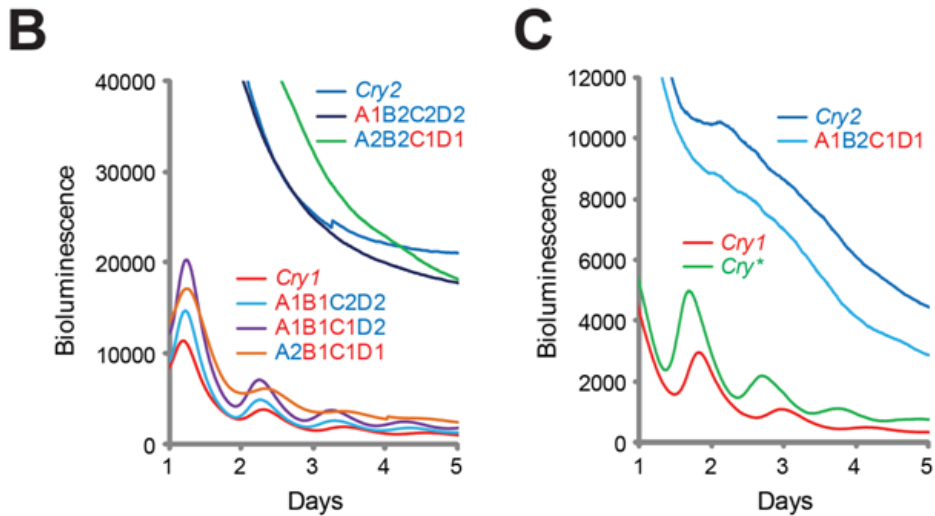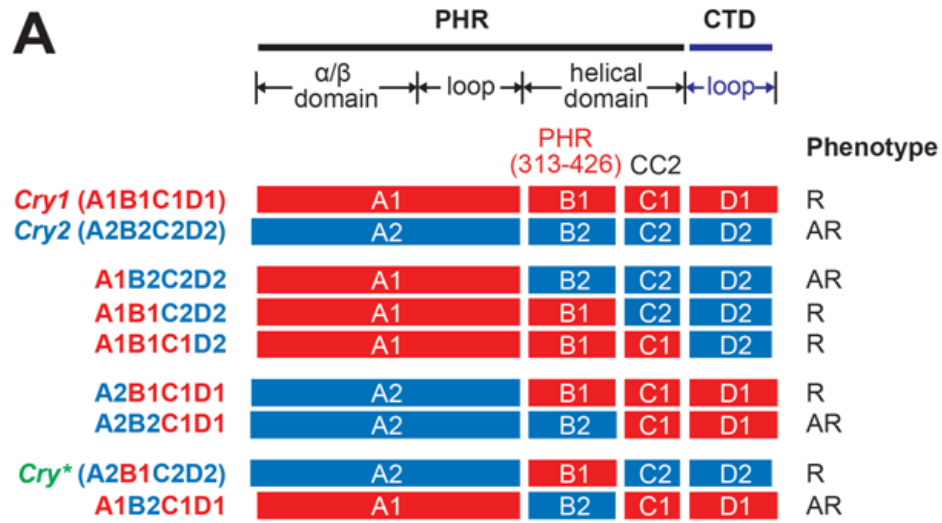
FIGURE 11. **CRY1-PHR(313–426) is critical for *Cry1* function.** (A) *Cry* expression constructs. *Cry1* (red) and *Cry2* (blue) were divided into four regions: 1) region A includes the N-terminal α/β domain and the inter-loop domain of the PHR; 2) region B is the CRY1-PHR(313–426) and includes the core α-helical domain of the PHR (from α13 to α18); 3) region C contains the rest of the α-helical domain, including sequences immediately after α18 and before the CTD where CC2 resides; and 4) region D is the CTD. For sequence details, see Figure 10. The chimeras were made by swapping these regions between CRY1 and CRY2. The schematics are drawn to scale; CRY2 has an extended N terminus, whereas CRY1 has an extended C terminus. Circadian phenotypes are shown on the right: R, rhythmic; AR, arrhythmic. (B) and (C), representative

FIGURE 11. **CRY1-PHR(313–426) is critical for *Cry1* function (contd.).**

bioluminescence records from *Cry1*$^{-/-}$:*Cry2*$^{-/-}$ fibroblasts expressing different *Cry* chimeras. Domain-swapped chimeras (in B and C) were tested for their ability to rescue rhythms in *Cry1*$^{-/-}$:*Cry2*$^{-/-}$ fibroblasts as described in the legend to Figure. 8. All the chimeras that contained the B region from *Cry1* (B1, red) were able to restore rhythms (B), implicating the B domain from *Cry1* in rhythm generation. The B region of *Cry1* is sufficient to render *Cry2* able to generate rhythms. Chimera A2B1C2D2 (*Cry**, green) restored rhythms, but A1B2C1D1 (light blue) failed to do so (in C), confirming a required role of CRY1-PHR(313–426) in *Cry1* function.

Spatial Orientation of Six Critical Residues and Potential Signal

Transduction– We further performed protein homology modeling to determine the

locations of the 6 critical residues in the modeled CRY1 structure. CRY1 and

CRY2 have conserved structures for regions A through C, with a root mean

square deviation (RMSD) less than 2.0 Å among structures predicted by different

programs using different templates. Most homology modeling programs failed to

predict a structure for the CTD, except for I-Tasser, which placed it in many

different orientations, implying intrinsic flexibility for this region. In a model

excluding the CTD, the identified critical residues are all solvent exposed with the

exception of I392 (Fig. 12B), which is located near the FAD-binding cavity. N425

is localized within a loop motif between helix α18 in region B and α19 in region C,

and is potentially involved in protein-protein interactions. The other four residues

(i.e., V316, K322, I372 and S404) are readily available for potential interaction

with the CTD, CC2, or other clock factors. S404 is localized within a recognition

loop between α17 and α18, which is recently implicated in interaction with the

CTD of *Drosophila* CRY (95).
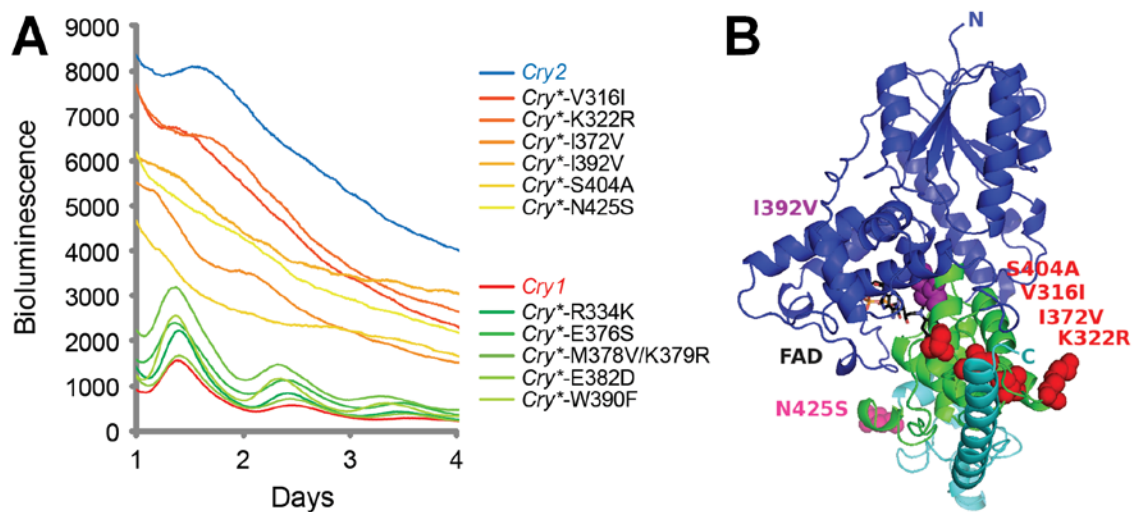
FIGURE 12. **Identification of critical amino acid residues within the CRY1-PHR(313-426).** (A) Representative bioluminescence records from *Cry1⁻/⁻:Cry2⁻/⁻* fibroblasts expressing *Cry* mutants. Within the CRY1-PHR(313–426), there exist 12 distinct amino acid residues that diverge between CRY1 and CRY2. *Cry** was mutated to the corresponding residues in CRY2 as indicated. Six of the CRY* mutants failed to rescue rhythms, indicating the critical role of the CRY1-PHR(313–426) in CRY1 function. (B) three-dimensional homology model structure of CRY1 without the CTD. The modeling was based on crystal structures of bacterial photolyase and Arabidopsis (6-4) PHR (UVR3).Region A, blue; B, CRY1-PHR(313–426), green; and C, cyan. The CTD is not shown. With the exception of Ile-392 (purple sphere), the other 5 critical residues identified within the CRY1-PHR(313–426), namely Val-316, Lys-322, Ile-372, Ser-404 (red spheres), and Asn-425 (pink sphere), are largely solvent exposed. FAD, black (O, red; N, blue; P, orange).

The CTD is Not Required for Circadian Rhythmicity– The CTD represents

the least conserved region among the CRYs. It is generally accepted that the

CTD is critical for CRY function (127). To test the functional importance of the

CTD, we generated a *Cry1* CTD-deletion construct, *Cry1(ΔCTD)* (Fig. 13A). To

our surprise, *Cry1(ΔCTD)* was able to rescue circadian rhythms in $Cry1^{-/-}{:}Cry2^{-/-}$

fibroblasts. Thus, contrary to expectation, our data suggest that the CTD is not

absolutely essential for CRY1 function (Fig. 13A). This result is consistent with a

previous study, which found that CTD is not absolutely required for repression

(1).

A coiled-coil 2 (CC2) motif within the C region, which is immediately

downstream of the CRY1-PHR(313-426) and upstream of the CTD, was

previously implicated in mediating interactions with other clock proteins (127).

Here we show that a larger C-terminal deletion, *Cry1(ΔCC2-CTD)*, which lacks

both CC2 and CTD, eliminated *Cry1*'s ability to maintain rhythmicity (Fig. 13B),

indicating an important role for CC2 in clock function.

The CTD Regulates Period Length of Circadian Rhythms– Although the

CTD of CRY1 is dispensable, rhythms rescued by *Cry1(ΔCTD)* showed

decreased rhythm amplitude compared to rhythms rescued by full-length *Cry1*

(see later results), suggesting that CTD modulates rhythm amplitude. Also,

interestingly, although *Cry1*-rescued cells displayed a long period (~27 hr),

characteristic of $Cry2^{-/-}$ cells, when *Cry1*-CTD is replaced by *Cry2*-CTD (chimera

A1B1C1D2), the rescued cells displayed shorter period lengths that are

comparable to wild type cells (~24 hr) (Fig. 14A). In fact, among all *Cry* chimeras

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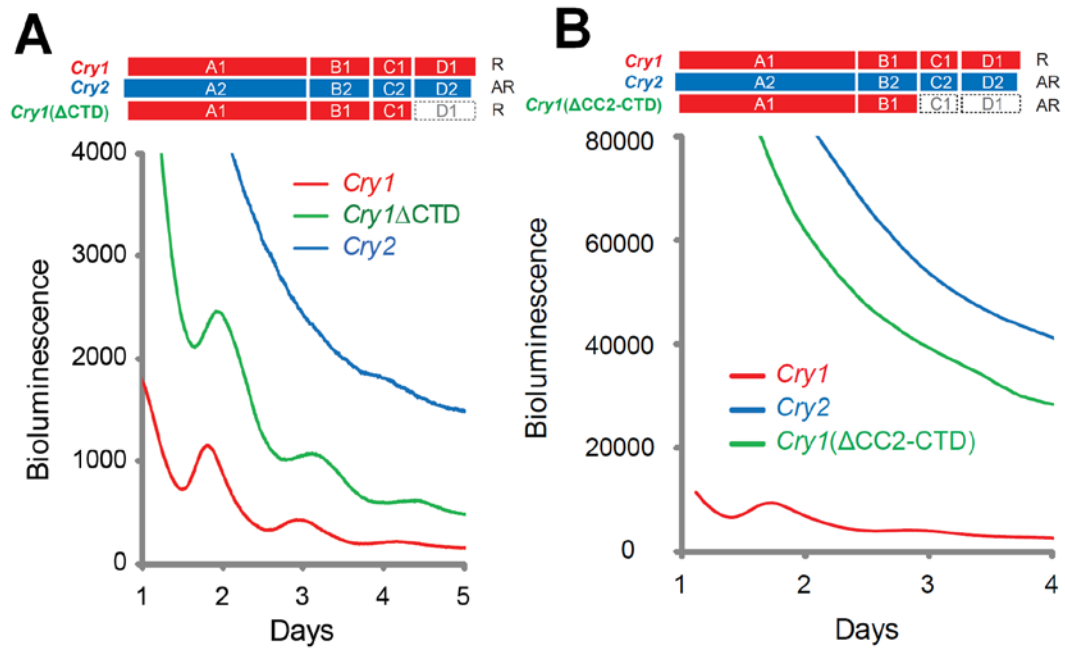
FIGURE 13. **The CTD is dispensable for CRY function.** (A) Schematic diagram of truncation construct *Cry1(ΔCTD)* in which the CTD is deleted (top panel). Representative bioluminescence records from *Cry1⁻ᐟ⁻:Cry2⁻ᐟ⁻* fibroblasts expressing different *Cry1(ΔCTD)* (bottom panel). Deletion of CTD did not render Cry1 unable to generate circadian rhythms, suggesting that the CTD is dispensable for CRY1 function. *Cry1(ΔCTD)*, green. (B) In the truncation construct, *Cry1(ΔCC-CTD)* (top panel), both the CTD and the C region containing CC2 are deleted from the full-length Cry1. This Cry1 truncation mutant failed to restore cellular rhythms in *Cry1⁻ᐟ⁻:Cry2⁻ᐟ⁻* fibroblasts (bottom panel; representative bioluminescence records) indicating an important role for CC2 domain in clock function.

containing the B region of CRY1 (and therefore conferring circadian rhythmicity), those that contain *Cry2*-CTD showed a period of ~24 hr, whereas those that contain *Cry1*-CTD showed a longer period (~27 hr). Taken together, our data suggest that although the CTD is dispensable for *Cry1* function, it plays important roles in modulating rhythm amplitude and period length.

   Modeling of the CTD– Our homology models for full-length CRY1 and CYR2 suggested plausible interactions between the CTD and the identified Cryptochrome differentiating domain involving the above-identified critical residues. Consistent with previous observations, the CTD assumes flexible structural configurations (71). Among possible arrangements of the CTD, those involving interactions with CRY1-PHR(313-426) are energetically favored, especially interactions with the side chains of V316, K322, I372, and S404 (Fig.14C), each shown to be critical for CRY function. The observation that these residues reside in critical regions (e.g., I392 and S404) and/or at an interface (e.g., V316, K322, I372 and S404) available for potential protein-protein interaction explains why mutating them impairs normal clock function.

   Differential Transcriptional Repression Activity of CRY1 and CRY2– In all our kinetic rhythm assay experiments, we noticed that there were low expression levels of the P(*Per2*)-d*Luc* reporter in rhythmic cells and high levels in arrhythmic cells (Figures 11-13). In those *Cry1*$^{-/-}$:*Cry2*$^{-/-}$ fibroblasts where rhythmicity was rescued by *Cry* constructs, bioluminescence reading (Y-axis) was observed to be low compared to those cells where rhythmicity was not restored. Lower bioluminescence readings indicate lower reporter activity, which in turn indicate

that the repressor activity of *Cry* constructs on BMAL1/CLOCK transcriptional activity is higher in rhythmic cells than arrhythmic cells. Therefore, this data suggested that rhythm amplitude may be related to potency of repression of BMAL1/CLOCK transcriptional activity. Since cryptochromes are the repressor of BMAL1/CLOCK transcriptional activity, the observation of differential reporter activity in rhythmic and arrhythmic cells prompted us to analyze the relationship between rhythm amplitude and repression activity of cryptochromes more quantitatively.

Cry1 Exhibits Stronger Repression Activity than Cry2 under Constitutive or Circadian Promoters– To examine the correlation between rhythm amplitude and transcriptional repression activity more quantitatively, we measured P(*Per2*)-d*Luc* expression in the presence of *Cry1* or *Cry2* in transiently transfected, non-synchronized cells. When assayed under non-rhythmic conditions in which *Cry* expression is controlled by a strong, constitutive promoter such as CMV or SV40, *Cry1* and *2* both displayed slightly different but strong levels of repression (Fig. 15), consistent with previous studies (1,24,118,119). To test for differences in repression activity of CRY1 and CRY2, we measured *Cry* repression under our conditions of genetic complementation in *Cry1$^{-/-}$:Cry2$^{-/-}$* fibroblasts, in which *Cry* is regulated by the *Cry1*-phase promoter. Under these conditions, CRY1 still displayed strong repression on the P(*Per2*)-d*Luc* reporter. CRY2, however, did not repress transcription to the same extent as CRY1, showing a repression activity 10 times weaker than CRY1 (Fig. 15).
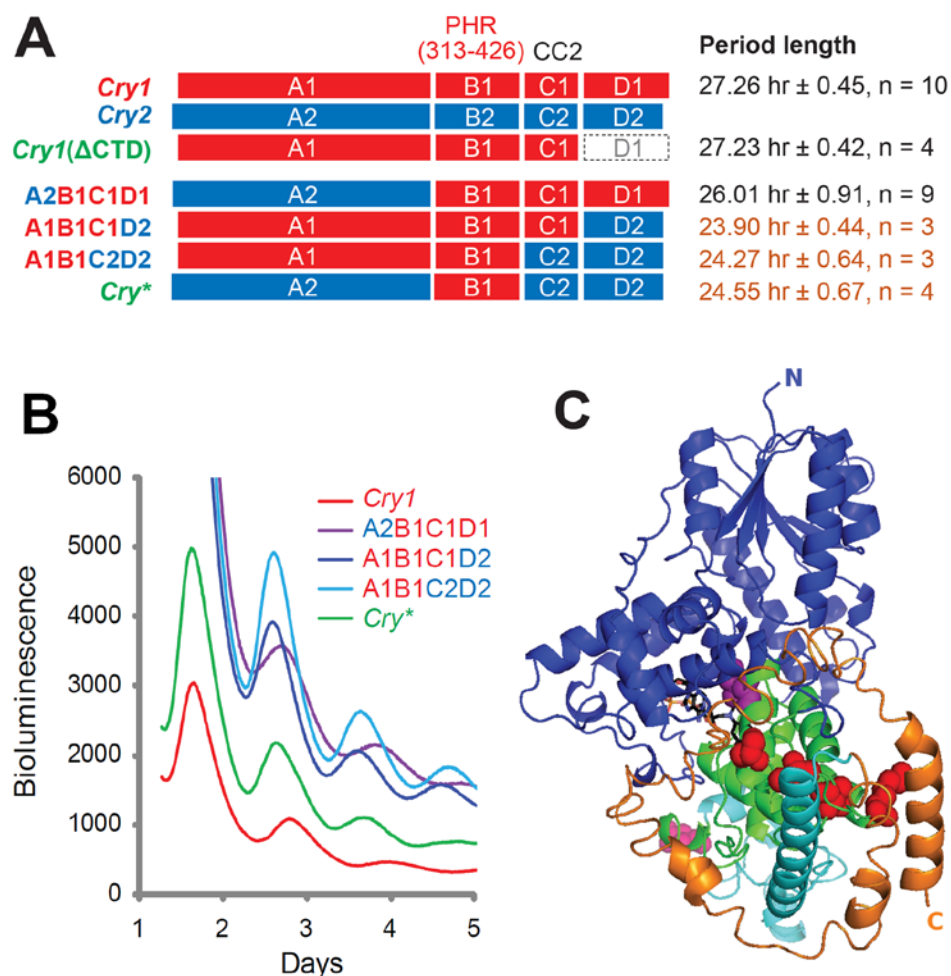
FIGURE 14. **The CTD modulates period length.** (A) Schematic diagram of various Cry constructs, including the truncation construct *Cry1(ΔCTD)* in which the CTD is deleted. Period length corresponding to each construct is shown on the right. *Mean ± S.D.* (error bar) of two independent experiments are shown. (B) Representative bioluminescence records from *Cry1⁻/⁻:Cry2⁻/⁻* fibroblasts expressing various *Cry* chimeras. The *Cry* rescue assay was performed as in Figure 8B. While the chimeras that contain the CTD from CRY1 (*Cry1* and A2B1C1D1) restored rhythms with a period length of ~27 hr (A), those that contain the CTD from CRY2 (A1B1C1D2, A1B1C2D2 and *Cry**) restored rhythms with a period length of ~24 hr (A), implicating the CTD in regulating period length. (C) three-dimensional homology model structure of full-length CRY1. The model was generated using the I-TASSER protein structure prediction server. Color scheme: region A, blue; B, CRY1-PHR(313–426), green; C, cyan; and D, CTD, orange. The CTD assumes a flexible structural configuration, and one of the predicted orientations is shown. In this configuration, the CTD resides in close proximity with the core CRY1-PHR(313–426), particularly with the 4 critical residues (red spheres).
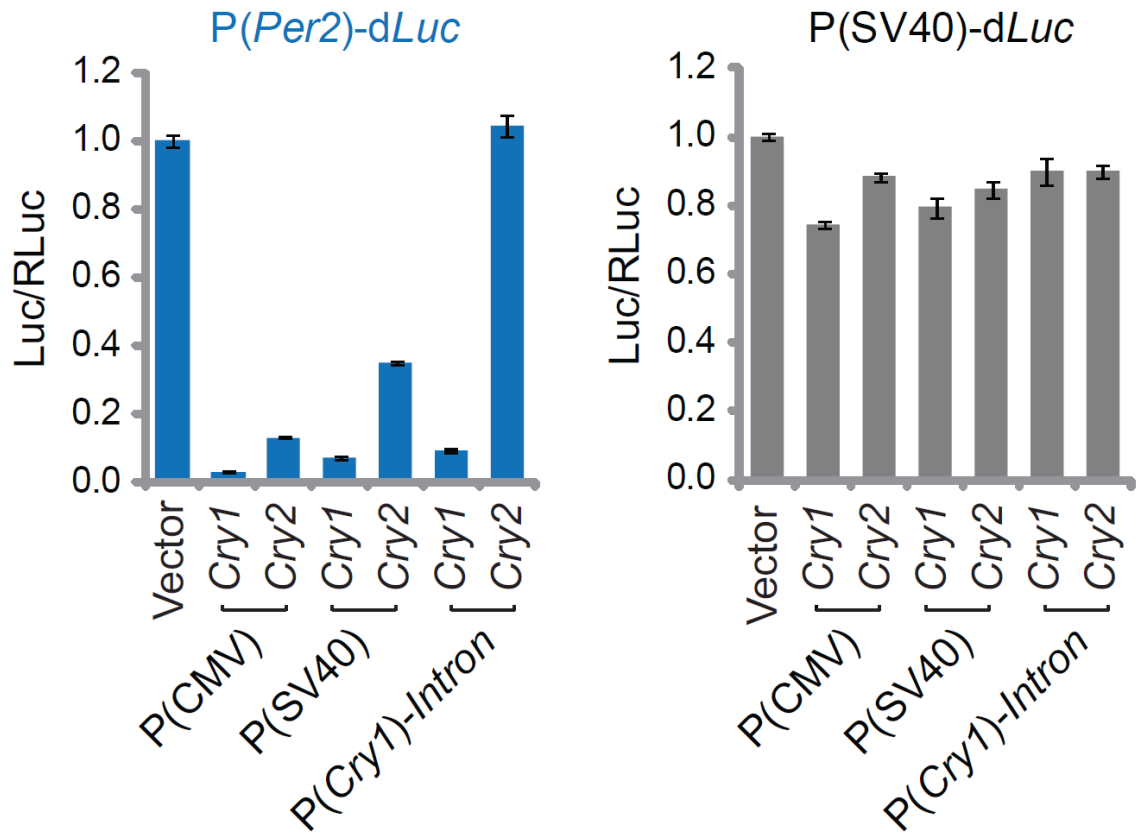
FIGURE 15. **Cry1 exhibits stronger repression activity than Cry2.** Dual luciferase reporter assay in $Cry1^{-/-}:Cry2^{-/-}$ fibroblasts. For $Cry1$ expression, three different promoters were tested. Each Cry construct was cotransfected with P(SV40)-dLuc (control) or P($Per2$)-dLuc reporter. A Renilla luciferase (RLuc) was added in each transfection to normalize transfection efficiency. Under the control of the $Cry1$-phase promoter, CRY1 acted as a much more potent repressor than CRY2. *Mean ± S.D.* (error bars) of two independent experiments are shown (n = 3 for each experiment).

Cry1-Mediated Repression is Specific to Circadian E-box Element– This difference in repression by CRY1 and CRY2 was observed with 3xE-box-P(SV40)-d*Luc* or 3xE'-box-P(SV40)-d*Luc* (Fig. 16A), suggesting that this differential repression is specific to E-box element and independent of reporters used in the assay. Similar differential repression was also observed when *Bmal1* and *Clock* were co-transfected in these cells (Fig. 16B). Therefore, we conclude that CRY1 is a much more potent transcriptional repressor than CRY2 when expressed under control of a *Cry1*-phase promoter and repression is specific to BMAL1/CLOCK transcriptional activity.

CRY Transcriptional Repression Positively Correlates with Rhythm Amplitude– The differential repression data prompted us to analyze the dependence of rhythm generation on transcriptional repression. To do this, we determined the repression activity of a subset of *Cry* chimeras and mutants used in our rescue studies. Under control of the *Cry1*-phase promoter, these *Cry* constructs showed various strengths of repression activity (Fig. 17). Importantly, we observed that all the constructs that were able to rescue the rhythms exhibited stronger repression activities, similar to *Cry1*, while those that failed to rescue have much weaker repression, similar to *Cry2* (Fig. 17 B). For example, *Cry1* (A1B1C1D1) and chimera A2B1C1D1 exhibited low but similar P(*Per2*)-d*Luc* expression, indicative of high repression. In contrast, A1B2C1D1 displayed significantly elevated reporter activity, similar to *Cry2*. In addition, mutation at each of the 6 critical residues within the CRY1-PHR(313-426) impaired repression (Fig. 17C). These results are consistent with reporter activities
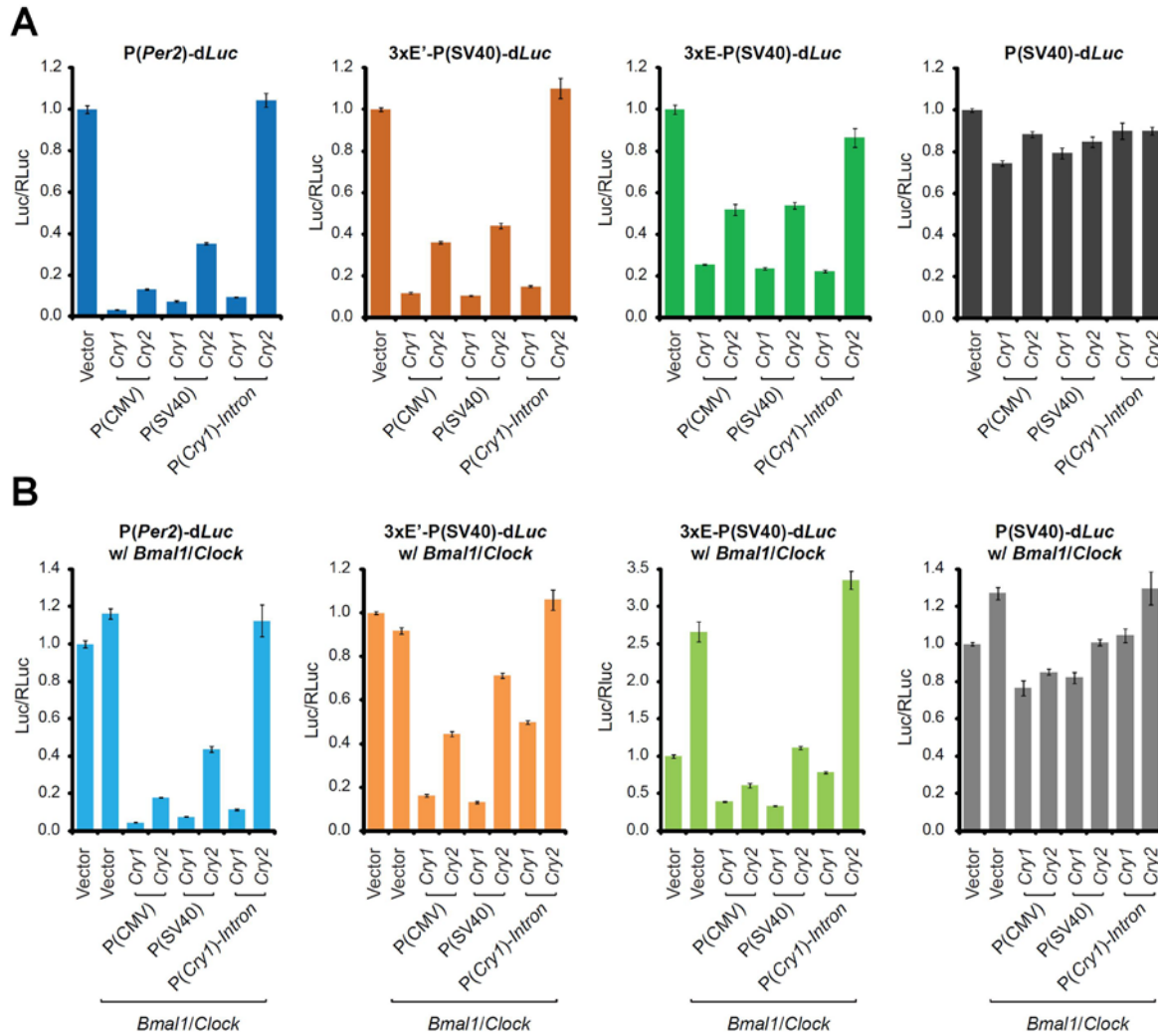
FIGURE 16. *Cry1* mediated repression is specific to circadian E-box element. Dual Luciferase reporter assay in $Cry1^{-/-}:Cry2^{-/-}$ fibroblasts was performed to test repression activity of *Cry1* and *Cry2* in the absence (A) or presence (B) of exogenous *Bmal1* and *Clock*. For *Cry* expression, three different promoters were tested: P(CMV), P(SV40) or P(*Cry1*)-*Intron*. Each *Cry* construct was cotransfected with P(SV40)-d*Luc* (control) or 3xE'-box-P(SV40)-d*Luc* or 3xE-box- P(SV40)-d*Luc* reporter. A Renilla *Luciferase* (R*Luc*) was added in each transfection to normalize transfection efficiency. Under all three promoters, particularly the *Cry1*-phase promoter, CRY1 acted as a much more potent repressor than CRY2 on circadian E-box element. Similar results were obtained when *Bmal1* and *Clock* are ectopically over-expressed. *Mean ± S.D.* (error bar) of two independent experiments are shown (*n* = 3 for each experiment).

observed in kinetic recordings (Figures 8, 11-13). Thus, strong repression activity is highly correlated with the capacity for rhythm generation.

Finally, we asked if repression activity is also correlated with rhythm amplitude. Using a previously described algorithm (48), we determined the rhythm amplitude of *Cry*-rescued circadian oscillations in *Cry1$^{-/-}$:Cry2$^{-/-}$* fibroblasts (Fig. 18A). We observed that rhythm amplitudes were low when the repression activities were relatively low; and conversely, rhythm amplitudes were high when the repression activities were relatively high. For example, compared to *Cry1*, *Cry1(ΔCTD)* showed attenuated transcriptional repression and accordingly lower rhythm amplitude. Overall, repression activity and rhythm amplitude bear a highly significant positive correlation, with 82% of the variance in rhythm amplitude explained by strength of *Cry* transcriptional repression (r^2 = 0.82, p < 0.001) (Fig. 18B). Thus, our data suggest that the strong repression conferred by CRY1, but not CRY2, on BMAL1/CLOCK-mediated transcription is the key to generating cell-autonomous circadian rhythms.
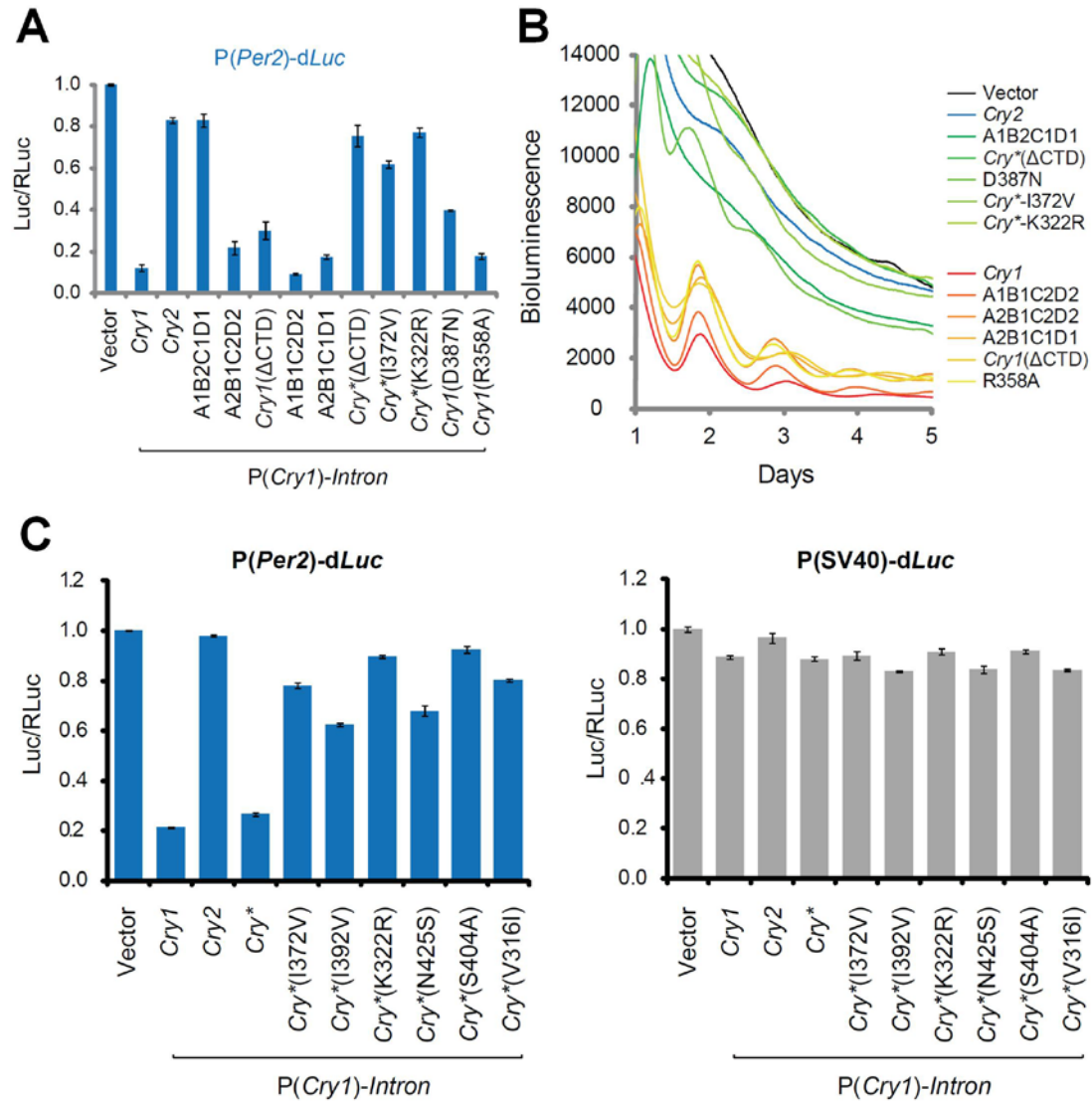
FIGURE 17. **Strong transcriptional repression activity is highly correlated with the capacity for rhythm generation.** (A) Repression activities of various *Cry* chimeras and mutants. Dual luciferase reporter assay was done as in Figure 15. The constructs that rescued rhythms exhibited stronger repression, similar to *Cry1*, whereas those that failed to rescue rhythms exhibited much weaker repression, similar to *Cry2*. *Mean ± S.D.* (*error bars*) of two independent experiments are shown ($n = 3$). (B) Representative bioluminescence records from $Cry1^{-/-}:Cry2^{-/-}$ fibroblasts expressing various *Cry* chimeras and mutants. The *Cry* rescue assay was performed as described in the legend to Figure 8. (C) Dual Luciferase reporter assay was performed to test repression activity of the CRY* single amino acid mutants. Each mutant contains a mutation in one of the 6 critical residues within the CRY1-PHR(313-426) of CRY* (as in Figure 12A). All mutants displayed weak repression (similar to *Cry2*), corroborating circadian phenotypes as shown in Figure 12A.

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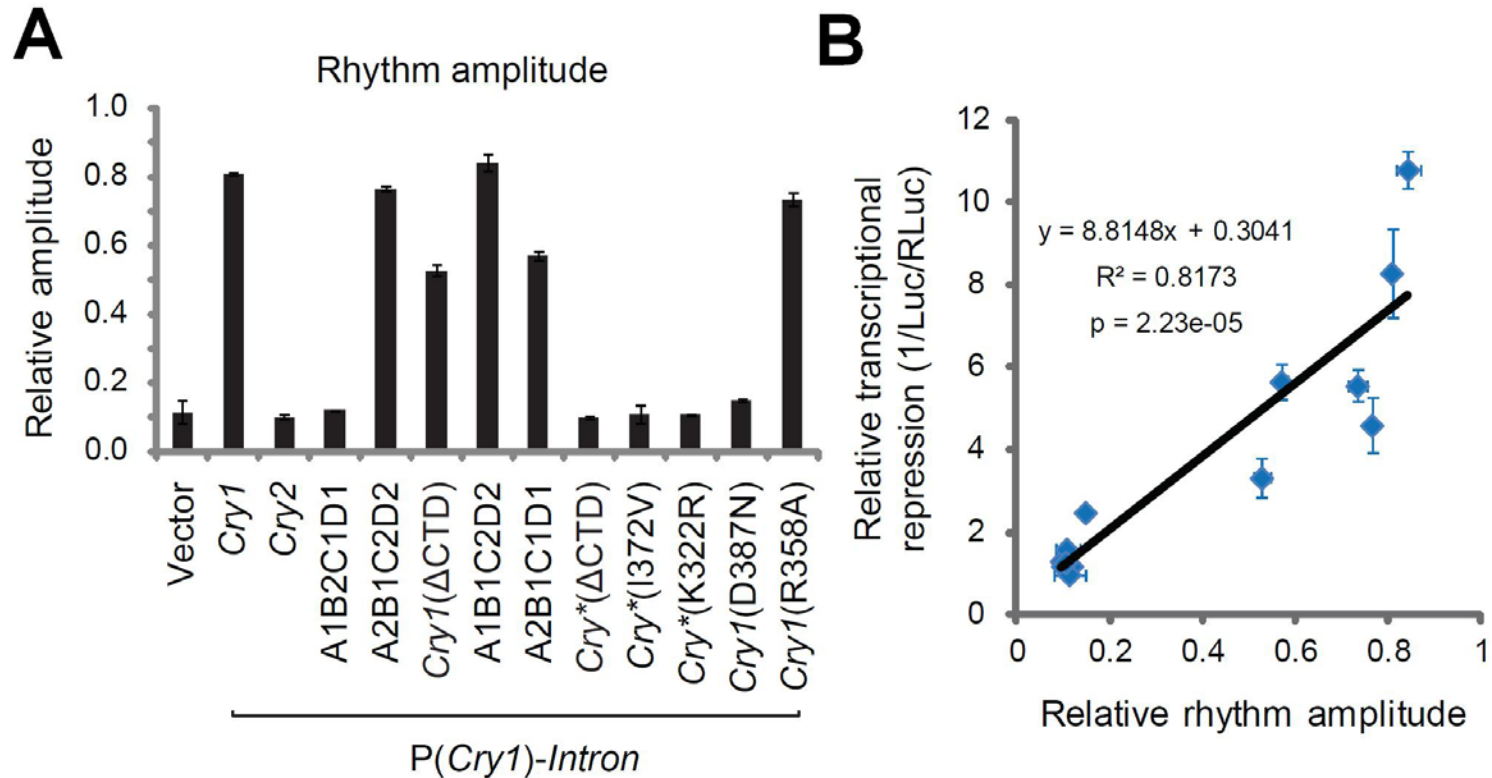
FIGURE 18. **Transcriptional repression positively correlates with rhythm amplitude.** (A) Relative amplitudes of rescued rhythms observed in Figure 17B. *Mean ± S.D.* (*error bar*) of two independent experiments are shown ($n = 3$). (B) Relative rhythm amplitude (*x* axis) is plotted against relative repression activity (*y* axis). Rhythm amplitude bears a positive correlation with transcriptional repression by various CRYs. *Mean ± S.D.* (*error bar*) of two independent experiments are shown ($n = 3$).

DISCUSSION

Unlike hourglass-type timers, oscillator-type timers such as the circadian clock regulate cyclic processes that repeat upon completion of a cycle. The mechanism underpinning this circadian oscillation in mammals is an autoregulatory transcriptional-translational negative feedback loop (22,117), in which transcriptional repression by the CRYs lies at the heart of this mechanism (18,24,119). To gain basic understanding of this biochemical mechanism, we sought to investigate the unique biochemical and structural aspects of the CRYs. Through a systematic analysis of protein structure-function relationships, we identified the distinct sequences that distinguish *Cry1* function from *Cry2*, and demonstrated that *Cry1*-specific transcriptional (strong) repression is required for mammalian clock function. This study provides insights into the unique biochemical and structural properties of CRY1, and presents new opportunities for future dissection of its precise role in the circadian clock mechanism.

Genetic Complementation of Cry1 in Cry-deficient Cells Provides a Functional Clock Model for Mechanistic Studies– In a recent study, we identified the full set of cis-elements responsible for the circadian expression pattern of *Cry1*, including primarily the E/E'-box and D-box elements in the promoter and RREs in the first intron of the *Cry1* gene (48). This allowed us to engineer a synthetic composite promoter that is both necessary and sufficient for establishing the *Cry1*-phase. Importantly, through genetic complementation in $Cry1^{-/-}$:$Cry2^{-/-}$ fibroblasts, we also demonstrated that *Cry1* expression at the

evening phase is required for generation and maintenance of cell-autonomous circadian rhythms.

This *Cry* rescue assay provided us with a unique opportunity to study CRY function in clock cells and confirmed that *Cry1* and *Cry2* indeed have differential functions in clock regulation. This assay also enabled us to uncover for the first time the different potency in transcriptional repression exhibited by *Cry1* and *Cry2*, which underlie their differential roles in clock function. In several prominent structure-function studies in which *Cry* expression was under a strong constitutive promoter (1,127-129), CRY protein (likely saturated) was assayed at steady-state levels, masking differences in repression activity between CRY1 and CRY2. Consistent with this notion, we show that, compared to the stronger CMV promoter, SV40-driven *Cry1* and *Cry2* exhibited a more noticeable difference in transcriptional repression (Fig. 15). In our study, *Cry* expression, under the control of the *Cry1*-phase promoter, is properly connected to the negative feedback loop involving both the E/E'-box and D-box elements and the RREs; under this condition, CRY expression levels would not reach saturation.

Sequence and Domain Structural Features that Distinguish CRY1 from CRY2– In this study we demonstrated that the functional difference between CRY1 and CRY2 lies primarily at the CRY1-PHR(313-426) and secondarily at the CTD. Mechanistically, the level of appropriately timed CRY1 repression is the key to generating robust rhythms. The CRY1-PHR(313-426) is critical for potent transcriptional repression. We observed a significant positive correlation between CRY repression activity and amplitude of the rhythms (Fig. 18). As the repression

activity goes up, so does the amplitude of the rhythms. Thus, from the evolutionary point of view, it is the elaboration of a new function for the conserved core domain of CRY that rendered it a core clock component.

Although the CTD is not absolutely required for circadian clock function, it participates in modulating basic clock function. Compared to wild type CRY1, the CRY1 chimera harboring the CTD of CRY2 (A1B1C1D2) shortened the period length (Fig. 14), indicating its role in period length regulation. Compared to CRY1, CRY* (A2B1C2D2) displayed slightly reduced, but by and large similar repression activity. Interestingly, however, compared to the full-length CRY1, CRY1(ΔCTD) displayed less transcriptional repression and generated lower amplitude rhythms, whereas CRY*(ΔCTD) exhibited dramatically reduced repression activity and failed to generate rhythms, similar to CRY2. Thus, our data suggest that CTD1 and CTD2 (from CRY1 and CRY2, respectively) play differential roles in fine-tuning the clock function, and that there might be a mechanism for signal transduction from identified Cryptochrome differentiating domain to CTD to accomplish the fine-tuning.

However, the mechanism of repression by CRY and potential signal transduction from the CRY1-PHR(313-426) to the CTD remain unknown. Current structural data on the CTD are confined to limited proteolysis and qualitatively interpreted solution NMR spectra (71), confirming predictions that CTD is largely disordered. A recent study described the crystal structure of full-length Drosophila CRY in which the CTD is found to interact with the FAD binding core domain (i.e., region B in our study). The CTD of dCRY contains only 20 residues,

whereas CTDs of mCRYs are much longer (80-100 residues) and diverge from dCRY, and thus, structurally more flexible. Our homology models of mCRYs confirmed the potential for interactions between CTD and the Cryptochrome differentiating domain. However, future structural and functional studies are required to elucidate the mechanism of coordinated function of CTD and the Cryptochrome differentiating domain of CRY proteins.

CRY1-specific Transcriptional Repression is Required for Circadian Clock Function– The basic concept of a circadian negative feedback loop in mammals was established in the late 1990s (22,116,117), and feedback repression is mediated primarily by CRYs, not PERs (24,118). Through studies of *Bmal1* and *Clock* mutants that interfere with CRY interaction, it was later demonstrated that CRY-mediated repression of BMAL1/CLOCK activity is required for clock function and maintenance of circadian rhythmicity (119). A hallmark of circadian clock function is the rhythmic expression of clock genes. Recently, we demonstrated that *Cry1* expression at the evening-time phase (i.e., not morning- or day-time) and therefore proper phasing in feedback repression by *Cry1* is important for normal circadian clock function (48). Here we further demonstrate that *Cry1*-specific repression is the key to generating circadian rhythms; *Cry1* was able to rescue the rhythms in $Cry1^{-/-}$:$Cry2^{-/-}$ fibroblasts, but *Cry2* failed to do so. In addition, $Cry1^{-/-}$ cells are largely arrhythmic, suggesting that endogenous *Cry2* alone is unable to support clock function (11,20). Thus, experimental data from both gain-of-function (this study) and loss-of-function studies in cellular clock models (11,130), as well as in circadian behavior of composite knockout mice

(108,112), establish that *Cry1* plays a more prominent role in clock function than *Cry2*. Despite the essential role of *Cry1* in cell-autonomous models, $Cry1^{-/-}$ mice nevertheless display persistent free-running rhythms (25,108,112). Therefore, there exists a gap in knowledge as to how transient rhythms in individual $Cry1^{-/-}$ neurons are organized into coherent rhythms in the SCN.

Future Perspective– Importantly, the mechanism by which CRY1 represses BMAL1/CLOCK complex activity remains elusive. Our findings that CRY1, but not CRY2, plays an essential role in clock function, and that CRY1 possesses unique biochemical features, especially within the key CRY1-PHR(313-426) domain, suggest that *Cry1* holds the key to our understanding of the feedback repression mechanism. A recent study showed that CRY1 and CRY2 bind to the CLOCK/BMAL1/E-box complex with the same affinity (131). Thus, it is possible that their functional difference lies at their different intrinsic repression activities or differential post-translational mechanisms, and future studies need to focus on the precise biochemical mechanism by which CRYs repress CLOCK/BMAL1 transcriptional activity. The functional assay established in this study provides new opportunities for future investigations into CRY1 structure-function relationships. Our findings shed new light on the functional importance of the CRY1-PHR(313-426) and the CTD in the clock mechanism. Several previous studies identified a subset of common motifs and sites, including nuclear localization sequences, coiled coils, phosphorylation sites of CK1ε, GSK3β, MAP kinase, and AMPK (1,126-129,132-135), and surely additional motifs remain yet to be identified. The functional significance of these

various sequences and structural features in CRY function will need to be tested using the assays developed in this study. These future studies will ultimately provide important insights into the biology of CRYs and their role in the negative feedback mechanism, as well as the functional evolution of the PHL/CRY family of flavoproteins.

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APPENDICES

Appendix A- Generation of phase-specific circadian reporter cell lines

The circadian clock is based on a biochemical negative feedback mechanism (Fig. 2). The core feedback loop consists of transcriptional activators BMAL1 and CLOCK, and repressors PERs and CRYs, which act on the circadian E/E'-box enhancer elements to produce rhythmic gene expression (with morning phase, e.g., *Rev-erbα*). The core loop regulates and integrates at least two other circadian cis-elements, the DBP/E4BP4 binding element (D-box; for day phase, e.g., *Per3*) and the ROR/REV-ERB binding element (RRE; for night phase, e.g., *Bmal1*) (18). Combinatorial regulation by multiple circadian elements can generate novel intermediate phases. For example, *Cry1* transcription is mediated by all three circadian elements (i.e., E/E'-box and D-box elements in the promoter and RREs in the first intron of the *Cry1* gene), giving rise to the distinct *Cry1* evening-time phase (48). Based on these mechanisms of gene regulation, we generated four different luciferase reporter constructs in which *Luciferase* gene is driven by different circadian elements.

Luciferases were first used in real-time luminescence recording of circadian gene expression in the early 1990s in plants and cyanobacteria, and have been commonly used in the mammalian system since 2000 (15,136-139). Luciferase reporters are generally less toxic and more sensitive than fluorescent reporters such as GFP, due to much lower background (140). The most commonly used bioluminescent reporter is firefly (Photinus pyralis) luciferase (*Luc+*) constructed in the pGL3 vector series (Promega), in which the coding region of the native Luc was modified for optimized transcription and translation.

The combination of firefly luciferase and its highly stable and cell-permeable

substrate, D-luciferin, is ideal for long-term recording. A destabilized *Luc* (d*Luc*)

is a modified version of *Luc+* with a PEST sequence fused at its C-terminus to

allow for rapid protein degradation. A further improved version, *Luc2*, is available

in the pGL4 vector series (Promega) with higher and less anomalous expression.

In a recent report, Brazilian click beetle luciferase (E*Luc*) was shown to exhibit a

much brighter signal than *Luc+*, suitable for single cell imaging (141).

Many recent studies have taken advantage of the mPER2::LUC fusion

knock-in reporter mouse (11,14,113,136,142-146). This reporter system allows

circadian phenotyping of cells and tissue explants including the SCN. In our

previous studies, we crossed this reporter mouse line with many of the

behaviorally characterized clock gene knockouts and examined the dynamics of

bioluminescence rhythms in SCN, liver and lung explants cultured ex vivo, and in

dissociated SCN neurons and fibroblasts cultured in vitro (14,113,142,145).

These studies allowed us to gain important insights into the molecular details of

clock operation at the cell and organismal levels.

Here we generated four different reporter constructs: P(*Per2*)-d*Luc* and

P(*Cry1*)-d*Luc* reporters containing both E/E'-box and D-box elements in the

regulatory region (18,147,148); P(*Cry1*)-*Intron*-d*Luc* representing combinatorial

regulation by all three elements (i.e., E/E'-box, D-box, and RRE) (18,48); and

P(*Bmal1*)-d*Luc* regulated exclusively by RRE (18,34,113,143). We cloned the

reporter cassettes into a lentiviral vector (pLV7-*Bsd*). Then, we introduced these

reporters into 3T3 cells by infecting them with lentivirus to produce the

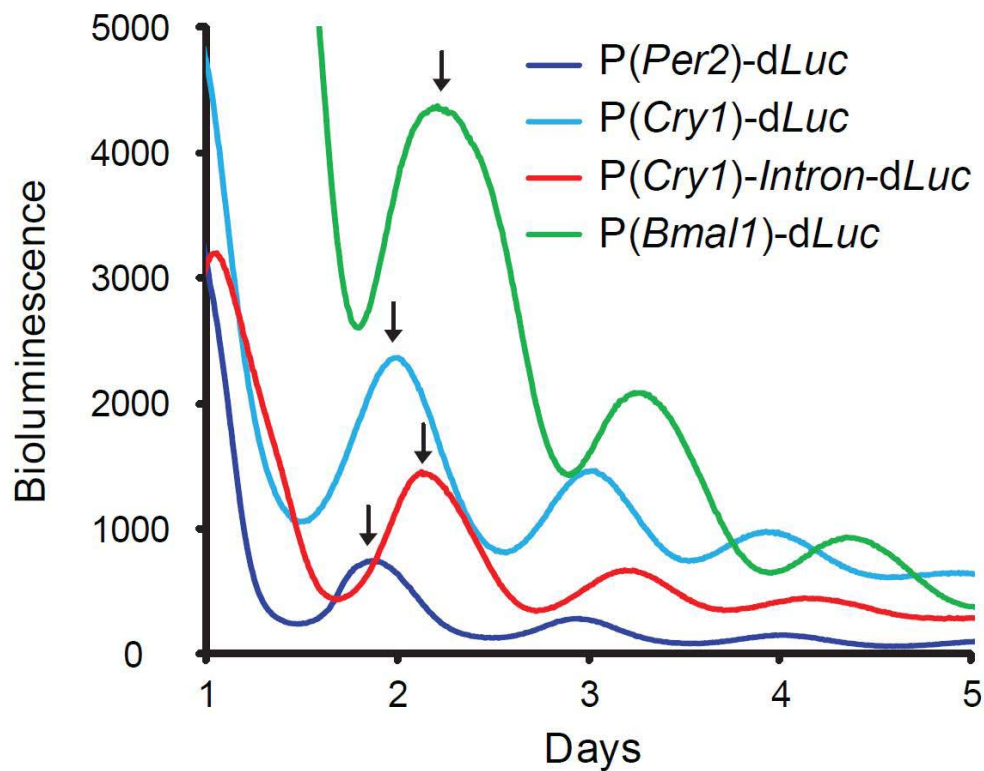anticipated distinct phases of reporter expression (Fig. 19).

FIGURE 19. **Phase-specific expression of bioluminescence reporters in 3T3 cells.** The lentiviral reporter vectors used in this experiment are pLV7-*Bsd*-P(*Per2*)-d*Luc*, P(*Cry1*)-d*Luc*, P(*Cry1*)-*Intron*-d*Luc*, and P(*Bmal1*)-d*Luc*. Each reporter exhibits a distinct phase of oscillation, as indicated by the arrows. While the *Per2* and *Cry1* promoters drive peak bioluminescence at morning-day phases and the Bmal1 promoter at night phase, combinatorial regulation by the P(*Cry1*)-*Intron* harboring E-box, D-box, and RRE elements confers evening phase of peak bioluminescence.

Unlike tissue or animal models, cell-based models are amenable to genetic and pharmacologic perturbations, and when necessary, also use in high-throughput screening (HTS) formats. Perturbation of gene function can be achieved by over-expression or RNAi-mediated knockdown. Selective small molecules can be used to interfere with protein function. Cell-autonomous clock models have greatly facilitated mechanistic studies. Hogenesch and colleagues used the 3T3 model to show that feedback repression by CRYs is required for clock function (119), and the U2OS model to probe the system-level properties of clock function (149). We employed the $Cry1^{-/-}:Cry2^{-/-}$ fibroblast model derived from mice to show that delayed feedback repression is necessary for clock function (48), and the $Bmal1^{-/-}$ fibroblast model to show that Rev-$erb\alpha$ and β play redundant roles in regulating RRE-mediated rhythmic gene expression, and that $Bmal1$ rhythm is not critically required for core clock function (113). Furthermore, chemical screening in reporter cells allowed identification and/or clarification of the functions of GSK-3β (period shortening when perturbed) (143), CK1σ and ε (period lengthening when perturbed) (150), and CK1α12.

In addition to the core feedback loop, the clock mechanism also integrates diverse signaling and regulatory factors. For identification of additional clock components and modifiers, cell-autonomous clock models are advantageous over genetic screening in mice due to the inherent lethality, pleiotropic effects, and developmental genetic compensation associated with mutant animal models (151). Cell-based screens, in combination with functional genomics approaches, have been effectively carried out using high-throughput recording systems to

screen genome-wide siRNA and shRNA libraries for identification of novel clock

factors (130,152). Similarly, one can employ chemical biological approaches and

screen for diverse small molecules to study their effects on clock function

(143,150,153,154). Although major clock genes and their functions have been

identified, these screens have identified additional components or modifiers that

are involved in regulation or modulation of the clock. Many of these modifiers

represent particular modalities of integrating signal transduction of synchronous

or asynchronous cues (clock inputs). These cellular models are more tractable

for rapid discovery of basic mechanisms, which then provide new entry points for

in vivo validation and exploration. These four cell lines will serve as great

resource to study the basic clock mechanisms *in vitro* before *in vivo* validation.

Appendix B- Mutation of FAD binding sites and Trp triad electron transfer pathway: consequences on *Cry1* function in circadian clock

Mutation analysis of FAD binding sites– The photolyase/cryptochrome super family of flavoproteins, found from bacteria to humans, use the same cofactor, FAD, to carry out dissimilar functions (50,51). A second cofactor has also been identified in some prokaryotic CPD PHRs, which serves as a light-harvesting antenna. From crystal structures of *E. coli* and *Anacystis nidulans* CPD PHRs, binding sites for the second cofactor 5,10-methenyltetrahydrofolic acid (MTHF) or 8-hydroxy-5-deazaflavin (8-HDF) were identified (155,156). Photolyases are DNA repair enzymes that revert UV-induced photoproducts into normal bases to maintain genetic integrity (52). In contrast, CRYs in plants are blue-light photoreceptors and regulatory proteins that control their growth and development. Whereas in animals, they regulate biological clocks (60). Ectopically expressed and purified human CRY1 and CRY2 from HeLa cells (157), as well as purified recombinant proteins from *E. coli* (73) were shown to contain FAD and pterin, presumably in the form of MTHF. However, both cofactors were found to be present at substoichiometric levels relative to the apoenzyme, limiting the characterization of their biophysical properties.

Since cryptochromes in mammals are not photoreceptors, rather they play a central role in the circadian clock, it is important to understand the functional contribution of the cofactor FAD in mammalian CRY function. Because of the prominent role CRY1 plays in the circadian clock function, we decided to examine the requirement of FAD by CRY1 for its function in circadian clock.

Since it is difficult to purify CRYs with their cofactors at stoichiometric levels (73,157), we took an indirect approach by employing site-directed mutagenesis followed by genetic complementation analysis of gene function to test the requirement of FAD. Recent modeling studies using the known crystal structure of *Arabidopsis* (6-4) photolyase demonstrated conserved structural features for FAD binding in mouse cryptochromes (126). Previously, it was shown that mutations in the conserved Asp387–Arg358 salt bridge (invariant in all PHL/CRY family members and positioned to stabilize the FAD radical), resulted in significant loss in transcriptional repression ability of CRY1 in an *in vitro* repression assay (126,158). However, these mutants were not tested in a true functional rhythm assay.

Here, we generated mutations in the conserved Asp-Arg salt-bridge by substituting i) Arg with Lys, Asp or Ala (i.e., *Cry1R358K*, *Cry1R358D*, *Cry1R358A*) and ii) Asp with Asn (*Cry1D387N*; *Cry^b*-like). In CRY1, Asn393 interacts with the redox-active N5 position of FAD. We also mutated Asn393 to Asp or Leu (*Cry1N393D, Cry1N393L*), mimicking insect- or plant-specific CRY, respectively. These mutants were then tested for their ability to restore circadian rhythms in *Cry1$^{-/-}$:Cry2$^{-/-}$* fibroblasts.

We found that, while all three Arg mutants were able to restore the rhythms, D387N substitution (Drosophila *Cry^b*-like) was sufficient to render *Cry1* unable to restore the rhythms (Fig. 20A). Our finding from this gain-of-function assay is consistent with results obtained in the *in vitro* transcriptional activity in transfected S2 cells (158). Compared with dCRY in which both R381A and

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D410A (or *Cry^b*) mutants abolished transcriptional repression activity and light responsiveness, only one mutation D387N of mCRY1 affected its transcriptional activity (158). Our mutation study also revealed that D387N mutation affected *Cry1* function; however, R358A, R358K and R358D mutations are tolerated for *Cry1* function.

In the case of Asn393 mutations, the key residue interacting with redox-active FAD, a mixed result was obtained; while N393D mutant was still able to generate and maintain rhythms in *Cry1^{-/-}:Cry2^{-/-}* fibroblasts, N393L abolished CRY1 function in rhythm generation (Fig. 20A).

Taken together, our mutation studies suggest that the FAD salt-bride partner Asp387 and the key residue Asn393 play important roles for CRY1 function, whereas Arg358 doesn't. These data can be interpreted in two ways;

i) *Cry1* requires FAD as a cofactor for its activity, and mutations at Asp-387 (D387N) and Asn-393 (N393L) abolished *the* ability of CRY1 to bind to FAD, whereas all Arg-358 mutants and N393D didn't have obvious adverse effects. Our data showing that D387N and N393L mutants are not able to establish rhythms in *Cry1^{-/-}:Cry2^{-/-}* fibroblasts supports this notion.

ii) As suggested previously (158), it is also possible that D387N and N393L mutations alter the structure of CRY1 protein and hence affect its function. Further studies are needed to confirm whether these mutations affect CRY1 structure or alter FAD binding.

Trp triad mutation analysis– Photolyases repair UV light-induced pyrimidine dimers via intermolecular as well as intramolecular redox pathways. Upon receiving sun light, photolyases get activated through intramolecular redox pathway. The photoantena MTHF absorbs a blue light photon and then excites FADH to an excited state, FADH*. Excitation of FADH results in its photoreduction by an electron transfer to excited FADH* via an internal chain of three tryptophans (FADH ←Trp-382←Trp-359← Trp-306; designated as Trp triad). Subsequently, through intermolecular redox pathway, an electron is transferred from reduced flavin, FADH$^-$ to DNA to repair pryrimidine dimers (159-161). Several other studies implicated that tryptophans corresponding to the Trp triad play a role in the regulation of light dependent or independent reactions mediated by animal cryptochromes (158,162). We set out to investigate whether Trp triad is involved in mammalian CRY1 function in circadian clock. We performed mutational analysis of the Trp triad (based on location in the structure, inside Trp-397 adjacent to FAD, middle Trp-374, and outside Trp-322) (52,126). We individually mutated these three tryptophans in CRY1 to either tyrosine, which is structurally different from tryptophan but still capable of electron transfer (163,164) or alanine, which is structurally different from tryptophan and also incapable of electron transfer (W397Y/A, W374Y/A and W322Y/A), and then tested the ability of the mutants to restore rhythms in *Cry1$^{-/-}$:Cry2$^{-/-}$* fibroblasts.
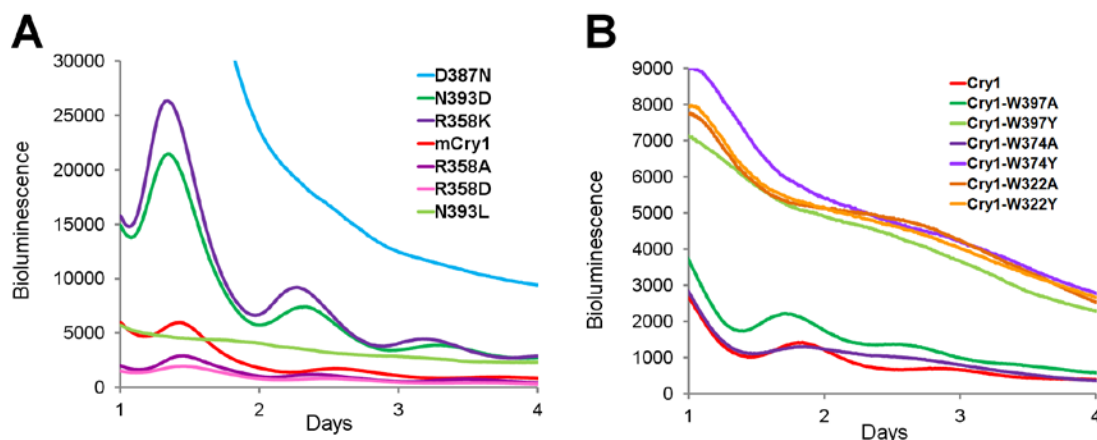
FIGURE 20. **Mutations of FAD binding site and Trp triad and *Cry1* function.**
A and *B*, representative bioluminescence records from $Cry1^{-/-}:Cry2^{-/-}$ fibroblasts
expressing *Cry1* mutants. *A, Cry1* was mutated to obtain mutation in Asp(387)-
Arg(358) salt-bridge which is positioned to stabilize the FAD radical, as indicated.
Another key residue Asn-393, which interacts with the redox-active FAD N5
position of FAD, was also mutated as indicated. D387N and N393L mutants
failed to rescue the rhythm indicating important role for *Cry1* function. *B*, all trp
triad mutants, except W397A, failed to rescue rhythms.

Even though W397A mutation still retained CRY1 function to support

scillation, the other two mutants, W374A and W322A, failed to restore rhythms in

$Cry1^{-/-}:Cry2^{-/-}$ fibroblasts, suggesting an important role for Trp triad in CRY1

function. Interestingly, even though W397Y, W374Y and W322Y mutants

contained redox-competent tyrosine, they were not able to restore the rhythms.

The inability of these three mutants to restore rhythms in $Cry1^{-/-}:Cry2^{-/-}$

fibroblasts may be caused by alteration in CRY1 protein structure due to the

mutations. Previous studies suggested that the Trp triad of *Drasophila* CRY palys

a role in maintaining structural integrity necessary for function, rather than

participating in the photochemistry of animal cryptochromes (158,162). Thus, it is

possible that our mutations also altered the structure of CRY1 protein, which may explain why the mutants we tested here are not functionally active to restore circadian rhythms in $Cry1^{-/-}:Cry2^{-/-}$ fibroblasts. Therefore, at this point it remains elusive whether Trp triad is required for CRY1 function.

Further studies are needed to confirm the role of Trp triad in structural integrity of mammalian cryptochromes. If it plays a role in maintaining structural integrity, different mutation strategy should be employed to maintain structural integrity of mutants and then test the functionality of the mutants in genetic complementation assay.

Appendix C- shRNA-mediated knockdown of *Cry* genes in U2OS cells

      Circadian clocks exist in cell lines cultured *in vitro*. Much of what we know about the biochemistry and cell biology of the clock mechanism is based on three cellular models: 3T3 mouse fibroblasts (18,119), U2OS human osteosarcoma cells (130,143,149,152), and mouse fibroblasts derived from mice (48,113,154). Some of these lines have been shown to be amenable to overexpression of DNA (cDNA) and knockdown of gene by RNA interference (RNAi). These cell lines have been successfully used to identify new components of many signaling pathways (for example, see (165-167)). When transfection efficiency is high, synthetic siRNA can be transiently transfected into cells to knock down gene expression. However, when transfection is technically difficult, an shRNA expression vector can be stably transduced into cells via lentiviral infection, so that shRNA produced by the cell is processed to siRNA for gene knockdown (KD). Although cumbersome and less reproducible, transiently transfected cells can also be grown in the continuous presence of antibiotics to generate stable cell lines. Lentiviral vectors are still preferred because of their stable integration into the cell's genome, as well as greater efficiency and versatility. The lentiviral vector system permits efficient delivery and stable integration into the host genome of both dividing and non-dividing mammalian cells, and therefore is not limited to certain cell types as in transient transfection.

      Recently, the U2OS model has become a preeminent cellular clock model largely because it meets the key requirements for high-throughput screening of commercially available human siRNA libraries (e.g., human origin, capable of

generating robust circadian rhythms). Here we tested the efficiency of shRNA

mediated knockdown of clock genes in U2OS cells. We generated shRNA

against constructs in pLL3.7 Gateway expression vector (113) for knockdown of

Cry1 and *Cry2* genes. We then infected two different U2OS reporter cell lines

(P(*Per2*)-d*Luc* and P(*Bmal1*)-d*Luc*) with lentivirus containing shRNA expression

cassettes.

Knockdown of *Cry1* and 2 resulted in opposite phenotype in this human

osteosarcoma cell line. Knocking down *Cry1* gene caused shortening of period

length, whereas knocking down *Cry2* lengthened the period length of circadian

rhythms. This opposite period length phenotype was independent of the reporter

as similar phenotypes were observed for both P(*Per2*)-d*Luc* and P(*Bmal1*)-d*Luc*

reporters (Fig. 21). Our knockdown phenotype are consistent with previously

observed knockout phenotypes at cellular, tissue and behavioral levels (14,108),

suggesting that shRNA-mediated knockdown in U2OS can be employed for

studying gene function, in which stable cell lines with KD of a particular gene can

be generated. Stable knockdown cell lines provide an opportunity to perform

different assays with the same source of cells, which is not readily possible for
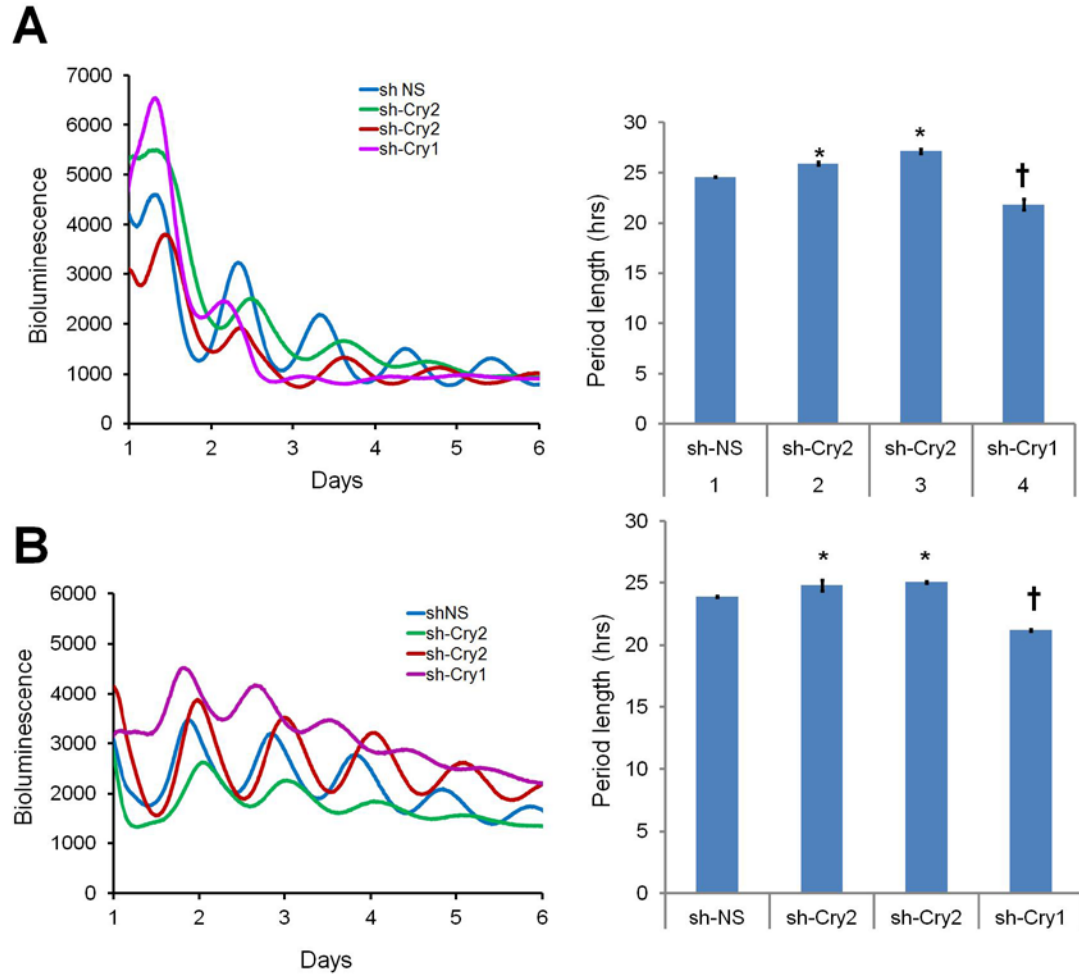
transiently transfected cells.

FIGURE 21. **shRNA mediated knockdown of *Cry* genes in U2OS cells.** *A* and *B,* (left panel) representative bioluminescence records from P(*Per2*)-d*Luc* and P(*Bmal1*)-d*Luc*U2OS reporter cells infected with lentivirus containing shRNA against either *Cry1* or *Cry2* gene. Period length corresponding to each shRNA is shown on the right panel. *Mean ± S.D.* (error bar) of two independent experiments are shown. *Cry2* knockdown results in lengthening of period length of circadian rhythm and *Cry1* knockdown results in shortening.